

OPTIMIZATION OF ROUTE OF ADMINISTRATION FOR COEXPOSURE TO OVALBUMIN AND PARTICLE MATTER TO INDUCE ADJUVANT ACTIVITY IN RESPIRATORY ALLERGY IN THE MOUSE

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Epidemiological and experimental studies have not only shown that air pollution induces increased pulmonary morbidity, and mortality, but also that air pollution components may potentiate allergic responses. The respiratory allergy model to ovalbumin in the mouse has been shown a useful tool to characterize the adjuvant potency of air pollution components. However, the choice for the most effective route of administration for testing small amounts of air pollution component is hampered by the diversity of routes of administration used. To test the adjuvant activity of airborne particles (Ottawa dust EHC-93), we studied the optimal route of respiratory administration: intranasally (in) and aerosol (aero) in comparison with responses observed by intraperitoneal (ip) with diesel exhaust particles (DEP) as a positive control. Our results show that the combination of in/aero with ovalbumin caused almost similar immunoglobulin (Ig)E and inflammatory responses compared to the ip/aero. In/in application induced less responses for IgE, less inflammation in the lung, and less increased numbers of eosinophils in the bronchoalveolar lavage (BAL). This response increased dramatically when ovalbumin was coadministered with DEP. Subsequently, EHC-93, which is made up of airborne particles, was tested via the in/in route of administration. EHC-93 induced similar IgE responses, inflammation, and eosinophilic response in BAL compared to DEP. In addition, EHC-93 increased the airway responsiveness of the ovalbumin-sensitized mice measured in unrestrained condition and not in nonsensitized control mice. It is concluded that intranasal sensitization with intranasal challenge with airborne particles (EHC-93) is an effective route of administration to show potency of adjuvant activity of airborne particles.

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For the last decade, interest in particulate matter has increased due to concern about decreased life expectancy (Brunekreef et al., 1995; Dockery et al., 1993; Dockery & Pope, 1994; Saldiva et al., 1995; Wordley et al., 1997; Schwartz & Neas, 2000) and increased prevalence of allergy and asthma. The latter concern cumulated in a number of epidemiological studies and studies in experimental animal models (D'Amato, 2002; Hajat et al., 2001; Kramer et al., 2000; Rusznak et al., 1997).

A predominant particle in air pollution is diesel exhaust particles (DEP), which have been subject of many studies (D'Amato, 2002; Hajat et al., 2001; Kramer et al., 2000; Rusznak et al., 1997). More recently, ambient airborne particles also became the subject of experimental studies (Cassee et al., 2002; Godleski et al., 2000; Goldsmith et al., 2002; Kodavanti et al., 2000; Saldiva et al., 2002). For these studies, particles have been recovered from filters of compactors (Chang et al., 2000) or collected by an ambient fine particulate concentrator (AFPC) (Sioutas et al., 1995; Goldsmith et al., 2002). The adjuvant activity in allergic responses of these particles has been studied most intensively in the ovalbumin model. However, many of these studies show a diversity of routes of administration. The intraperitoneal (Miyabara et al., 1998; Suzuki et al., 1993; Heo et al., 2001), intranasal (Takafuji et al., 1987; Fujimaki et al., 1997; Nilsen et al., 1997), aerosol (Takano et al., 1997; Goldsmith et al., 2002), intratracheal (Ichinose et al., 1997; Al Humadi et al., 2002), and subcutaneous (van Zijverden et al., 2000; Lovik et al., 1997) routes of administration and their combinations have all been used.

In a number of European studies, airborne particles are being studied for a variety of characteristics. Therefore, collected airborne particles have been distributed over various laboratories, making the samples too small for aerosol application. Moreover, for studies on the adjuvant activity of air pollution components the respiratory tract is the preferential route, as the port of entry for allergens and air pollution is the airways. For the present study we made an inventory of the routes of administration using diesel exhaust particles (DEP) (Ghio et al., 2000) to compare the adjuvant potential of particles in the ovalbumin model. To test the outcome of this study we finally examined the well-known ambient air particles of Ottawa dust (EHC-93) (Fujii et al., 2001; Prieditis & Adamson, 2002; Ulrich et al., 2002; Vincent et al., 2001), which has been proven to induce cytokine release by bronchial epithelial cells, acute lung toxicity, inflammatory responses, and cardiovascular effects, but has not yet been investigated for the potency of adjuvant activity in respiratory allergic responses.

This investigation was performed in the framework of an EU study entitled Respiratory Allergy and Inflammation due to Ambient Particles (RAIAP)* in which EHC-93 will be used as a positive standard for testing ambient particles from four European cities on adjuvant activity on allergy responses.

*See <http://www.raiap.org> for additional information on this EU project.

MATERIALS AND METHODS

Animals

Male BALB/cByJ.ico mice 6 to 8 wk old were obtained from Charles River, L'Ardresle-Cedex, France. The animals were housed in Macrolon Type II and III cages. Food pellets and tap water were available ad libitum, and all animals were allowed to adapt for at least 1 wk before the experiments started. All experiments were performed according to international and institutional guidelines for animal care.

Allergens Sensitization and Challenge

Ovalbumin The mice were placed at random in groups ($n = 4-8$). For intraperitoneal (ip) sensitization the mice were injected with 20 μ g ovalbumin emulsified in 2.25 mg aluminum hydrochloride in a total volume of 100 μ l at day 0. For the challenge on days 35, 38, and 41, mice were exposed for 20 min to nebulized ovalbumin solution (aerosol 1%, aero) in 7 ml phosphate-buffered saline (PBS) or PBS as a control using an ultrasonic nebulizer. For intranasal (in) sensitization, mice were slightly anesthetized with halothane at days 0 and 14. At the moment of awakening, 0.4 mg/ml ovalbumin (grade V, Sigma Chemical, St. Louis, MO) in 50 μ l saline was administered into both nostrils (2, 20, and 40 μ g/mouse). For the challenge on days 35, 38, and 41, mice were again intranasally exposed to ovalbumin at the same concentration or exposed to nebulized ovalbumin solution (0.1 and 1.0% aero). To study the effects of particles, the suspension of particles and ovalbumin were mixed at a final concentration of 0.4 mg/ml ovalbumin and 3 mg/ml particles. In case of aero or ip injection, DEP was given separately by intranasal application. Animals were sacrificed at day 42 (see Figure 1).

Particles DEP originated from Dr. J. Mauderly, Lovelace Respiratory Research Institute, Albuquerque, NM. This DEP was generated by 1980 model 5.7-L Oldsmobile V-8 engines mounted on test stands simulating a mid-sized passenger car. Ottawa dust (EHC-93) was a gift of Dr. R. Vincent (Environmental and Occupational Toxicology Division, Environmental Health Directorate, Health Canada, Ottawa, Ontario, Canada (Ball et al., 2000)).

In a *Limulus* amoebocyte lysate (LAL) test (Limusate, Sigma Aldrich Chemie BV, Zwijndrecht, the Netherlands) as described by the manufacturer, the endotoxin content of the DEP suspension was below the detection level (<0.125 ng endotoxin/ml) except for EHC-93 (50 ng endotoxin/ml).

DEP and EHC-93 were weighed, suspended in phosphate-buffered saline (PBS, 3 mg/ml), and ultrasonicated with a Branson sonifier 250. Ovalbumin solution was added to this suspension (final concentration 0.4 mg ovalbumin/ml PBS) and the suspension was homogenized by vigorous shaking again.

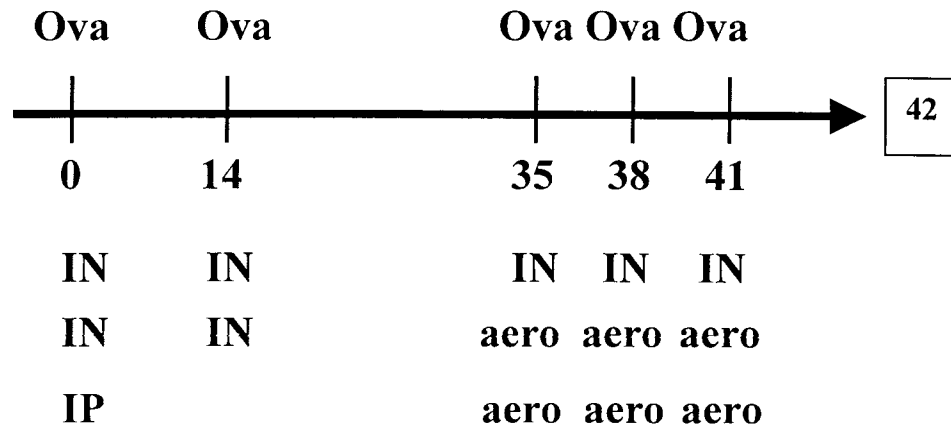


FIGURE 1. Sensitization and challenge protocols used.

Autopsy

At autopsy, animals were anesthetized with ketamine/Rompun [0.1 ml/100 g body weight of a mix of 0.85 ml of 100 mg/ml ketamine (Aesculaap, Boxel, the Netherlands) and 0.65 ml of 20 mg/ml Rompun (Bayer, Leverkusen, Germany) and blood was taken from the abdominal aorta. A cannula was inserted in the trachea, and lung lavage was performed with 1 ml PBS (37°C). The lungs were lavaged three times with the same fluid and identical volumes. Then the lungs were intratracheally fixed with formalin.

Bronchoalveolar Lavage

Fluid collected from the bronchoalveolar lavage (BAL) of each animal was centrifuged at $400 \times g$, 4°C, for 10 min. Cell-free fluid from the lavage was used for analysis of cytokines like interleukin-4 (IL-4), IL-5, and interferon- γ (IFN γ). Sediments were used for determination of total cell number (mixing 0.5 ml of the cell suspension with 9.5 ml isoton, Beckman Coulter BV, Mijdrecht, the Netherlands) and for producing cytopsin preparations on which differential cell counts were performed after staining with May-Grünwald Giemsa. For each cytopsin preparation, 400 cells were counted.

Serum Samples

After collection, blood was drawn and kept at 4°C for about 2–4 h for clotting. The blood samples were centrifuged for 15 min at $1200 \times g$ at 4°C. Serum was removed and stored in small aliquots at –20°C until use.

ELISA for Specific Ovalbumin IgE

The enzyme-linked immunosorbent assay (ELISA) were performed in serum according to Steerenberg et al. (1999, 2003). In short, the ELISA was performed in flat-bottom 96-well polystyrene microtiter plates with high binding capacity (MaxiSorp, number 439454, Nunc, Denmark, or number

655092, Greiner, Germany). All incubations were performed with 100 μ l aliquots. After adding a solution, the plates were covered and shaken for 30 s. Each incubation was followed by extensive washing (six cycles) on an automatic plate washer with PBS containing 0.1% Tween-20. The incubations at 37°C were performed in a thermostatically controlled incubator. A conjugate of ovalbumin–digoxigenin (DIG) solution was prepared by labeling the ovalbumin with DIG using a reagent kit (next subsection).

Microwells were coated overnight at 4°C with monoclonal rat anti-mouse immunoglobulin (Ig)E (Jackson, PA) in Na_2CO_3 buffer (0.1 M; pH 9.6). Blocking reagent [PBS containing 0.1% (v/v) Tween-20 and 1% (w/v) dried milk] was added and incubated for 1 h at 37°C. Mouse serum samples (1:4 diluted) were incubated for 1 h at 37°C in PBS. The binding of IgE was measured by incubating the ovalbumin extract–DIG solution (0.01 μ g/ml in blocking reagent) for 1 h at 37°C, followed by an incubation of anti-DIG Fab fragments *poly* labeled with peroxidase (PO; number 1633716, Boehringer Mannheim, Germany; 50 U/ml; 1:625), and incubated for 3 min at 20°C in the dark with tetramethylbenzidine/dimethyl sulfoxide (TMB/DMSO) substrate. The enzyme reaction was stopped by adding 50 μ l 2 M H_2SO_4 , and the optical density was read at 450 nm on a multichannel photometer. A pooled positive serum with antibodies to ovalbumin was derived from mice sensitized and challenged in a different way: intraperitoneal sensitization by injection with 20 μ g ovalbumin emulsified in 2.25 mg aluminum hydrochloride in a total volume of 100 μ l at day 0 and challenged on days 35, 38, and 41 by exposure to a nebulized ovalbumin solution [aerosol: 1% in 7 ml phosphate-buffered saline (PBS)] using an ultrasonic nebulizer for 20 min. The extinction value of this reference serum varied from minimal 1.4 to maximal 1.7 optical density. To compare the different experiments, extinction values of samples were expressed as percentage of the standard.

Labeling Proteins with DIG

Ovalbumin was labeled with digoxigenin 3-*O*-succinyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide ester (DIG-NHS) using a DIG antibody labeling kit (DIG antibody labeling kit, number 1367200, Boehringer, Mannheim, Germany). Nonreacted DIG-NHS was separated on a Sephadex *G-25 column. The labeled protein was collected in fractions of 1 ml. After determining the protein concentration (BCA protein assay kit, Pierce, Rockford, IL), the fractions with the highest protein concentration were pooled and the protein concentration was determined. The solution had a protein concentration of 80 mg/ml (ovalbumin–DIG) in PBS.

ELISA for Specific Ovalbumin IgG1 and Ig2a

Microwells were coated overnight at 4°C with 10 μ g ovalbumin/ml PBS (grade V, Sigma Chemical, St. Louis, MO). Blocking reagent [ethylenediamine tetraacetic acid (EDTA) buffer: 3.03 g Tris-aminomethane, 0.372 g EDTA, 14 g NaCl in 450 ml water, and adjusted for pH 7.2] was added for 60 min at

37°C. Mouse serum samples were diluted (IgG1 1:10⁶ and IgG2a 1:50) in 0.5% bovine serum albumin (BSA/PBS) and were incubated (with shaking) for 2 h at room temperature (RT). After washing the wells were incubated (shaking during 1.5 h at RT) with biotinylated rat monoclonal anti-mouse IgG1 (diluted 1:2000 in PBS) and IgG2a (diluted 1:6000 in 0.5%BSA/PBS) (Zymed, Laboratories, San Francisco CA). The wells were washed and incubated with streptavidin conjugated to poly-horseradish peroxidase (CLB, Amsterdam, the Netherlands) in 100 µl 0.5% BSA/PBS for 45 min (shaking) at RT. This was followed by washing of the wells and incubated for 3 min at 20°C in the dark with tetramethylbenzidine/dimethyl sulfoxide (TMB/DMSO) substrate. The enzyme reaction was stopped by adding 50 µl 2 M H₂SO₄, and the optical density was read at 450 nm on a multichannel photometer.

Interleukins

Interleukin (IL)-4, IL-5, and interferon gamma (IFN-γ) were purchased from R&D (ITK Diagnostics, Uithoorn, the Netherlands), and the assays were performed according to the manufacturers' description.

Histopathology

Lungs were fixed in 4% phosphate-buffered formaldehyde and embedded in paraplast. Five-micrometer sections were cut and routinely stained with hematoxylin and eosin (HE) and with May-Grünwald Giemsa. The histopathological lesions were semiquantitatively and blinded scored as absent (0), minimal (1), slight (2), moderate (3), marked (4), or strong (5). In this score both the incidence and the severity of the lesions were incorporated.

Determination of Airway Responsiveness

Airway responsiveness (AR) was measured in unrestrained mice by barometric plethysmography using whole-body plethysmography (Buxco, EMKA Technologies, Paris) as described by Hamelmann et al. (1997). In short, mice ($n = 5$) were placed in individual chambers and the baseline readings were taken and averaged for 3 min. Aerosolized PBS or methacholine (MCh) in increased concentrations (3 to 7.5 mg/ml) was nebulized during 3 min and the readings were taken and averaged for 3 min after nebulization. Airway reactivity was expressed as fold increase for each concentration of MCh (Penh_{MCh}) compared with the Penh values after PBS challenge (Penh_{PBS}). For the quantification of the dose response to methacholine, the linear regression of Penh on log base 2 was calculated for individual mice. The data are reported as the geometric mean with the lower and upper limit of 95% confidence interval.

Statistical Analysis

The induced serum titers were compared after sensitization and challenge with ovalbumin alone and with serum titers obtained after ovalbumin in combination with tested particles. The statistical differences between groups

were determined using an analysis of variance (ANOVA). To derive the 95% confidence interval of each parameter for each treatment group, Student's two-sample *t*-test was used. The BAL cell numbers, expressed as percentages, and the histological parameters were statistically tested with the Wilcoxon test. The data were expressed as means $\pm$ SEM (standard error). The result was considered significant when a *p* value $< .05$ was found.

RESULTS

Effect of Different Routes of Application of Ovalbumin on IgE Response and Lung Lesions

Mice ($n = 4$) ip sensitized (20 $\mu\text{g}/\text{mouse}$) and challenged by aero (1%) showed specific IgE to ovalbumin (Figure 2). Intranasal administration of ovalbumin (20 $\mu\text{g}/\text{mouse}$) during sensitization and aero challenge (20 $\mu\text{g}/\text{mouse}$) resulted in a similar IgE response (Figure 2). Intranasal administration of ovalbumin (20 $\mu\text{g}/\text{mouse}$) both in the sensitization and in the challenge phase induced less IgE in comparison to the previous described routes of administration (Figure 2). Histopathological examination showed the largest perivascular and peribronchiolar inflammation after ip/aero administration, less after in/in application and the least after in/aero administration (Table 1). The prominent inflammatory cell found after immunization was the eosinophilic granulocyte. Hypertrophy of the bronchiolar mucous cells was prominently present in the combination ip/aero.

Effect of DEP on the IgE Response and Lung Lesions After in or aero Application of Ovalbumin

Because the ip route is not the preferential route of administration for studies in the respiratory tract, a comparison was made between the in/aero and the in/in route of administration with different doses of ovalbumin while ovalbumin was coadministered with DEP (one concentration).

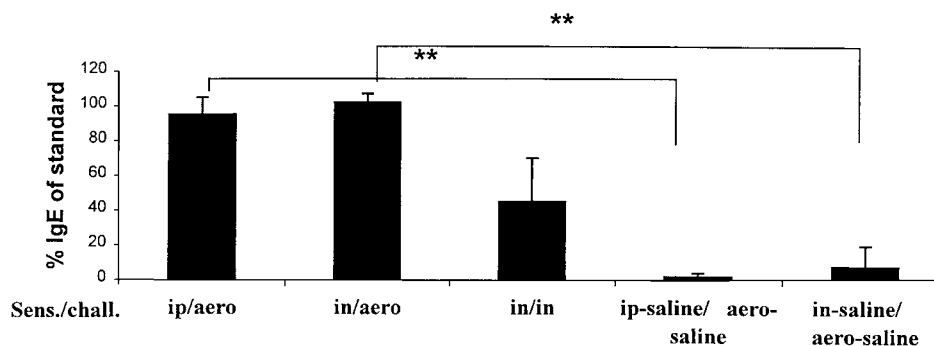


FIGURE 2. IgE response in mice after various routes of administration for sensitization (20 $\mu\text{g}/\text{mouse}$) and challenge of ovalbumin (aero, 1%; in, 20 $\mu\text{g}/\text{mouse}$). Statistically significant differences from saline exposed group: ** $p < .01$.

TABLE 1. Mean score ($\pm$ SEM) of histological lung lesions in mice exposed to ovalbumin by different routes of administration

Treatment	ip/aero	in/aero	in/in	PBS in/ PBS aero	PBS ip/ PBS aero
Peribronchiolar infiltrate	2.5 $\pm$ 0.3	0	0.8 $\pm$ 0.8	0	0
Perivascular infiltrate	0.8 $\pm$ 0.5	0	1.0 $\pm$ 1.0	0	0.3 $\pm$ 0.3
Hypertrophy mucous cells	2.8 $\pm$ 0.3	1.5 $\pm$ 0.3	1.3 $\pm$ 1.0	0.3 $\pm$ 0.3	0.3 $\pm$ 0.3
Eosinophils influx	0.5 $\pm$ 0.3	0.3 $\pm$ 0.3	0.5 $\pm$ 0.5	0	0

Note. Histological parameters were individually and quantitatively scored [absent (0), minimal (1), slight (2), moderate (3), marked (4), and strong (5)] and averaged per treatment group ($n = 4$); in, intranasal; ip, intraperitoneal; aero, aerosol.

Effect of DEP Via in/aero Intranasal sensitization of ovalbumin at a dose of 2 and 20 μ g/mouse in combination with an aero challenge of 0.1 and 1% concentration respectively induced a minimal IgE antibody response to ovalbumin (Figure 3). When ovalbumin was coadministered with DEP, a significant increase in IgE specific for ovalbumin was shown in the combination of 20 μ g intranasally and 1% ovalbumin aero. For this exposure the inflammatory response in the lungs revealed a strong inflammation compared to the ovalbumin alone (Table 2). DEP alone induced slight hypertrophy of mucous cells and slight alveolitis. The presence of eosinophils in the lung reflected the number of eosinophils in the BAL only in the 20- μ g group. The number of eosinophils in the BAL for mice treated with ovalbumin alone and ovalbumin + DEP was 0.6×10^4 and 13.9×10^4 /ml, respectively (not shown).

Effect of DEP Via in/in Administration of ovalbumin was studied at 3 concentrations: 2, 20, and 40 μ g/mouse, with and without coadministration

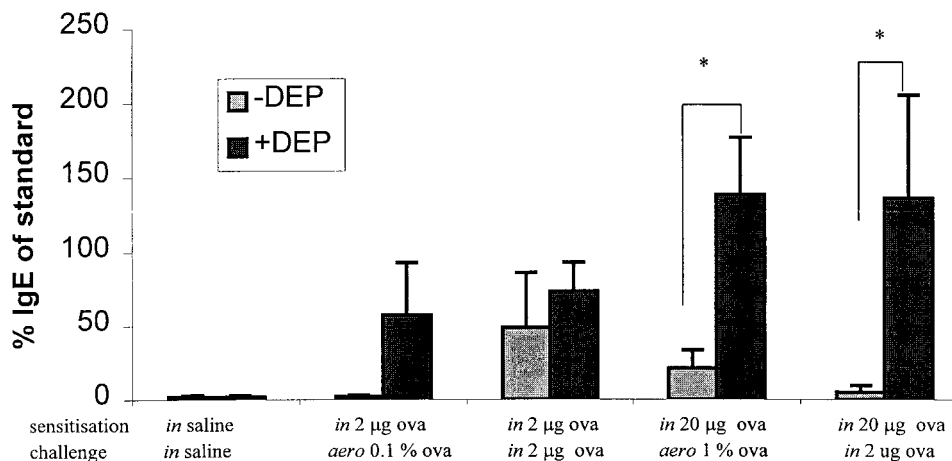
**FIGURE 3.** IgE response of mice intranasally sensitized and challenged with 2 or 20 μ g ovalbumin or in sensitized with 2 or 20 μ g ovalbumin and aero challenged with 0.1 or 1% ovalbumin, and coexposed to DEP (in/aero and in/in). Statistically significant differences from ovalbumin only exposed group: * $p < .05$.

TABLE 2. Mean score ($\pm$ SEM) of histological lung lesions in mice, intranasally (in) sensitized and aerosol (aero) challenged with different doses of ovalbumin, in combination with DEP

Sensitization, ovalbumin (in)	–	–	2 μ g	2 μ g	20 μ g	20 μ g
Sensitization, DEP (3 mg/ml)	–	+	–	+	–	+
Challenge, ovalbumin (aero)	–	–	0.1%	0.1%	1%	1%
Peribronchiolar infiltrate	0.2 $\pm$ 0.2	0.4 $\pm$ 0.3	1.2 $\pm$ 0.2 ^a	1.6 $\pm$ 0.3	1.6 $\pm$ 0.3 ^a	3.0 $\pm$ 0.4 ^a
Perivascular infiltrate	0.2 $\pm$ 0.2	1.4 $\pm$ 0.3 ^a	0.2 $\pm$ 0.2	2.0 $\pm$ 0.4 ^a	1.4 $\pm$ 0.7	4.2 $\pm$ 0.2 ^b
Hypertrophy mucous cells	0.4 $\pm$ 0.5	2.0 $\pm$ 0.0	0.8 $\pm$ 0.4	3.0 $\pm$ 0.0 ^b	2.2 $\pm$ 0.5	4.6 $\pm$ 0.3 ^b
Alveolitis	0	2.2 $\pm$ 0.4 ^b	0	2.4 $\pm$ 0.3 ^b	0.2 $\pm$ 0.2	2.6 $\pm$ 0.5 ^a
Quantity of DEP	0	3.6 $\pm$ 0.5	0	3.6 $\pm$ 0.6	0	4.0 $\pm$ 0.6
Eosinophils influx	0	0	0	0.8 $\pm$ 0.4	1.0 $\pm$ 0.5	2.4 $\pm$ 0.5

Note. Histological parameters were individually and quantitatively scored [absent (0), minimal (1), slight (2), moderate (3), marked (4), and strong (5)] and averaged per treatment group ($n = 4$); in, intranasal; aero, aerosol. Statistical analysis: control group versus DEP, OVA (2 μ g) and OVA (20 μ g); group OVA (2 μ g) versus OVA (2 μ g) + DEP; group OVA (20 μ g) versus group OVA (20 μ g) + DEP.

^aSignificant at $p < .05$.

^bSignificant at $p < .01$.

of DEP. A concentration of 20 μ g showed the largest difference in IgE response between ovalbumin and ovalbumin coadministered with DEP (data not shown). Histopathological examination showed a strong inflammatory response that was most prominent in the 20- μ g and 40- μ g groups (Table 3; 40- μ g group not shown). No influx of eosinophils was found after administration of DEP alone. Moreover, DEP did not induce hypertrophy of mucous cells and alveolitis. The presence of eosinophils in the lung reflected the number of eosinophils in the BAL (only the 20- μ g group was examined). The number of eosinophils in the BAL was 0.5×10^4 for mice treated with ovalbumin alone, and $23.8 \times 10^4/\text{ml}$ for ovalbumin + DEP (Figure 4).

TABLE 3. Mean score ($\pm$ SEM) of histological lung lesions in mice, intranasally exposed to different doses of ovalbumin in combination with DEP

Sensitization, ovalbumin (in)	–	–	2 μ g	2 μ g	20 μ g	20 μ g
Sensitization, DEP (3 mg/ml)	–	+	–	+	–	+
Challenge, ovalbumin (in)	–	–	2 μ g	2 μ g	20 μ g	20 μ g
Peribronchiolar infiltrate	0.4 $\pm$ 0.4	0	2.0 $\pm$ 0.6	2.8 $\pm$ 0.2	1.0 $\pm$ 0.5	3.0 $\pm$ 0.4 ^a
Perivascular infiltrate	0.8 $\pm$ 0.4	1.8 $\pm$ 0.2	2.0 $\pm$ 0.6	3.6 $\pm$ 0.3 ^a	1.6 $\pm$ 0.3	4.2 $\pm$ 0.7 ^a
Hypertrophy mucous cells	0.6 $\pm$ 0.7	0	1.8 $\pm$ 0.7	3.0 $\pm$ 0.0	1.0 $\pm$ 0.5	3.2 $\pm$ 0.4 ^a
Alveolitis	0.8 $\pm$ 0.7	0	0	0	0	1.8 $\pm$ 0.5
Eosinophils influx	0	0	1.2 $\pm$ 0.7	1.2 $\pm$ 0.2	0.2 $\pm$ 0.2	4.0 $\pm$ 0.4 ^b

Note. Histological parameters were individually and quantitatively scored [absent (0), minimal (1), slight (2), moderate (3), marked (4), and strong (5)] and averaged per treatment group ($n = 4$); in, intranasal. Statistical analysis: control group vs. DEP, 2 and 20 μ g OVA; OVA (2 μ g) vs. OVA (2 μ g) + DEP; OVA (20 μ g) vs. OVA (20 μ g) + DEP.

^aSignificant at $p < .05$.

^bSignificant at $p < .01$.

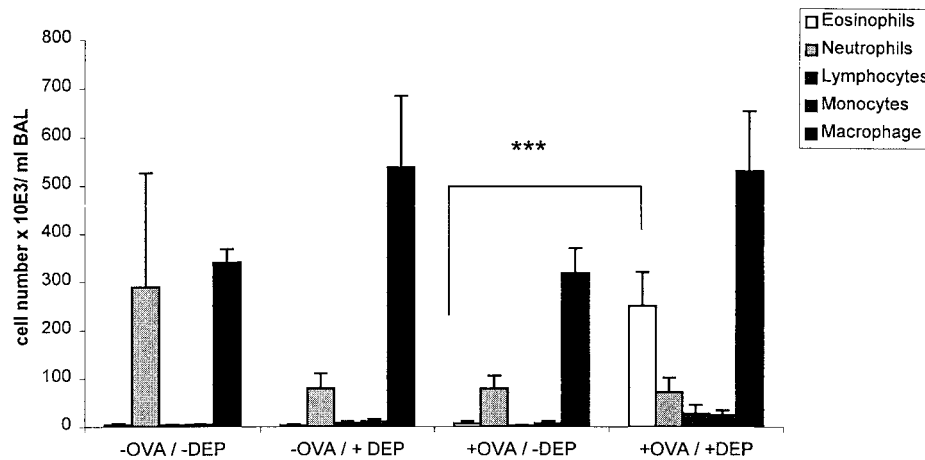


FIGURE 4. BAL cell analysis of mice intranasally sensitized and challenged with 20 µg ovalbumin and coexposed to DEP. Statistically significant differences from control +OVA–DEP shown as *** $p < .001$.

Effect of EHC-93 Via in/in

In contrast to DEP collected immediately from an engine, we have studied whether more complex and airborne particles can be examined via the in/in route of administration. Therefore, EHC-93 was tested in this developed system. EHC-93, administered in a way similar to DEP, induced a significantly increased IgE response to ovalbumin, as well as an increased IgG1 and IgG2a response (Figure 5). Histopathological examination showed again a strong inflammatory response and hypertrophy of mucous bronchiolar cells. Beside macrophages, an influx of eosinophils was present to a larger extent compared to the inflammation due to administration of ovalbumin only (Table 4 and Figure 6).

Not only a significant increase in the number of BAL cells but also the number of eosinophils were increased after coadministration of EHC-93 compared to the results in mice immunized with ovalbumin only (Figure 7). Finally, we found a significant increase in the Penh only after coadministration of ovalbumin and EHC-93 (Figure 8).

In none of our studies was a reproducible increase of IL-4 or IL-5 or decrease of interferon- γ in BAL found (data not shown).

DISCUSSION

In this study we have shown that coexposure of particles and ovalbumin evokes a Th2-like response characterized by IgE and an influx of eosinophils in BAL and lung tissue in BALB/c mice for different routes of application. It was shown that intranasal application is a very easy and noninvasive route of application to test small amounts of ambient particles for their adjuvant activity.

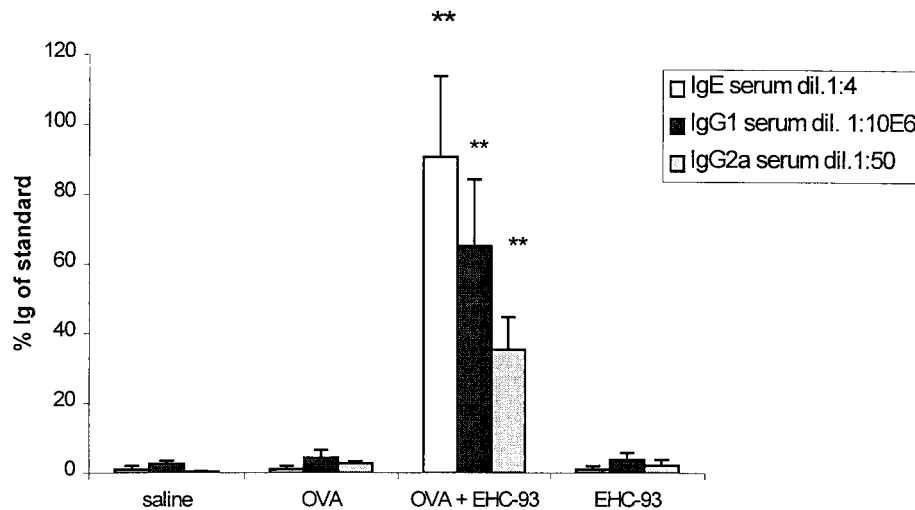


FIGURE 5. Immunoglobulins response of mice, intranasally sensitized and challenged with 20 μ g ovalbumin and coexposed during sensitization and challenge to EHC-93. Immunoglobulins are expressed as percentage of a positive standard; serum dilutions of IgE, IgG1, and IgG2a used are 1:4, 1:10⁶, and 1:50, respectively. Significant differences from OVA-exposed group shown as ** $p < .01$.

This study was initiated in the framework of a European study (RAIAP) in which small amounts of particles from four European cities were distributed over five laboratories to examine a number of characteristics, such as adjuvant potency. A huge amount of literature illustrates that DEP have the potency to increase the allergic response in the ovalbumin model in the mouse.

To test the adjuvant potency of ambient particles, however, the route of administration in this model is ambiguous. To study the best route of administration we compared three combinations of which the ip/aero and in/aero

TABLE 4. Mean score of histological lung lesions in mice, intranasally exposed to ovalbumin and EHC-93

Sensitization, ovalbumin (in)	–	–	+	+
Sensitization, EHC-93	–	+	–	+
Challenge, ovalbumin (in)	–	–	+	+
Peribronchiolar infiltrate	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2	0.6 $\pm$ 0.4	2.2 $\pm$ 0.4
Perivascular infiltrate	0	1.0 $\pm$ 0.3	0.8 $\pm$ 0.2	3.2 $\pm$ 0.4 ^b
Hypertrophy mucous cells	0	0	2.0 $\pm$ 0.0 ^b	3.4 $\pm$ 0.2 ^b
Alveolitis	0	0.2 $\pm$ 0.2	0.8 $\pm$ 0.2	0.8 $\pm$ 0.2
Eosinophils influx	0	0	0.2 $\pm$ 0.2	2.6 $\pm$ 0.5 ^a

Note. Histological parameters were individually and quantitatively scored [absent (0), minimal (1), slight (2), moderate (3), marked (4), and strong (5)] and averaged per treatment group ($n = 4$); in, intranasal. Statistical analysis: control group versus EHC-93, OVA; OVA versus OVA + EHC-93.

^aSignificant at $p < .05$.

^bSignificant at $p < .01$.

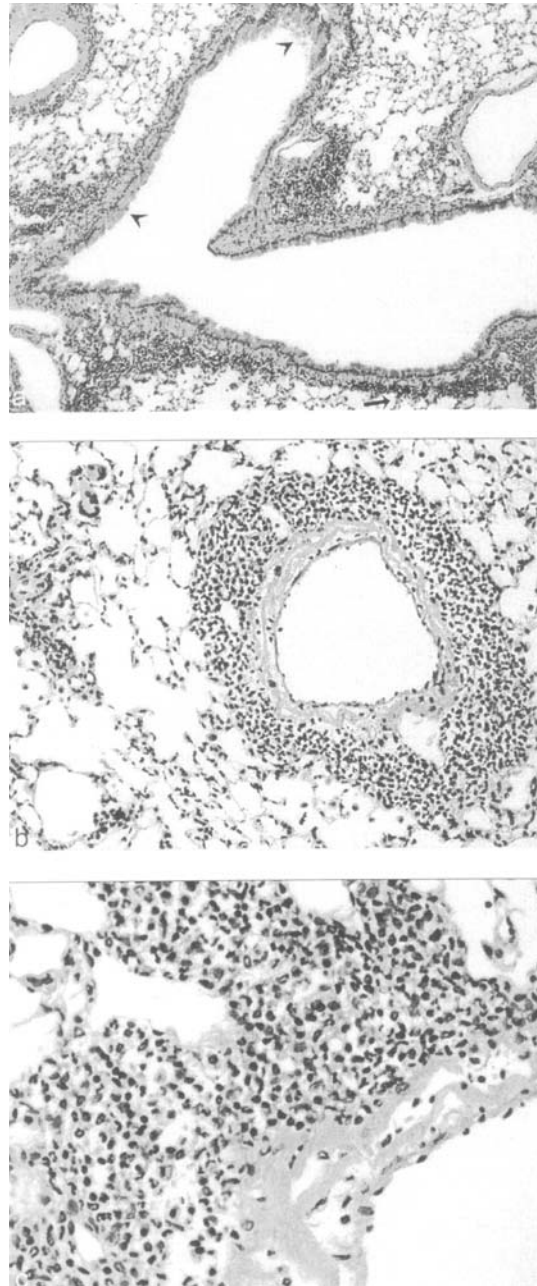


FIGURE 6. Histological pulmonary response after intranasal exposure to ovalbumin and EHC-93. (a) Overview of the airways with peribronchiolar infiltrate (arrow, score 3), hypertrophy of mucous cells (arrowhead, score 3) and perivascular infiltrate (center, score 3); H&E, 55 $\times$. (b) Perivascular infiltrate, evaluated with a score 4, and minimal alveolitis with some giant cells (top, left); H&E, 140 $\times$. (c) Detail of perivascular infiltrate with and a moderate influx of eosinophilic granulocytes; H&E, 300 $\times$.

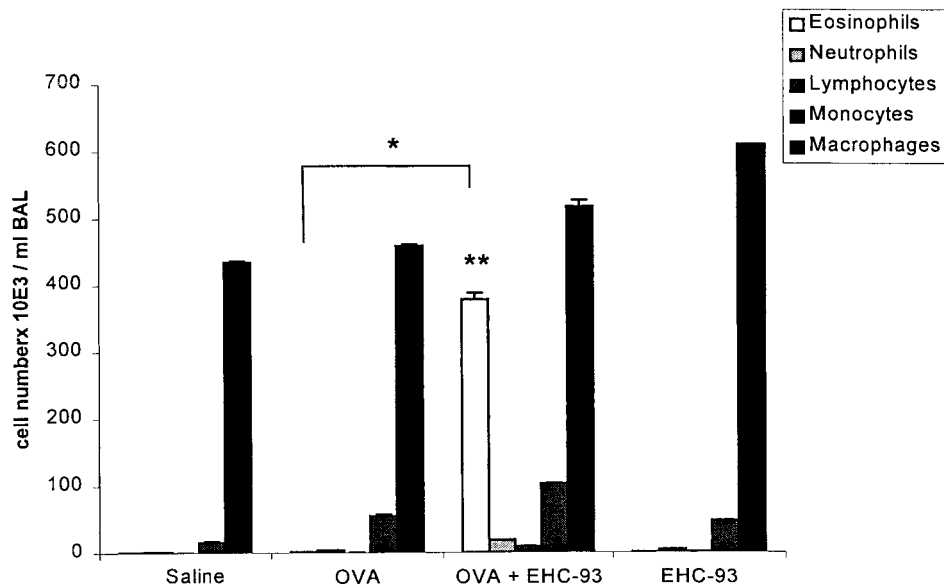


FIGURE 7. BAL cell analysis of mice intranasally sensitized and challenged with 20 μ g ovalbumin and coexposed during sensitization and challenge to EHC-93. Significantly different from OVA-exposed group shown as: * $p < .05$, ** $p < .01$.

route showed the highest increase of IgE and ip/aero the most severe inflammatory response in the lung in comparison with the in/in route (Figure 2). It was hypothesized that when DEP potentiated the response after in/in coadministration with ovalbumin the responses would be at a level similar to those of the ip/aero group. For the in/aero application it was found that 20 μ g Ova (in) and 1% ovalbumin (aero) was an optimal immunization route to show a significant difference in specific IgE to ovalbumin, inflammation, and eosinophils in BAL and lung between groups coexposed with ovalbumin plus DEP versus and ovalbumin alone. Similar differences were found between the forementioned groups after in/in administration. The latter results are comparable with intranasal results in the literature (Takafuji et al., 1987; Fujimaki et al., 1997; Nilsen et al., 1997).

To examine EHC-93 for its potentiation of the allergic response to ovalbumin, we have chosen the in/in route, in which we are able to make mixtures of small amounts of particles and ovalbumin. The respiratory and cardiovascular toxicity of EHC-93 have been described already (Fujii et al., 2001; Prieditis & Adamson, 2002; Ulrich et al., 2002; Vincent et al., 2001). EHC-93 has potentiated cytokine messenger RNA expression in human bronchial epithelial cells, such as GM-CSF, IL-1 β , and IL-8 (Fujii et al., 2001). In combination with ozone it has elevated IL-6 and IL-8 in BAL of rats and it has increased the inducible nitric oxide synthase (Ulrich et al., 2002). Moreover, it has induced increased plasma levels of endothelins (ET-1 to ET-3)

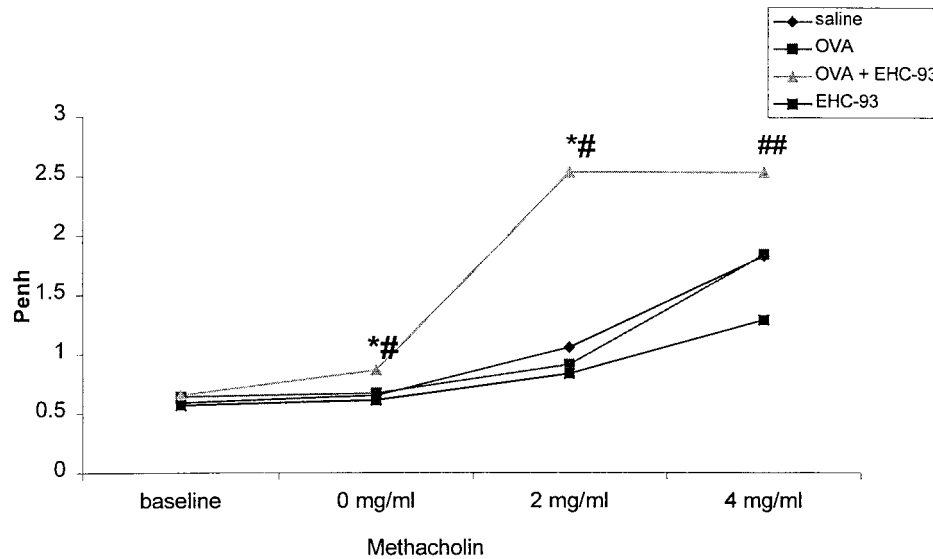


FIGURE 8. Airway responsiveness after stimulation with 0, 2, and 4 mg/ml methylcholine in mice intranasally sensitized and challenged with 20 μ g ovalbumin and coexposed to EHC-93. Significant differences shown as * $p < .05$ vs. OVA-exposed group, and by # and ## for significantly different from EHC-93 only ($p < .05$ and $p < .01$, respectively).

indicating cardiovascular toxicity in rats (Vincent et al., 2001). In the present study, EHC-93 significantly increased the IgE response to ovalbumin, and the pulmonary inflammatory response with a prominent influx of eosinophils. Moreover, coexposure of EHC-93 and ovalbumin resulted in an increased Penh, reflecting constriction of airways.

From the study of Prieditis and Adamson (2002) it was indicated that the atmospheric contaminant metals Zn and Cu are most likely to cause lung injury and inflammation as compared to metals such as Ni, Fe, Pb, and V at the same concentrations. It appears that a similar toxicity occurs when both redox (Cu) and nonredox (Zn) reactions are involved (Prieditis & Adamson, 2002). Concentrated ambient particles (CAPs) inhalation also led to tissue-specific increases in the activities of the antioxidant enzymes superoxide dismutase and catalase (Gurgueira et al., 2002). This coincides with the hypothesis that oxidants might be involved in the adjuvant activity of particles, as others have shown that antioxidant such as pretreatment with *N*-acetylcysteine or bucillamine inhibited the adjuvant activity of DEP (Whitekus et al., 2002). EHC-93 itself does not induce eosinophils; only a small increase of pulmonary neutrophilic granulocytes was demonstrated. However, this phenomenon of oxidative stress might be the initiation of an increased response to allergens that lead secondarily to an increased allergic response when particles are coadministered with ovalbumin. Although the observed response after coadministration of ovalbumin and EHC-93 may be characterized as a Th2 response, the presence of IL-4 in BAL was only mar-

ginally increased, while in most studies the IFN γ concentration in the BAL was similar to the saline group (Takano et al., 1997; Maejima et al., 2001; Miyabara et al., 1998; Heo et al., 2001). After coadministration of ovalbumin and EHC-93, not only was an increase in the IgE and IgG1 antibody response observed, but also the production of IgG2a was significantly increased. This may indicate that not only the Th2 response is involved but the Th1 response as well. Similar results were found by Takano et al. (1997) at wk 9, showing a 10-fold increase of IgG2a. However, their system seems to be less sensitive, because the increase of IgE, IgG1, and IgG2a was not seen at 6 wk.

In conclusion, in this study we have shown that coexposure of EHC-93 and ovalbumin evokes a profound response characterized by IgE, IgG1, and IgG2a and an influx of eosinophils in BAL and lung tissue in BALB/c mice. It was shown that intranasal application is a very easy and noninvasive route to test small amounts of ambient particles for their adjuvant activity.

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