

# Ultrafine Carbon Black Particles Cause Early Airway Inflammation and Have Adjuvant Activity in a Mouse Allergic Airway Disease Model

Colin de Haar,<sup>1</sup> Ine Hassing, Marianne Bol, Rob Bleumink, and Raymond Pieters

Department of Immunotoxicology, Institute for Risk Assessment Sciences, Utrecht University, 3584 CM Utrecht, The Netherlands

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To gain more insight into the mechanisms of particulate matter (PM)-induced adjuvant activity, we studied the kinetics of airway toxicity/inflammation and allergic sensitization to ovalbumin (OVA) in response to ultrafine carbon black particles (CBP). Mice were exposed intranasally to OVA alone or in combination with different concentrations of CBP. Airway toxicity and inflammation were assessed at days 4 and 8. Immune adjuvant effects were studied in the lung draining peribronchial lymph nodes (PBLN) at day 8. Antigen-specific IgE was measured at days 21 and 28, whereas allergic airway inflammation was studied after OVA challenges (day 28). Results show that a total dose of 200  $\mu$ g CBP per mouse, but not 20  $\mu$ g or 2  $\mu$ g, induced immediate airway inflammation. This 200  $\mu$ g CBP was the only dose that had immune adjuvant activity, by inducing enlargement of the PBLN and increasing OVA-specific production of Th2 cytokines (IL-4, IL-5, and IL-10). The immune adjuvant activity of 200  $\mu$ g CBP dosing was further examined. Whereas increased OVA-specific IgE levels in serum on day 21 confirms systemic sensitization, this was further supported by allergic airway inflammation after challenges with OVA. Our data show a link between early airway toxicity and adjuvant effects of CBP. In addition, results indicate that local cytokine production early after exposure to CBP is predictive of allergic airway inflammation. In addition this model appears suitable for studying the role of airway toxicity, inflammation and other mechanisms of particle adjuvant activity, and predicting the adjuvant potential of different particles.

**Key Words:** ultrafine particles; adjuvant; allergy; intranasal; inflammation.

## INTRODUCTION

Exposure to airborne particulate matter (PM) has been associated with adverse health effects in epidemiological studies. These effects can be divided into direct effects, like cardiovascular disease, respiratory inflammation, exacerbation

of asthma symptoms (Donaldson *et al.*, 2003; Li *et al.*, 2003; Tao *et al.*, 2003), and long-term effects like the development of lung cancer, chronic bronchitis, and allergic airway sensitization (D'Amato, 2002; Donaldson *et al.*, 2003; Peterson and Saxon, 1996; Popp *et al.*, 1989; Salvi, 2001). PM is a complex mixture of particles of different size, chemical composition, and structure. Which of the various characteristics is involved in the different PM health effects, and their underlying mechanisms, are still not clear.

In both humans (Diaz-Sanchez *et al.*, 1999; Fujieda *et al.*, 1998) and animals (Lambert *et al.*, 2000; Nilsen *et al.*, 1997; Steerenberg *et al.*, 2003; van Zijverden *et al.*, 2001; Whitekus *et al.*, 2002) inhalation or intranasal exposures to various PM (*e.g.*, diesel exhaust particles [DEP], CBP, or residual oil fly ash [ROFA]) in combination with an antigen is capable of increasing levels of antigen-specific IgE. Because IgE is involved in type 1 allergic responses, it is involved in allergic asthma as well as other allergic diseases. Therefore IgE is used to assess the adjuvant potential of particles (Nilsen *et al.*, 1997; Takafuji *et al.*, 1987; Takano *et al.*, 1997; van Zijverden *et al.*, 2001) on allergic sensitization. Some of these studies also show the development of allergic airway inflammation, and airway hypersensitivity after antigen challenge (Fernvik *et al.*, 2002; Lambert *et al.*, 1999; Steerenberg *et al.*, 2003).

The mechanisms behind this adjuvant activity of particles are still not clear. Apart from the role of particles as antigen depot (Gupta, 1998), the induction of local inflammatory responses may also be of importance. Various particle characteristics have been associated with particle-induced airway inflammation. Lambert *et al.* (2000), showed that transition metals are involved in ROFA-induced airway inflammation and adjuvant activity. Other factors that might be important are PAH from DEP (Diaz-Sanchez, 1997), biologics like endotoxins attached to freshly isolated PM (Soukup and Becker, 2001), or a combination of both (Yanagisawa *et al.*, 2003). Particle size and surface area are also important factors in particle airway-toxicity (Brown *et al.*, 2000) and have recently been shown, with the popliteal lymph node assay, to be important factors in adjuvant activity (Nygaard *et al.*, 2004).

<sup>1</sup> To whom correspondence should be addressed at Institute for Risk Assessment Sciences, Department of Immunotoxicology, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands. Fax: +31–302535077. E-mail: C.deHaar@iras.uu.nl.

Because our previous studies (van Zijverden *et al.*, 2001) have shown that ultrafine CBP and DEP have adjuvant activity after combined intranasal exposure with OVA, we wanted to investigate the airway inflammatory effects early after intranasal exposure. We have used ultrafine CBP in the current study, because we were interested in the toxicity and adjuvant activity of particles per se. Furthermore, the particle core of most of the real-life PM, like, for instance, DEP, consist of carbon, making these particles relevant to real-life exposures. Ultrafine CBP has also been shown to induce an airway inflammation in rat models that was independent of any soluble factors (Brown *et al.*, 2000). So, in the current study we examined particle-induced toxicity and local airway inflammation using different doses of CBP with a constant dose of OVA, and in addition assessed whether immune sensitization occurred.

Th2 cytokines have been shown to play an important role in both the sensitization and the challenge phases of allergic airway disease. We therefore determined cytokine production by cells obtained from the lungs and lung draining lymph nodes at day 8 (sensitization) and at day 28 (after challenge). In addition, we measured antigen-specific serum IgE levels, and allergic airway inflammation upon antigen challenge.

Our data show that CBP-induced local airway damage and inflammation is detectable 24 h after the last intranasal exposure (day 4), and that Th2 skewing of the immune response against the co-administered OVA is already apparent on day 8. These CBP+OVA effects were only present in mice treated with 200 µg CBP, and this high-dose group also showed increased OVA-specific IgE and allergic airway inflammation after antigen challenge.

## MATERIALS AND METHODS

**Mice.** Specific pathogen-free female BALB/cANNCrl mice (6–8 weeks old) were obtained from Charles River (Sulzfeld, Germany) and randomly assigned to specific treatment. Mice were maintained under barrier conditions in filter-topped Macrolon cages with wood chip bedding, at a mean temperature of  $23 \pm 2^\circ\text{C}$ , relative humidity 50–55%, and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments were conducted according to the guidelines of the animal experiments committee of the Veterinary Faculty of Utrecht University. Six to eight mice were used in each experimental treatment group.

**Intranasal sensitization and challenge.** Carbon black (CBP) particles were obtained from Brunswich Chemicals (Amsterdam, The Netherlands) and particle diameter was determined using electron microscopy (approximately 30–50 nm). Particles were suspended in phosphate buffered saline (PBS) at a concentration of 3.3 mg/ml, and sonicated using a Branson 1510 Ultrasonic bath (Branson, Danbury, CT) for 2 h. After sonification, the suspensions were 1:10 and 1:100 diluted in PBS to achieve the desired concentration series.

OVA (Sigma-Aldrich, Zwijndrecht, The Netherlands) was prepared in PBS, and endotoxin was removed with Pierce detoxigel (Perbio Science, Etten-Leur, The Netherlands). Endotoxin levels were 0.6 ng/mg OVA, and no endotoxin could be detected in CBP as determined by Limulus Amebocyte Lysate (LAL) assay (LAL Kinetic-QCL Kit, Bio-Whittaker, Walkersville, MD). OVA was

added to the CBP suspensions to a final concentration of 0.5 mg/ml. Mice were anesthetized by intramuscular injection of 40 µl ketamine (25 mg/ml)/xylazine (5 mg/ml), and exposed to 20 µl of OVA (0.5 mg/ml) or 200 µg, 20 µg, 2 µg CBP+OVA (3.3, 0.33, 0.033 mg/ml CBP in combination with 0.5 mg/ml OVA, respectively) in PBS by intranasal droplet application on days 0, 1, and 2.

Mice were sacrificed at day 4 and day 8 by an overdose of pentobarbital, or they were challenged to allow study of asthma-like allergic airway inflammation. On days 25, 26, and 27, a challenge was performed by intranasal droplet application of 20 µl OVA (0.5 mg/ml) in PBS or PBS only to mice anesthetized by intramuscular injection of 40 µl ketamine (25 mg/ml)/xylazine (5 mg/ml). Mice were sacrificed on day 28 by an overdose of pentobarbital.

**Bronchoalveolar lavage.** Bronchoalveolar lavage (BAL) was performed by flushing the lungs with  $3 \times 1$  ml sterile PBS 24 h after the last intranasal exposure. The first lavage was kept separate from the other two. BAL was centrifuged at  $900 \times g$  for 5 min, and the BAL fluid (BALF) from the first lavage was stored at  $-80^\circ\text{C}$  and used for further analysis. The cells from all three lavages were pooled. Total BAL cell numbers were counted with a Coulter counter (Coulter Electronics, Luton, UK), and differential cell count was performed under FACS staining.

Lactate dehydrogenase (LDH), total protein, and tumor necrosis factor alpha (TNF- $\alpha$ ) levels were determined in first BALF fraction using a TOX-7 kit (Sigma-Aldrich Zwijndrecht, The Netherlands), a Pierce BCA kit (Perbio Science, Etten-Leur, The Netherlands), and a TNF- $\alpha$  ELISA kit (Biosource Europe, Fleurus, Belgium), all according to the manufacturers' protocols.

**Isolation and culture of lymph node and lung tissue cells.** Lungs were perfused with 10 ml sterile PBS through the right ventricle to remove blood from the lungs. Lungs were isolated and chopped with sterile blades and digested with collagenase IV and DNase I, as described previously (Vermaelen *et al.*, 2001). Peribronchial lymph nodes (PBLN) were isolated and minced using frosted objective slides. Single cell suspensions from both organs were taken up in complete RPMI 1640 with Glutamax-I (Invitrogen Life Technologies) supplemented with 10% FCS (Valeant) and 2% penicillin-streptomycin.

Cell suspensions were plated in round-bottom 96-well plates ( $2 \times 10^6$  cells/ml) and restimulated with 100 µg OVA for 4 days. Levels of IL-4, IL-5, IL-10, and IFN- $\gamma$  in culture supernatants were measured by ELISA (BD Pharmingen, Hamburg, Germany) according to the manufacturer's protocol.

**Flow cytometry.** Cells from BAL, lungs, and PBLN were stained with FITC-labeled anti-GR-1, PE-labeled anti-B220, and APC-labeled anti-CD11c. Cells from lungs and PBLN were also stained with FITC-labeled anti-CD4, PE-labeled anti-CD8, PerCP-labeled anti-CD3, and APC-labeled CD19. Cells were incubated with Fc block (2.4G2 Ab) for the reduction of nonspecific Ab binding, and staining was performed at  $4^\circ\text{C}$ . Antibodies used were from BD Pharmingen (San Diego, CA) and cells were measured using a FACScan and analyzed using CellQuest software (BD Bioscience). Dead cells and debris were excluded from BAL samples using propidium iodide staining (Sigma-Aldrich) and detection in FL-3.

**Determination of OVA-specific IgE.** Serum was collected on days 21 and 28 after the start of intranasal exposures. OVA-specific IgE antibody levels were determined by means of a sandwich ELISA. Briefly, plates (high bond 3950; Costar, Cambridge, MA) were coated overnight at  $4^\circ\text{C}$  with 2 µg/ml rat anti-mouse IgE antibody (Pharmingen) in 0.05 M bicarbonate buffer (pH 9.6), and blocked with PBS containing 0.05% Tween 20 (PBS-T) and 3% milk powder (Campina Melkunie) (1 h,  $37^\circ\text{C}$ ). Serum was serially diluted in PBS-T containing 1% bovine serum albumin (PBS-T 1% BSA) and incubated for 1 h at  $37^\circ\text{C}$ . This was followed by a 1 h incubation with biotinylated OVA. After incubation with poly-HRP-streptavidin (Sanquin, Amsterdam, The Netherlands) (1 h, RT), TMB substrate was added (15 min, RT). The coloring reaction was stopped by adding 2 M  $\text{H}_2\text{SO}_4$ , and absorbance was measured at 450 nm and compared between treatment groups.

**Lung histology.** Lungs were inflated with 1 ml of 4% formalin in PBS, and paraffin-embedded. Sections (5 µm thick) were stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS) and examined by light microscopy.

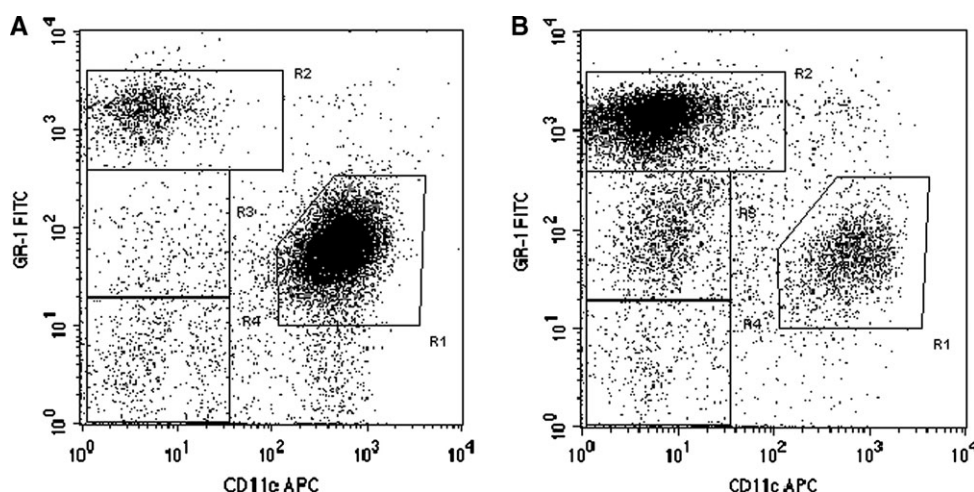


**FIG. 1.** Broncheolar lavage (BAL) cells were differentiated using GR-1 and CD11c staining and flow cytometry. Mice were exposed to ovalbumin (OVA) (A) or 200 µg carbon black particles (CBP)+OVA (B). On day 8 BAL was performed on the airways and the cells were stained for GR-1 and CD11c. The combination of these markers was used to discriminate the different cell types in the BAL. The percentages of macrophages (R1), neutrophils (R2), eosinophils (R3), and lymphocytes (R4) were assessed, and percentages were combined with total cell counts in Figure 2A.

**Statistical analysis.** Data are presented as mean values  $\pm$  standard error of the mean (SEM), and are representative of at least two independent experiments. Because our data were not normally distributed, they did not meet the assumptions needed for analysis of variance (ANOVA). Therefore we used the nonparametric Kruskal-Wallis test for multiple independent samples, followed by a Mann-Whitney U-test to compare the different treatment groups. When concentrations were not detectable (ND), they were set at 0 for statistical analysis (see Table 2). All tests were performed using SPSS software 12.0 for Windows. Data are considered significant when  $p$  values are  $<0.05$ .

## RESULTS

### *CBP+OVA Exposure Induces Concentration-Dependent Acute Airway Damage and Inflammation*

The direct proinflammatory effect of intranasal OVA or CBP+OVA exposure on the airways was assessed by analyzing BALF, BAL cells and by histological examination of lung tissue. Differentiation of BAL cells was determined by a flow cytometry staining method (De Heer *et al.*, 2004) that discriminates neutrophils, eosinophils, macrophages, and lymphocytes on the basis of GR-1 and CD11c labeling (Fig. 1).

The kinetics of CBP+OVA-induced airway damage and inflammation was studied on day 4 and day 8. On both days total protein content, TNF- $\alpha$  and LDH levels in BALF, as well as the proportions of neutrophils and eosinophils were determined, all of which are indicators of particle-induced airway inflammation. Only on day 4 were LDH levels increased in the 200 µg CBP+OVA-exposed animals (OD 0.161 compared to 0.068), suggesting direct toxicity (data not shown). Because both airway inflammation and adjuvant activity could be detected on day 8, only data from this time point are presented here.

Although doses of 200 µg, 20 µg, and 2 µg CBP combined with OVA were tested, only exposure to 200 µg CBP+OVA

induced significantly ( $p = 0.004$ ) higher numbers of BAL cells compared to the OVA control (Fig. 2A). BAL contained many neutrophils, whereas eosinophils and macrophages were only slightly increased. Other inflammatory parameters like total protein (Fig. 2B) and TNF- $\alpha$  (Fig. 2C) levels in the BALF were also increased only in mice exposed to 200 µg CBP.

Carbon black particles were seen in the airways Lumen and inside alveolar macrophages (histology, data not shown). The RAS of the alveolar macrophages, which can be used as a parameter for phagocytosis (Stringer *et al.*, 1995) was increased dose dependently. Whereas both 200 µg and 20 µg of CBP+OVA induced RAS levels that were significantly higher ( $p = 0.004$ ) than those of the OVA controls, 2 µg of CBP+OVA did not (Fig. 2D). Furthermore, the RAS of the macrophages of the 200-µg-dosed animals were significantly increased compared to the 20-µg-dosed animals ( $p = 0.041$ ).

### *Dose-Dependent CBP+OVA Adjuvant Activity on PBLN*

On day 8 the total number of lymphocytes in the PBLN (Fig. 3) was significantly increased 4–5-fold in the 200 µg CBP+OVA-exposed group compared to the OVA control ( $p = 0.004$ ) and the 20-µg- and 2-µg-exposed groups ( $p = 0.015$  and  $p = 0.002$ ). The increase in PBLN cell number was mainly attributable to the increase in CD19 cells (7–8 times increase), whereas the number of CD4 and CD8 cells was doubled that of the other treatments. The 20-µg and 2-µg exposures did not increase the number of PBLN cells compared to the OVA control.

The production of different cytokines by PBLN cells after *ex vivo* restimulation with OVA is shown in Table 1. Compared to the OVA-only exposed mice, all three concentrations of CBP+OVA induced significantly higher levels of the

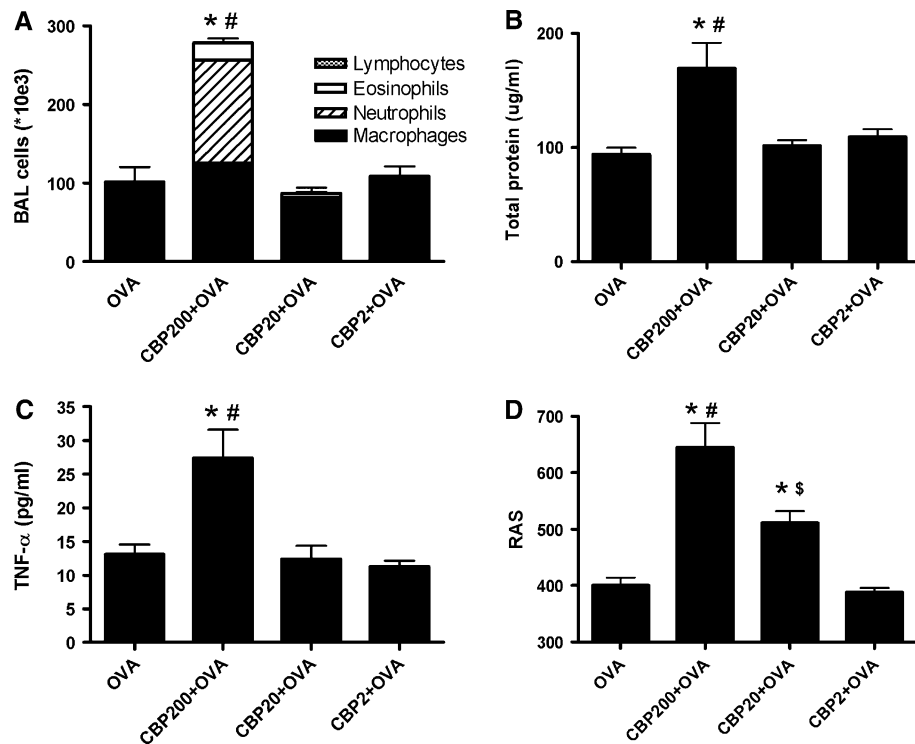


**FIG. 2.** The effects of different intranasal exposures on airway inflammation measured in BAL(F). Mice were exposed to 200  $\mu$ g, 20  $\mu$ g, or 2  $\mu$ g of carbon black particles (CBP) combined with OVA, or to OVA only. On day 8 the airways were lavaged with phosphate buffered saline (PBS). The total number of BAL cells and the contribution of different cell types were measured (A). Furthermore, total protein (B) and tumor necrosis factor (TNF)- $\alpha$  (C) were assessed in the BALF. The right angle scatter (RAS) of macrophage populations was analyzed by flow cytometry as a measure of CBP phagocytosis. In all groups  $n = 6$ , \*Significantly different from the OVA control ( $p < 0.005$ ), #Significantly different from CBP 20  $\mu$ g and 2  $\mu$ g ( $p = 0.005$ ). \$Significantly different from CBP 2  $\mu$ g ( $p < 0.002$ ).

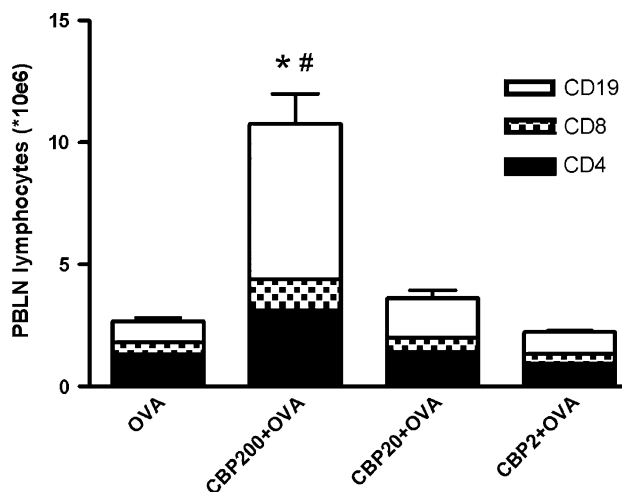


**FIG. 3.** Peribronchial lymph node (PBLN) cells of mice intranasally exposed to OVA or different concentrations of CBP+OVA on day 8. The PBLN were isolated and single cell suspensions were prepared. Then, total cell numbers were counted, and the contribution of different cell types was analyzed by flow cytometry. Bars represent mean  $\pm$  SEM. (\* $p = 0.004$  in comparison with OVA only groups and \*\* $p < 0.02$  in comparison with the 20  $\mu$ g and 2  $\mu$ g CBP+OVA groups  $n = 6$ ).

Th2-associated cytokines IL-4, IL-5, IL-10, and IL-13, with the levels being 10–200 times higher in 200  $\mu$ g CBP+OVA exposures than in 2- $\mu$ g and 20- $\mu$ g CBP+OVA exposures. The Th1-associated cytokine IFN- $\gamma$  was only significantly increased in mice exposed to 200  $\mu$ g CBP+OVA. When the different concentrations of CBP+OVA are compared, we see that, for all cytokines measured, there is no difference between the 2  $\mu$ g and 20  $\mu$ g, whereas levels after 200  $\mu$ g CBP+OVA exposure are all significantly increased compared to the 2  $\mu$ g and 20  $\mu$ g exposures.

Because the 2  $\mu$ g and 20  $\mu$ g CBP did not increase the total number of lymph node cells, or any of the measured lymphocytes subtypes and showed a very limited production of cytokines, we assume that these concentration do not have adjuvant activity.

#### CBP+OVA Sensitization Induces Systemic IgE Production

To further study local and systemic sensitization only the 200- $\mu$ g CBP dose combined with OVA and OVA alone were used. OVA-specific IgE levels were measured on day 21, and again on day 28 (after intranasal challenges at days 25, 26, and 27). Figure 4 shows that OVA-specific IgE levels were significantly ( $p = 0.035$ ) increased at day 21 in serum of

TABLE 1  
Cytokine Production after Restimulation with OVA (day 8)

| Organ | Treatment  | IL-4 (pg/ml)      | IL-5 (pg/ml)         | IL-10 (pg/ml)        | IFN- $\gamma$ (pg/ml) |
|-------|------------|-------------------|----------------------|----------------------|-----------------------|
| PBLN  | OVA        | 0.4 $\pm$ 0.1     | 0.2 $\pm$ 0.2        | 1.1 $\pm$ 0.7        | 0.8 $\pm$ 0.8         |
|       | CBP2+OVA   | 2.0 $\pm$ 1.0*    | 60.6 $\pm$ 31.0*     | 30.6 $\pm$ 20.7*     | 0.1 $\pm$ 0.1         |
|       | CBP20+OVA  | 3.4 $\pm$ 0.6*    | 55.9 $\pm$ 30.9*     | 43.7 $\pm$ 19.4*     | 0.2 $\pm$ 0.2         |
|       | CBP200+OVA | 25.2 $\pm$ 8.7*** | 719.8 $\pm$ 217.6*** | 700.8 $\pm$ 250.1*** | 16.2 $\pm$ 8.0***     |

Note. Data are presented as mean value  $\pm$  SEM. OVA: ovalbumin; PBLN: peribronchial lymph nodes; IL: interleukin; INF: interferon; CBP: carbon black particles.

\*Significantly different from OVA group,  $p \leq 0.05$  ( $n = 6$ ).

\*\*Significantly different from CBP 2  $\mu$ g and 20  $\mu$ g groups,  $p \leq 0.01$  ( $n = 6$ ).

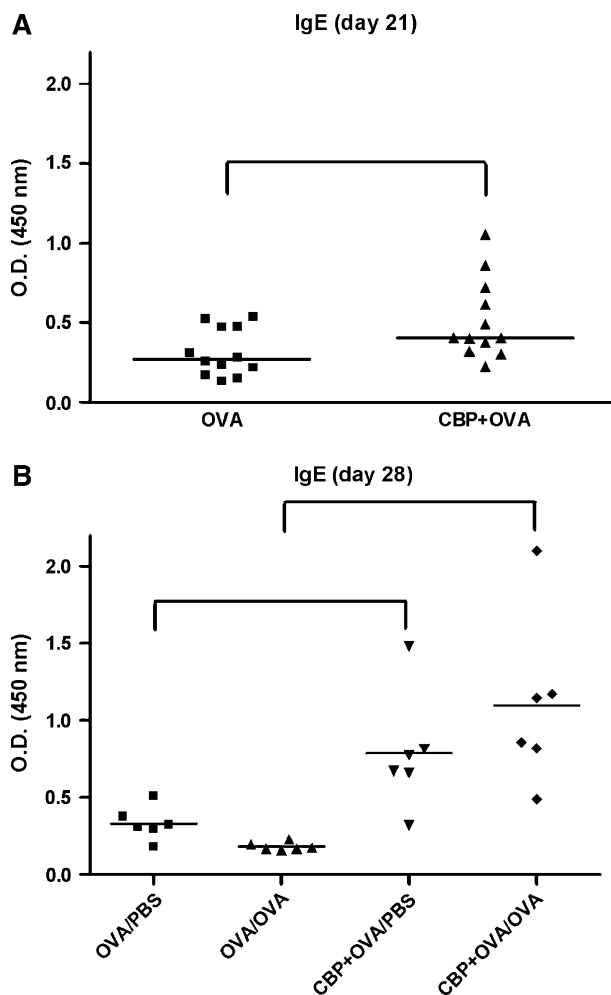


FIG. 4. Mice were intranasally exposed to OVA or 200  $\mu$ g CBP+OVA on days 1, 2, and 3, and OVA-specific IgE antibody levels were measured in serum on day 21 (A). Next, mice were intranasally challenged with PBS or OVA on days 25, 26, and 27. Twenty-four hours later (day 28) OVA-specific IgE antibody levels were measured in serum (B). Data are expressed as O.D. values using a 1:8 dilution for all serum samples. Individual and mean values are shown. Brackets represent significant differences between IgE levels of CBP+OVA and OVA only sensitized mice ( $p < 0.05$  in comparison to OVA sensitization  $n = 6$ ).

CBP+OVA-exposed mice (Fig. 4A). The IgE levels of mice sensitized with CBP+OVA were even higher on day 28 ( $p < 0.02$ ) (Fig 5B), whereas OVA-specific IgE levels were not significantly different after a challenge with PBS or OVA (Fig 5B).

#### *PBLN and Lung Lymphocytes Are Stimulated after Intranasal OVA Challenge*

The PBLN also play an important role during immune challenge, because in these lymph nodes clonal expansion of memory T cells takes place, and these cells subsequently migrate to the airways as effector T cells (Harris *et al.*, 2002). Challenge with OVA caused an increase in PBLN cell numbers in both OVA ( $p = 0.02$ ) and CBP+OVA ( $p = 0.02$ ) sensitized mice when compared to their respective control groups (OVA/PBS and CBP+OVA/PBS (Fig. 5A). Both CD4 and CD8 populations were increased in both groups, yet the CD19 population was the major contributor to the increased total cell number (Fig. 5A).

The cytokine profile after challenge matched the skewed profile of the 200- $\mu$ g CBP+OVA-dosed group observed at day 8, but with even more pronounced and higher levels of Th2 cytokines IL-4, IL-5, and IL-10 compared to IFN- $\gamma$  (Table 2). Like the IgE-levels, PBLN cell numbers in mice treated with CBP+OVA and challenged with PBS were higher than those of mice treated with OVA and challenged with PBS. In both treatment groups, the cytokine production by PBLN cells after PBS challenge was very low. Only the levels of IL-5 were significantly ( $p < 0.021$ ) higher in the CBP+OVA/PBS group compared to the OVA/PBS groups (Table 2).

After expansion in the PBLN, effector T cells are able to migrate to the lungs, where they can be reactivated by local antigen-presenting cells. Figure 5B shows that the number of lung lymphocytes was significantly higher in CBP+OVA/OVA-treated animals than in OVA/OVA-treated or CBP+OVA/PBS-treated mice ( $p = 0.029$ ), and that no differences were found between OVA/PBS and OVA/OVA, or between OVA/PBS and CBP+OVA/PBS treatments, respectively. The higher lung lymphocyte numbers were caused

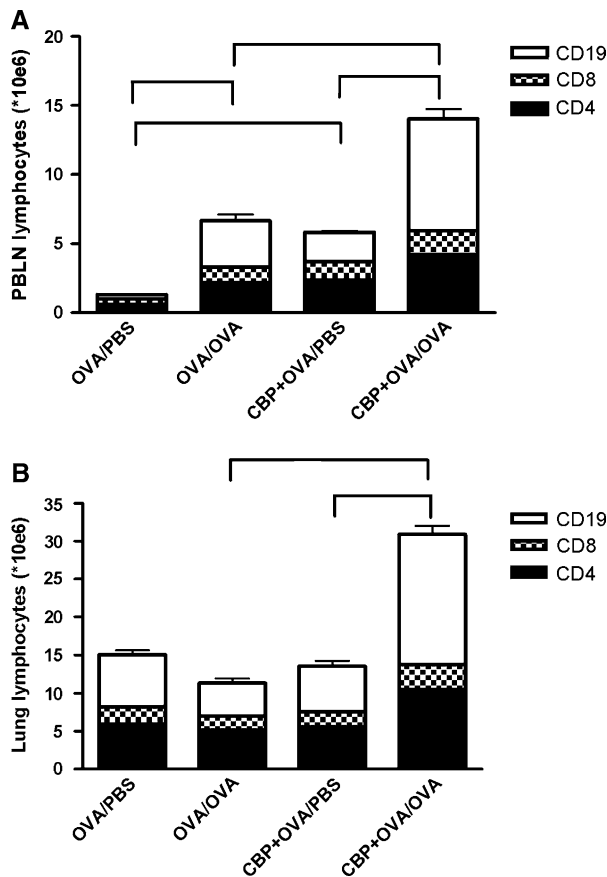


FIG. 5. Peribronchial lymph nodes and lung lymphocytes in mice, which were sensitized by intranasal application of OVA or 200 µg CBP+OVA and intranasally challenged with PBS or OVA on days 25, 26, and 27. Depicted are the total number of lymphocytes and the contribution of the different subtypes in the PBLN (A) and lung (B) as detected 24 h after the last challenge. Brackets represent significant differences ( $p < 0.05$ ,  $n = 12$  (day 21) or  $n = 6$  [day 28]).

mainly by higher numbers of CD4 and CD19 (2–3 times increased). Lung lymphocytes from both OVA- and CBP+OVA-treated mice produced clearly higher levels of cytokines after OVA challenge than after PBS challenge

(Table 2), but in the case of CBP+OVA, the production of IL-5 and IL-10 was four to five times higher than in OVA-treated mice. In both OVA-challenged groups the cytokine profile was reminiscent of the Th2-skewed immune response (Table 2). When the production of cytokines was compared between PBLN and lung lymphocytes, the profile was comparable, although the increases in cytokine production by PBLN were substantially higher.

#### Intranasal Challenge with OVA Induces Asthma-Like Airway Inflammation in CBP+OVA-Sensitized Mice

Antigen-specific airway challenge of Th2-sensitized mice is known to induce strong eosinophilic airway inflammation (van Rijt *et al.*, 2002). The total number of cells in the BAL was increased tenfold ( $p = 0.019$ ) in mice sensitized with CBP+OVA and challenged with OVA (Fig. 6A). Although all cell types were increased, the eosinophils were increased the most in the CBP+OVA/OVA-treated mice, with cell numbers that were 50 times higher compared to all other groups. The airway inflammation was also confirmed by histological examination, showing perivascular and peribronchial infiltrates, and goblet cell hyperplasia in CBP+OVA/OVA-treated mice (Fig. 6E), whereas the other groups showed no histological changes (Fig. 6B–D). Together, our findings indicate that antigen-specific inflammation with predominantly eosinophilic influx is induced only in CBP+OVA-sensitized mice.

While CBP containing macrophages were still observed in CBP+OVA-sensitized mice on day 28 (Fig. 6D black arrows), no influx of inflammatory cells was observed, confirming that the CBP+OVA-induced direct airway inflammation was resolved.

## DISCUSSION

Whereas toxic and inflammatory lung effects of particle exposure, as well as adjuvant effects of particles, have been studied extensively (Brown *et al.*, 2001; Gilmour *et al.*, 2004;

TABLE 2  
Cytokine Production after Restimulation with OVA (day 28)

| Organ | Treatment   | IL-4 (pg/ml)         | IL-5 (pg/ml)        | IL-10 (pg/ml)       | IFN- $\gamma$ (pg/ml) |
|-------|-------------|----------------------|---------------------|---------------------|-----------------------|
| PBLN  | OVA/PBS     | 1.4 $\pm$ 0.6        | ND                  | ND                  | ND                    |
|       | OVA/OVA     | 4.3 $\pm$ 0.4*       | 32.0 $\pm$ 32.0     | 18.3 $\pm$ 18.3     | ND                    |
|       | CBP+OVA/PBS | 0.2 $\pm$ 0.2        | 25.8 $\pm$ 9.23**   | ND                  | ND                    |
|       | CBP+OVA/OVA | 188.4 $\pm$ 115.5*** | 3821 $\pm$ 1049***  | 4374 $\pm$ 1955***  | 24.20 $\pm$ 19.1***   |
| Lung  | OVA/PBS     | 2.8 $\pm$ 1.3        | 247.4 $\pm$ 36.7    | ND                  | ND                    |
|       | OVA/OVA     | 22.5 $\pm$ 7.5*      | 1040 $\pm$ 343.3*   | 254.9 $\pm$ 85.4*   | 15.8 $\pm$ 9.4        |
|       | CBP+OVA/PBS | 4.8 $\pm$ 1.2        | 108.8 $\pm$ 70.8    | ND                  | 30.9 $\pm$ 8.9**      |
|       | CBP+OVA/OVA | 35.2 $\pm$ 5.3*      | 5198 $\pm$ 557.3*** | 1056 $\pm$ 121.3*** | 50.7 $\pm$ 17.6       |

Note. Data are presented as mean value  $\pm$  SEM. PBS: phosphate buffered saline; ND: below detection limit.

\*Significantly different from PBS challenge,  $p \leq 0.032$  ( $n = 6$ ).

\*\*Significantly different from OVA sensitization,  $p \leq 0.021$  ( $n = 6$ ).

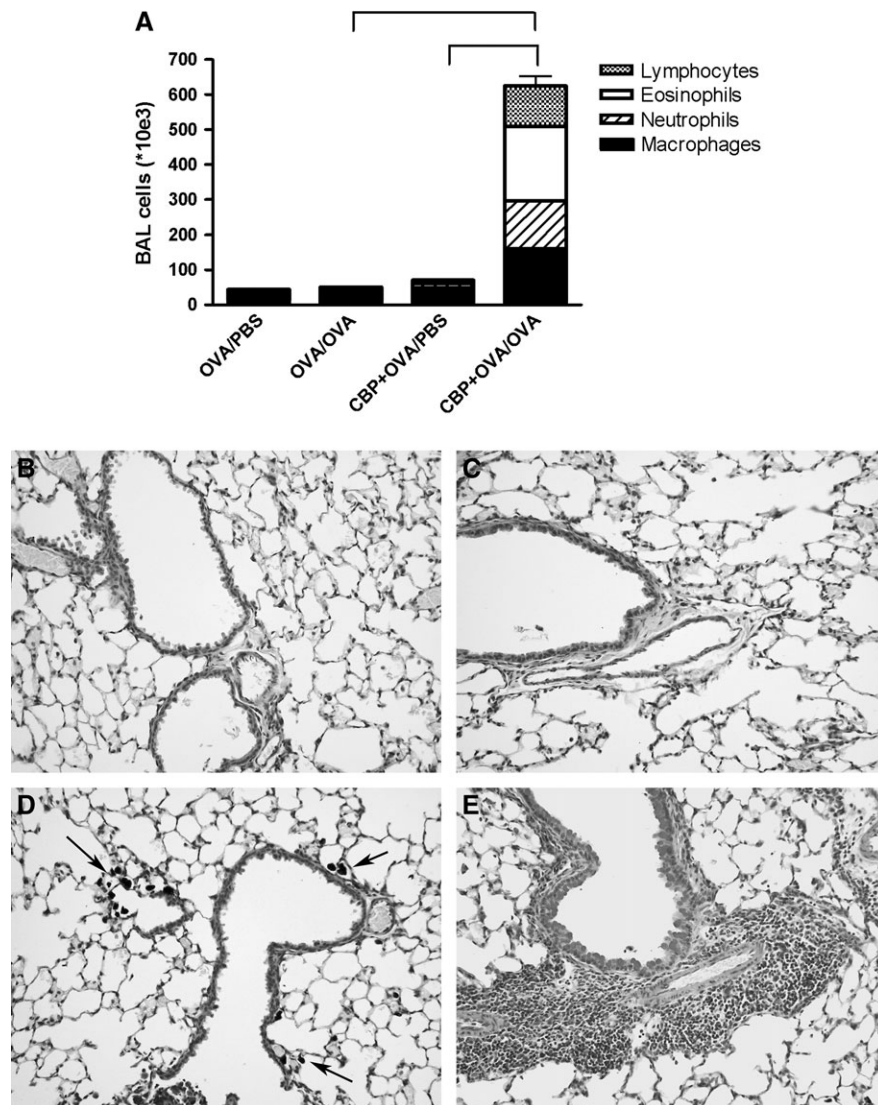


**FIG. 6.** Allergic airway inflammation was measured by flow cytometry on BAL and morphologic analyses of lungs. Mice were sensitized by intranasal application of OVA or 200  $\mu$ g CBP+OVA and intranasally challenged, using either PBS or OVA, on days 25, 26, and 27. Twenty-four hours after the last challenge, BAL cell numbers were counted and the inflammatory cell subtypes were analyzed by flow cytometry using GR-1 and CD11c staining. Brackets represent significant differences ( $p < 0.05$ ,  $n = 6$ ). To examine lung histology, lungs were fixed in formalin and embedded in paraffin, and histological sections were stained with periodic acid–Schiff (PAS) and photographed at 100 $\times$  magnification. Mice exposed to OVA/PBS (B) and OVA/OVA (C) appear normal. CBP+OVA/PBS (D) shows CBP-containing macrophages (black arrows) but no inflammation, whereas CBP+OVA/OVA induced strong airway inflammation with hyperplasia of goblet cells and infiltrates of inflammatory cells around bronchi and blood vessels.

Nilsen *et al.*, 1997; Takafuji *et al.*, 1987; van Zijverden *et al.*, 2001), only a few studies showed a possible link between particle-induced toxicity, local inflammation, and adjuvant activity (Lambert *et al.*, 1999). Because induction of local airway damage and inflammation would provide the signals (Gallucci and Matzinger, 2001) that the immune system needs to be fully activated, the inflammatory potential of particles may be a crucial factor in particle adjuvant activity. To investigate whether particle-induced airway damage and inflammation are involved in CBP adjuvant activity, we examined the kinetics of particle-induced airway inflammation and

immune sensitization to OVA using different concentrations of ultrafine CBP. For these studies we used intranasal exposures based on previous studies that have shown systemic sensitization after combined particle and antigen exposures (Nilsen *et al.*, 1997; van Zijverden *et al.*, 2001). Results show that, one day after the last sensitization treatment (day 4), intranasal exposure to 200  $\mu$ g CBP combined with OVA induces an immediate inflammatory response. Four days later (day 8) the airway inflammation was still detectable, PBLN cell numbers were increased, and an apparent type 2-skewed immune responses against the co-administered OVA was detected by

OVA-induced cytokine production *ex vivo*. Lower concentrations of CBP combined with OVA used in the sensitization studies did not induce airway inflammation, did not increase PBLN total cell numbers or lymphocyte subtypes, and induced very limited levels of cytokines compared to the 200- $\mu$ g CBP+OVA dose. We therefore used the 200- $\mu$ g CBP+OVA dose during sensitization to further study systemic sensitization and allergic airway inflammation after antigen-specific airway challenge. This dose of CBP+OVA caused increased levels of OVA-specific IgE on day 21. A challenge of the animals with OVA induced allergic airway inflammation, which was characterized by strong eosinophilia. Animals that received a vehicle challenge (PBS) showed no airway inflammation, confirming that the inflammation was not an ongoing inflammatory response induced during sensitization, but antigen-induced allergic airway inflammation.

Various rat studies have shown that CBP induce acute airway inflammation early after intratracheal exposure (Brown *et al.*, 2000; Gilmour *et al.*, 2004). The inflammation in these studies was characterized by increased levels of LDH, total protein, TNF- $\alpha$ , neutrophils, eosinophils, and lymphocytes in the BALF. In the present study, 200- $\mu$ g CBP+OVA exposure also caused an increase in LDH, total protein levels, and inflammatory cells, all findings indicative of airway damage-induced local inflammation. Because only the 200- $\mu$ g CBP+OVA concentration induced local inflammation and had immune adjuvant activity, our data confirm data reported by other investigators (Lambert *et al.*, 1999; Saxon and Diaz-Sanchez, 2005; Whitekus *et al.*, 2002) and suggest that airway damage plays an important role in the adjuvant activity of air pollution.

Whereas, the 200- $\mu$ g dose of CBP may be considered high compared environmental exposures to particles in humans, toxicity and induction of inflammation of the airways is shown in humans after exposure to particles (Ghio and Huang, 2004). The suggested role of particle toxicity on particle adjuvant activity in animal studies may therefore be one possible mechanism of increased allergic disease at sites with high air pollution.

Various different particle characteristics like polycyclic aromatic hydrocarbons (PAHs) (Diaz-Sanchez *et al.*, 1997), transition metals (Lambert *et al.*, 2000), surface area (Nygaard *et al.*, 2004), oxidative potential (Whitekus *et al.*, 2002), and biologics (Soukup and Becker, 2001) are known to influence airway responses to particles and adjuvant activity. Because the ultrafine CBP used in the present studies does not contain PAHs or biologics, and because transition metals are probably not involved in the CBP toxicity (Brown *et al.*, 2000), we suggest that physicochemical particle characteristics like surface area, charge, and oxidative potential may be involved.

Based on present findings, we suggest that particles like CBP induce airway hypersensitivity to bystander antigens as follows. Initially, inhaled particles such as CBP reach and interact with the lung epithelial cells and alveolar macrophages depending on

their size and charge. Ultrafine particles that have oxidative potential can directly damage these cells if the antioxidant effect of the epithelial lining fluid falls short (Greenwell *et al.*, 2002). Apart from directly damaging the cells, uptake of the particles can also activate epithelial cells and alveolar macrophages to produce reactive oxygen species, activate nuclear factor kappa B (NF $\kappa$ B) and synthesize and secrete proinflammatory cytokines like TNF- $\alpha$  (Lambert *et al.*, 2001; Li *et al.*, 2003; Tao and Kobzik, 2002). Disruption of the epithelial barrier, which will further increase in response to increased levels of proinflammatory cytokines (Coyne *et al.*, 2002), will allow antigens like OVA to become more easily available to antigen-presenting dendritic cells (Lambert *et al.*, 1999).

Dendritic cells are constantly taking up antigen from the airways. After uptake of the antigen, a portion of the dendritic cells will migrate to the local lymph node to present the antigen to naïve T cells, without fully maturing. Although the presentation of antigen by dendritic cells is enough to induce some T-cell proliferation, it will normally lead to the induction of tolerance. Increased levels of co-stimulation are needed to induce immune sensitization against the presented antigen (Lambrecht and Hammad, 2003). Increased migration, maturation of dendritic cells, including increased MHCII antigen presentation and upregulation of co-stimulatory molecules, is induced by proinflammatory cytokines (Banchereau and Steinman, 1998). This finding is supported by a study in which co-exposure to TNF- $\alpha$  and antigen induced a state of immune sensitization comparable to that observed after exposure to ROFA and antigen (Lambert *et al.*, 2001). The importance of oxidative stress and ensuing toxicity is strengthened by studies showing that particle-induced TNF- $\alpha$  release from macrophages *in vitro* (Brown *et al.*, 2004) and inflammatory responses *in vivo* (Dick *et al.*, 2003) can be partially inhibited by antioxidants. Another study (Whitekus *et al.*, 2002) demonstrated that the adjuvant effect of DEP on IgE production could also be partially inhibited by antioxidants. Whereas the depot function is not mentioned in the mechanism described here, it may make an important contribution to the immune adjuvant potential. Both the concentration of antigen and its prolonged presence may help to further increase the response.

In the present study the early inflammation in the airways, after the 200- $\mu$ g CBP+OVA exposure was soon followed by a type 2-skewed immune sensitization. This type 2-skewed response was apparent from cytokine production profiles in the PBLN, both early after induction of sensitization (day 8) and again after challenge (on day 28). Interestingly, although the increase in cytokine levels was less pronounced on day 8 than on day 28, the Th2 cytokine profiles on those days were comparable. Using the mouse model described here, the cytokine profile observed in PBLN cells *ex vivo* stimulated with OVA, apparently may be used early after sensitization to predict the skewing of the immune response that may eventually develop.

Our data with regard to cytokine production by lymphocytes from PBLN and lungs demonstrate and confirm data reported by others that airway exposure to particles and antigens elicits a Th2 cytokine profile (Fujimaki *et al.*, 1994). Locally produced cytokines are important for the clinical manifestations of the allergic airway inflammation. Taking this view, it is interesting that lung lymphocytes isolated from mice sensitized with CBP+OVA and challenged with OVA produce high amounts of the Th2 cytokines, in particular, IL-5. Notably IL-5 is crucial in eosinophilic airway inflammation (Hamelmann *et al.*, 2000). In addition and intriguingly, in time, lung lymphocytes produce more IL-10 than IL-5 (and IL-4). Whereas IL-10 is often regarded as a Th2 cytokine and is shown to be critical for the development of asthma-like responses like IL-5 production, eosinophilia, and mucus secretion (Yang *et al.*, 2000), IL-10 is also known to have regulatory effects, especially on different innate cells such as macrophages. Moreover, IL-10 is shown to inhibit particle-induced cytokine production from macrophages (Im and Han, 2001). Both IL-10 effects mentioned here may be involved in our model, helping the induction of a Th-2 response (early after CBP+OVA exposure) and possibly inhibiting innate immune cells at the effector phase of the allergic response.

In conclusion, the current kinetic data on particle-induced inflammation and immune sensitization suggest a causal relation between CBP toxicity and immune adjuvant activity. Furthermore, the cytokine profile on day 8 in the airways, in particular in the PBLN, seems predictive of the allergic airway sensitization. This has important implications not only for studying underlying mechanisms, but also for designing assays to predict the sensitizing potential of particles.

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