

Dose Dependency of Adjuvant Activity of Particulate Matter from Five European Sites in Three Seasons in an Ovalbumin–Mouse Model

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Various particulate matter (PM) samples were tested for their adjuvant potency in an animal model of allergy (ovalbumin) in the European Union study entitled “Respiratory Allergy and Inflammation Due to Ambient Particles.” Coarse and fine ambient particles were collected during spring, summer, and winter in Rome, Oslo, Lodz, Amsterdam, and De Zilk. De Zilk, at the Dutch seaside, has mainly westerly winds and served as a negative pollution control. EHC-93 (Ottawa dust) was used as a positive control. We studied the adjuvant potency of the particle antibody responses to ovalbumin and histopathological changes in the lung. After a sensitization phase by coexposure to EHC-93 and ovalbumin, the antibody response to ovalbumin and inflammatory responses in the lung were huge. There was more adjuvant activity in reaction to 9-mg/ml samples than to 3-mg/ml samples. A best-fit analysis of these samples shows that the ambient coarse and fine particles at these sites, in combination with allergens, have severe to mild adjuvant activity in the order Lodz, Rome, Oslo, and Amsterdam. A high dose of the fine fraction was more potent than a high dose of the coarse fraction, except at De Zilk, where the reverse was true. Spring and winter PM was more potent than summer PM. Depending on the site, either a water-soluble or a water-insoluble fraction was responsible for the adjuvant activity. A concentration of 3 mg/ml is effective for screening high-activity samples, as is a concentration of 9 mg/ml for screening low-activity samples in the ovalbumin–mouse model.

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Some studies imply that living in urban areas with intense traffic is positively associated with symptoms of allergic rhinitis and respiratory complaints and with a prevalence of pollen sensitization (Ciccone et al., 1998; Duhme et al., 1998; Weiland et al., 1994). The European Community Respiratory Health Survey has shown wide geographic variation of the prevalences of reported symptoms highly suggestive of asthma, treatment for asthma, and current hay fever or nasal allergies of adults. In the last decade, attention focused on the emission of particulate matter (PM) air pollution derived from industrial

processes (Kim et al., 2001) and from traffic around homes (Duhme et al., 1998; Edwards et al., 1994; Nitta et al., 1993; Oosterlee et al., 1996; van Vliet et al., 1997; Weiland et al., 1994; Wjst et al., 1993). PM from industrial processes (e.g., coal/oil fly ash) originates from incomplete combustion of carbon-containing fuels. In addition to carbon, it contains water-soluble metal salts (especially sulfates of vanadium, nickel, and iron) and metals complexed at the surface of insoluble components (Dreher et al., 1997; Gavett et al., 1997). Traffic-related PM air pollution is primarily composed of elemental and organic carbon species and contains very little sulfate and metal (Westerholm & Egeback, 1994). Ambient PM can be classified in three size ranges: coarse [mass median aerodynamic diameter (MMAD) $>2.5 \mu\text{m}$ to $<10 \mu\text{m}$], fine (MMAD $0.1 \mu\text{m}$ to $2.5 \mu\text{m}$) and ultrafine particles (MMAD $<0.1 \mu\text{m}$).

In a previous study, we showed that PM collected from various cities differs in potency of adjuvant activity in a mouse-ovalbumin model (Steenenberget al., 2004). Only 4 of 13 samples were reactive in this model. In the present study, we have tripled the dose to determine whether the threshold varies.

In search of the constituents that can be more closely associated with the adverse effects of PM, some studies have investigated the water-soluble and water-insoluble fractions of PM with different outcomes (Greenwell et al., 2002; Monn et al., 2003; Huang et al., 2003b; Soukup & Becker, 2001; Walters et al., 2001). Therefore, this study focuses on the question whether the water-soluble fraction or the insoluble one is responsible for the adjuvant effect of the PM. This was investigated within the European Union (EU) study entitled "Respiratory Allergy and Inflammation Due to Ambient Particles" (RAIAP).

MATERIALS AND METHODS

Animals

Six- to 8-wk-old male BALB/cByJ.ico mice were obtained from Charles River (L'Ardresle, France). The animals were housed in Macrolon Type II 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.

Allergen Sensitization and Challenge

Ovalbumin

The mice were randomly allocated to a group ($n = 7$). For intranasal sensitization at days 0 and 14, the mice were slightly anesthetized with halothane (Table 1). At the moment of awakening, 0.4 mg/ml ovalbumin (grade V, Sigma Chemical, St. Louis, MO) in a total of $50 \mu\text{l}$ saline was administered in both nostrils. For the challenge on days 35, 38, and 41, mice were again intranasally exposed to ovalbumin (OVA) at the same concentration. To examine the adjuvant effect of particles, in this study the suspension of particles and OVA were administered

TABLE 1
Experimental design

Group $n = 7$	Sensitization days 0 and 14	Challenge days 35, 38, and 41
1	NaCl	NaCl
2	OVA + EHC-93	OVA
3	OVA	OVA
4	OVA + coarse particles	OVA
5	OVA + fine particles	OVA

Note. EHC-93, Ottawa dust; OVA, ovalbumin; autopsy was performed on day 42.

only during sensitization to a final concentration of 0.4 mg/ml OVA and 9 mg/ml PM, which resulted in a dose of $20 \mu\text{g}$ ovalbumin/mouse and $450 \mu\text{g}$ PM/mouse. The animals were sacrificed on day 42.

Particles

Particle collection. A high-volume cascade impactor with a multistage, round, slit-nozzle impactor was used to collect PM fractions on polyurethane foam (PUF) by impaction (Demokritou et al., 2002). This instrument operates at a flow rate of 900 L/min . The selected stages have actual measured cut-points at 8.8 , 2.52 , and $0.12 \mu\text{m}$.

Sampling locations and the periods of sampling. Sampling locations and the periods of sampling are described in "Description of Sampling Framework and the Precise Locations in 4 Cities and a Seaside Site" (Cassee et al., 2001) (see <http://www.raiap.org>). Briefly, each site has been visited 3 times for 4 to 6 wk each time. The sampling site in Oslo is located along a throughway called Ring 2 (section Kirkeveien/Griffenfeldts gate) approximately 20 m from the curbside of the road. The monitoring site in Lodz is situated in downtown Srodmiemie, Lodz, in heavy traffic. The monitoring site in Rome is at the Termini railway station in the center of Rome. The monitoring site in Amsterdam is near the Olympic stadium, south of the center and northeast of a busy motorway (Ring South). With a prevailing westerly wind, De Zilk, which has low traffic emissions and natural allergens such as pollen, was selected as a control site. The site is only about 800 m from the sea and is surrounded by sand dunes and fields. The nearest traffic route is 500 m eastward.

Particle removal from collection substrate. To analyze the chemical composition of the PM collected, it had to be extracted from the PUF. The methanol extraction method used is described by Salonen et al. (2000). A detailed description of the procedure is given by Cassee and Boere (2001). Dried PM samples were weighed on an analytical balance. Then they were stored at -20°C to prevent compositional decay of PM due to evaporation or chemical reactions.

To assign adjuvant activity to a specific PM fraction, the PM samples were divided into water-soluble and water-insoluble fractions. The PM samples were suspended in 9-mg/ml saline

solution and incubated overnight while shaking. After incubation, the samples were separated into 2 fractions by centrifugation ($12,000 \times g$) for 5 min. The supernatant was used for intranasal application without further preparation, and the pellet was resuspended in the same amount of saline, centrifuged again ($12,000 \times g$, 5 min), and resuspended into the desired concentration (9 mg/ml).

The EHC-93 (Vincent et al., 2001; 1997a, 1997b; Bouthillier et al., 1998) was a gift of Dr. R. Vincent (Environmental and Occupational Toxicology Division, Environmental Health Directorate, Health Canada, Ottawa, Ontario, Canada). The EHC-93, which was used as the standard reference material, contained 50 ng endotoxin/ml (in a 3-mg/ml PM suspension). The dose response of EHC-93 adjuvant activity was tested to find the PM concentration with maximal adjuvant activity.

Autopsy

Before autopsy, animals were anaesthetized with ketamine/Rompun [0.1 ml/100 g body weight of a mix of 0.85 ml 100 mg/ml ketamine (Aesculaap, Bostel, 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 phosphate-buffered saline (PBS) solution per mouse (pH 7.2, 37°C). The lungs were lavaged three times with the same fluid and identical volumes and then fixed with formalin.

Serum Samples

Blood was taken from the abdominal aorta and kept at 4°C for about 2–4 h to allow for clotting. The blood samples were centrifuged at 4°C for 15 min at $1200 \times g$. Serum was removed and stored in aliquots at –20°C until use.

Bronchoalveolar Lavage Fluid

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 lactate dehydrogenase (LDH) activity and cytokine analysis including interleukin-4 (IL-4), IL-5, IL-10, interferon γ (IFN γ), and tumor necrosis factor α (TNF α). Sediments were used to determine the total cell number (mixing 0.5 ml of the cell suspension with 9.5 ml isotone; Beckman Coulter, Mijdrecht, the Netherlands) and for producing cytospin preparations on which differential cell counts were performed after staining with May–Grünwald Giemsa. Per cytospin preparation, 400 cells were counted.

ELISA for Specific Ovalbumin IgE, IgG1, and IgG2a

Briefly, the enzyme-linked immunosorbent assay (ELISA) was performed with serum in flat-bottomed, 96-well, polystyrene microtiter plates with high binding capacity (MaxiSorp, number 439454, Nunc, Denmark, or number 655092, Greiner, Germany) (Steerenberg et al., 2003a). Mi-

crowells were coated and stored overnight at 4°C with monoclonal rat antimouse immunoglobulin (Ig) E, IgG1, and IgG2a (Jackson, PA) in Na₂CO₃ buffer (0.1 M; pH 9.6). Mouse serum samples (1:4 diluted) were incubated for 1 h at 37°C in PBS solution. The binding of IgE, IgG1, and IgG2a was measured as follows. The OVA extract–DIG solution (0.01 μ g/ml in blocking reagent) was incubated for 1 h at 37°C. Then anti-DIG Fab fragments, *poly*-labeled with peroxidase (PO) (number 1633716; Boehringer, Mannheim, Germany; 50 U/ml; 1:625), were incubated for 3 min at 20°C in the dark with a tetramethyl benzidine and dimethyl sulfoxide substrate. We stopped the enzyme reaction by adding 50 μ l of 2 M H₂SO₄, and read the optical density at 450 nm on a multichannel photometer. We derived a pooled positive serum with antibodies to OVA from mice sensitized and challenged in a different way: intraperitoneal sensitization by injection with 20 μ g OVA emulsified in 2.25 mg aluminium hydrochloride in a total volume of 100 μ l at day 0. The challenge on days 35, 38, and 41 consisted of exposure to a nebulized OVA solution (aerosol: 1% in 7 ml PBS in an ultrasonic nebulizer) activated for 20 min. Extinction values of samples were expressed as percentage of the standard.

Interleukins and interferon. The interleukins TNF α and IL-5 were purchased from BioSource (Woerden, the Netherlands); IL-4 and IFN γ were purchased from R&D, ITK Diagnostics (Uithoorn, the Netherlands). The assays were performed according the manufacturers' instructions. We expect that increased adjuvant activity will be accompanied with increased and decreased levels of IL4 and IFN γ , respectively. An increased number of eosinophils will be accompanied by an increased level of IL-5, and TNF α is responsible for an increased inflammatory response.

Histopathology

A syringe with 4% phosphate-buffered formaldehyde (pH 7.2), inserted into the trachea, was used to inflate the excised lungs. After full inflation, the trachea was ligated, and the lungs were immersed in fixative and embedded in paraplast. Five-micrometer sections were prepared and stained with hematoxylin and eosin (HE). The histopathological lesions (peribronchiolar and perivascular infiltrate, hypertrophy of bronchiolar mucous cells, alveolitis, and eosinophils influx) were semi-quantitatively and blindly scored as absent (0), minimal (1), slight (2), moderate (3), marked (4), or strong (5). The incidence as well as the severity of the lesions was incorporated into this score. Examples of the scoring values are illustrated in Steerenberg et al. (2003b).

Statistical Analysis

The induced serum titers and the number of BAL cells after sensitization and challenge with OVA alone were compared with the serum titers and number of BAL cells or cytokines obtained after challenge with OVA in combination with PM. An analysis of variance (ANOVA) was used to determine the statistical differences between groups. Student's two-sample

t-test was used to derive the 95% confidence interval of each parameter for each treatment group. The histological parameters within one experiment were statistically tested with the Wilcoxon test. A result was considered significant if the *p* value was less than .05.

All effect parameters were associated with the site (Amsterdam, Lodz, Oslo, and Rome), season (spring, winter, and summer), and PM size (PM10, PM2.5, and blank) (see Table 6). Ovalbumin with one of the test substances (made in one batch) was administered to each of the seven mice. We decided to use a log₁₀ transformation (after adding the usual number 0.5 to prevent difficulties with zero) of all variables except for pathology. The variables of pathology—although they are formally ordinal variables on a scale from 0 to 5—were considered to be linear and have been used untransformed. ANOVA was used in the formal analysis with the factors site, season, and test substance, and all two-way interactions. The three-way interaction was included in the residual error (as each of the seven mice was treated with one batch of each test substance in turn). A *p* value less than .05 was considered significant. When factors or interactions were found to be significant, further tests were done; the least significant difference (LSD) was used as the test criterion (i.e., performing a Student's *t*-test for each sublevel of the factor with a threshold of .05).

As data at the site De Zilk were only collected during one season, we compared the means of the De Zilk data with the means of the other sites and sizes in a Student's *t*-test with pooled variants from foregoing ANOVAs (see Table 7).

The mean response was calculated over all nine values from the experiments for each site, season, and concentration level (0 mg/ml, 3 mg/ml, and 9 mg/ml). We fitted an ANOVA model (on the log scale) for each experimental level (3 mg/ml and 9 mg/ml) against the zero level with the factors season; site; and combined concentration, season, and site data (see Figures 5 and 6). To calculate the concentration of the PM needed for a response to antibodies, a cutoff point that was comparable to the mean response of the group receiving only

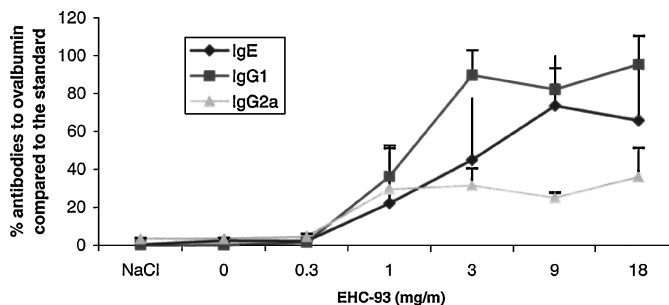


FIG. 1. Immunoglobulin production in mice coexposed during the sensitization phase to ovalbumin and various concentrations of EHC-93 during the sensitization phase.

ovalbumin plus twice the standard variation was chosen (see Table 9).

RESULTS

Dose Response to EHC-93

Antibodies to Ovalbumin

All three immunoglobulin subclasses (IgE, IgG1, and IgG2a) showed a concentration-dependent increase that reached a maximum within the range studied (0–18 mg/ml) (Figure 1).

Histopathology

Statistically significant increases of peribronchiolar and perivascular infiltrates due to EHC-93 at 1 mg/ml were observed (Table 2). At concentrations of 3 mg/ml of EHC-93 and more, an increased influx of eosinophils and hypertrophy of mucous cells were present. Alveolitis increased with greater concentrations of PM, but did not reach the level of significance. It is evident that more PM was encountered in the lungs of the group exposed to 18 mg/ml of EHC-93 than in the groups exposed to lower concentrations. Some clusters of alveolar macrophages (AMs) were observed, and some of them had large quantities of phagocytized PM with a black appearance, while other AMs in

TABLE 2

Mean score of histological lung parameters of mice coexposed to ovalbumin and various doses of EHC-93

Sensitization, OVA +/–	–	+	+	+	+	+	+
Sensitization, EHC-93 mg/ml	–	–	0.3	1.0	3.0	9.0	18
Challenge, OVA +/–	–	+	+	+	+	+	+
Peribronchiolar infiltrate	0.3 ± 0.3	0.3 ± 0.2	0.9 ± 0.3	1.3 ± 0.5 ^a	2.1 ± 0.4 ^a	2.1 ± 0.3 ^a	2.3 ± 0.3 ^a
Perivascular infiltrate	0.3 ± 0.3	0.4 ± 0.2	0.9 ± 0.3	1.4 ± 0.4 ^a	2.9 ± 0.4 ^a	2.7 ± 0.4 ^a	3.0 ± 0.6 ^a
Hypertrophy of mucous cells	0	1.3 ± 0.2	1.3 ± 0.2	2.3 ± 0.5	3.1 ± 0.3 ^a	3.6 ± 0.3 ^a	3.6 ± 0.4 ^a
Alveolitis	0	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	0.4 ± 0.3	0.9 ± 0.3	1.0 ± 0.5
Eosinophils influx	0	0	0	0.3 ± 0.3	2.6 ± 0.4 ^a	2.4 ± 0.4 ^a	2.0 ± 0.6 ^a

Note. EHC-93, Ottawa dust; OVA, ovalbumin. Data ± SEM; *n* = 7 per group. Histological lesions were semiquantitatively scored as absent (0), minimal (1), slight (2), moderate (3), marked (4), and strong (5). The histological parameters within one experiment were statistically tested with the Wilcoxon test.

^aSignificantly different from the OVA group, *p* < .05.

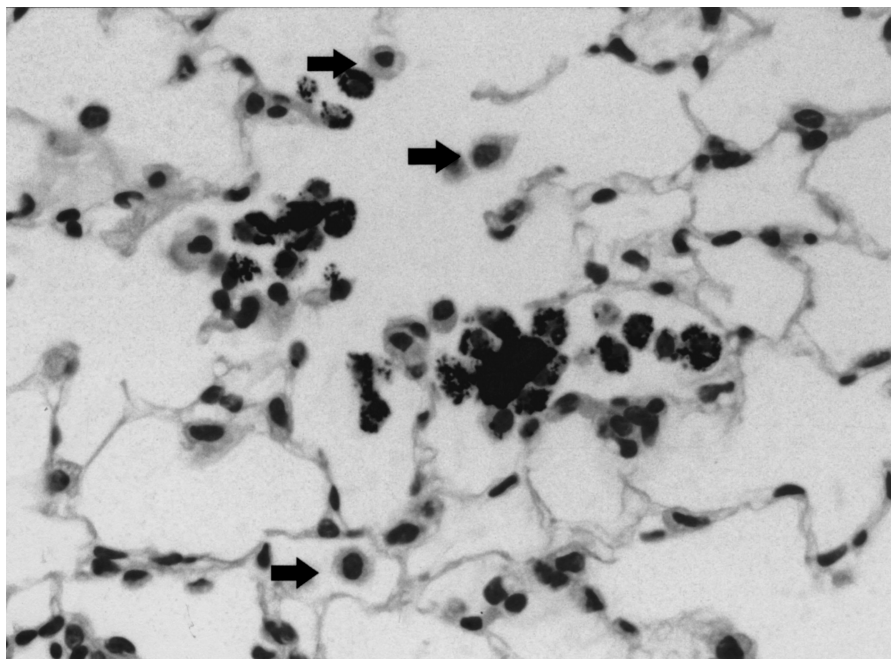


FIG. 2. Lung of a mouse exposed to 18 mg/ml EHC-93 (900 μ g) at days 0 and 14. Many alveolar macrophages have phagocytized particulate matter, giving a black appearance. However, some macrophages (arrow) are free of particles and show no phagocytized material. H&E, original magnification 370 $\times$.

the immediate vicinity had only a few single particles or none at all (Figure 2).

Bronchoalveolar Cells

The numbers of eosinophils, neutrophils and monocytes were significantly increased at doses of 3 mg/ml and 9 mg/ml of EHC-93 (Figure 3). At a dose of 18 mg/ml only the number of neutrophils was significantly increased.

Cytokine Production

Interleukin-4 was not detectable in the BAL fluid of mice treated with saline solution only. A minimal concentration of

3 mg/ml of EHC-93 resulted in a low concentration of IL-4 being detected (Figure 4a). Interferon γ was present in high concentrations in animals that were exposed only to saline solution. The concentrations increased slightly after coexposure to EHC-93 at 0.3 mg/ml and ovalbumin, but they decreased steadily in the groups exposed to greater concentrations of EHC-93 in comparison with the saline-treated group. However, this decrease was not statistically significant. Tumor necrosis factor α did not change during sensitization, regardless of coexposure (Figure 4b). Interleukin-5 showed a statistically significant increase after exposure to a concentration of 1 mg/ml of EHC-93.

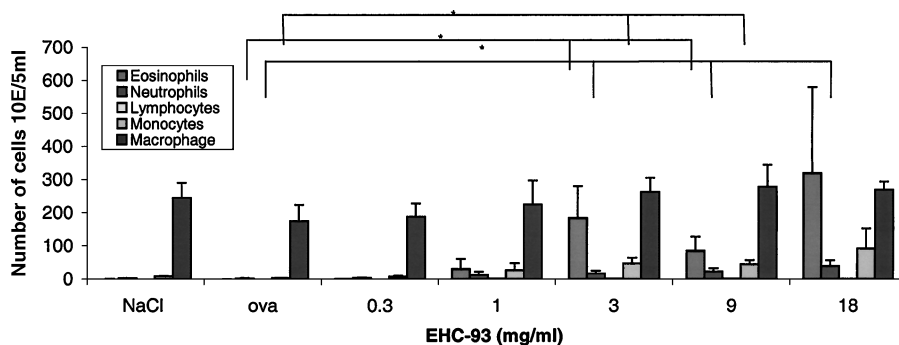


FIG. 3. Number of bronchiolar cells from mice coexposed during the sensitization phase to ovalbumin and various concentrations of EHC-93. An analysis of variance (ANOVA) was used to determine the statistical differences between groups. Student's two-sample *t*-test was used to derive the 95% confidence interval of each parameter for each treatment group. Asterisk indicates significant at $p < .05$.

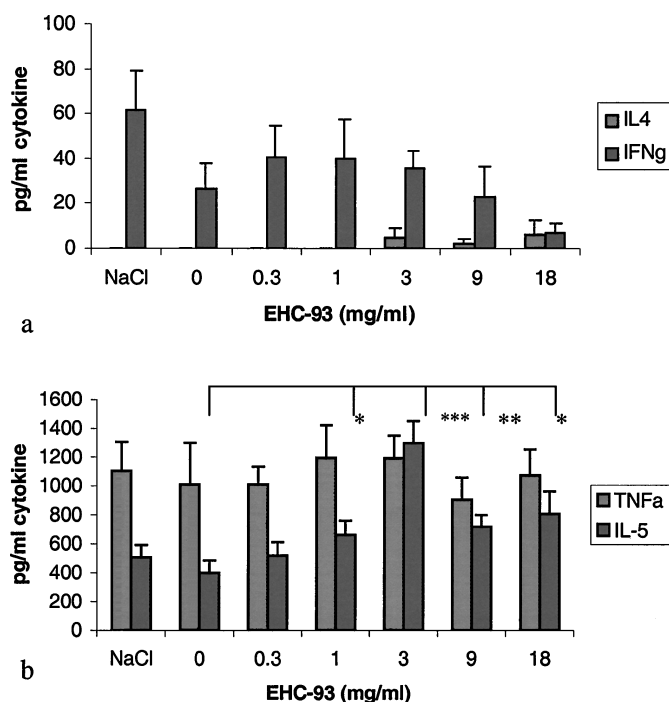


FIG. 4. Cytokines (a) IL-4 and IFN γ and (b) TNF α and IL-5 in BAL of mice coexposed during sensitization with ovalbumin and various doses of EHC-93. An analysis of variance (ANOVA) was used to determine the statistical differences between groups. Student's two-sample *t*-test was used to derive the 95% confidence interval of each parameter for each treatment group. Asterisks indicate significant at: **p* < .05, ***p* < .01, ****p* < .001.

Adjuvant Effect of Coarse and Fine Particles From Five European Sites

Immunoglobulins

Although the concentration of 18 mg/ml showed the strongest responses, a 9-mg/ml concentration of particles was chosen as the amount of PM from the 5 European sites was limited. At this concentration, most of the PM samples showed statistically significant adjuvant activity, that is, 6 of 13 coarse-particle samples and 9 of 13 fine-particle samples for IgE and IgG1 (Table 3). The concentrations of IgG2a were increased in 3 of 13 coarse-particle samples and 5 of 13 fine-particle samples. Particles from De Zilk induced a statistically significant adjuvant activity.

Histopathology of the Lung

Most of the coarse samples (9 of 13) from various sites and seasons showed strong adjuvant activity after coexposure with OVA (Table 3). Conversely, coadministration of fine samples in only 5 of 13 samples showed adjuvant activity. The adjuvant activity of the samples from de Zilk is remarkable, considering that these samples were meant as a negative control. This

adjuvant activity resulted in a slight to moderate inflammatory reaction in the lung, comparable to the inflammatory response induced by EHC-93.

Bronchoalveolar Cells

The coarse and fine fractions of the samples from Lodz in spring and summer induced increases of eosinophils, neutrophils, and monocytes (Table 4). The coarse and fine fractions from Rome in spring induced an increase of neutrophils. Coexposure of other PM samples and OVA did not result in increased numbers of bronchoalveolar cells of any kind.

Cytokines

The coarse and fine fractions from five sites in three seasons produced no IL-4 responses (Table 5). Interferon- γ concentrations were lower in the BAL of mice coexposed to the fine fraction (*p* < .05) than concentrations from mice exposed to ovalbumin only. Some PM samples in either the coarse or fine fraction induced statistically significantly (*p* < .05) more TNF α or IL-5 than did ovalbumin only.

Statistical Considerations

Statistical evaluation revealed that subclasses of antibodies to ovalbumin (IgE, IgG1, and IgG2a) were correlated with the variables site, season, or size (data not shown). Additional analysis showed that the sites could be ranked from high to low as follows: Lodz > Rome $\geq$ Oslo > Amsterdam (Table 6). Furthermore, for adjuvant activity concerning the immunoglobulins, the fine fraction was statistically significantly stronger than the coarse fraction (Table 6). For the histopathological parameters, the sites were ranked almost the same way (Table 6). No trends were found for the other parameters. The results for the antibody responses to ovalbumin in spring and winter were more closely associated with each other than they were with the responses in summer (Table 6). In most of the cases, the adjuvant activity of the De Zilk particles was lower than that of Lodz particles, but comparable to that of Rome and Oslo particles, and generally greater than the adjuvant activity of Amsterdam particles (Table 7). Both coarse and fine particles were active; however, the coarse fraction produced more adjuvant activity than the fine fraction (Table 7).

Crude, Water-Soluble, and Water-Insoluble Particulate Matter

On the basis of immunoglobulins and histopathology, we chose three samples with strong adjuvant activity (Lodz and Rome, coarse, spring; and Lodz, fine, spring) and one with weak adjuvant activity (Oslo, coarse, spring) to study the hypothesis that the water-soluble fraction of PM is responsible for the adjuvant activity. From the crude samples, we found that the antibody response was similar to our data (Table 8). Different reactions were found for the water-soluble and water-insoluble fractions. For the samples taken in the spring in Lodz,

TABLE 3

Immunoglobulins and pathological responses in mice coexposed to ovalbumin and coarse and fine PM samples (9 mg/ml), compared to the ovalbumin-only group

	Season	IgE		IgG1		IgG2a		Pathology	
		Coarse	Fine	Coarse	Fine	Coarse	Fine	Coarse	Fine
Rome	Spring	+	+	+	+	=	+	+	=
	Summer	=	=	=	=	=	=	=	=
	Winter	+	+	+	+	+	+	+	+
Oslo	Spring	=	+	=	+	=	+	+	+
	Summer	=	+	=	+	=	=	+	=
	Winter	=	+	=	+	=	=	=	=
Lodz	Spring	+	+	+	+	+	+	+	+
	Summer	=	=	=	=	=	=	+	=
	Winter	+	+	+	+	=	+	+	+
Amsterdam	Spring	=	=	=	=	=	=	=	=
	Summer	=	+	=	+	=	=	+	=
	Winter	+	=	+	=	=	=	=	=
De Zilk	—	+	+	+	+	+	=	+	+
Number of positive samples vs. total number of samples (13)		6	9	6	9	3	5	9	5

Note. Ig, immunoglobulin. = Similar, + significantly ($p < .05$) increased immunoglobulin concentration and pathology relative to the ovalbumin-only group. An analysis of variance (ANOVA) was used to determine the statistical differences between groups. Student's two-sample t -test was used to derive the 95% confidence interval of each parameter for each treatment group. The histological parameters within one experiment were statistically tested with the Wilcoxon test.

TABLE 4

Responses in numbers of cells in BAL of mice coexposed to ovalbumin and coarse or fine PM samples (9 mg/ml) compared to the ovalbumin-only group

Place	Season	Macrophages		Eosinophils		Neutrophils		Monocytes	
		Coarse	Fine	Coarse	Fine	Coarse	Fine	Coarse	Fine
Rome	Spring	+	=	+	=	+	+	=	=
	Summer	=	=	=	=	=	+	=	=
	Winter	=	=	=	=	=	=	+	=
Oslo	Spring	=	=	=	=	+	=	+	+
	Summer	=	=	=	=	=	+	=	=
	Winter	=	=	=	=	=	=	=	=
Lodz	Spring	+	=	+	+	+	+	+	+
	Summer	=	=	+	+	+	+	+	+
	Winter	=	=	=	+	=	=	=	+
Amsterdam	Spring	=	=	=	=	=	=	=	=
	Summer	=	=	+	=	=	=	=	=
	Winter	=	=	=	+	=	=	=	=
De Zilk		=	=	=	=	+	=	=	=
Increased response		2	0	4	4	5	5	4	4

Note. = Similar response, + significantly ($p < .05$) increased number of cells, compared to the ovalbumin-only group. An analysis of variance (ANOVA) was used to determine the statistical differences between groups. Student's two-sample t -test was used to derive the 95% confidence interval of each parameter for each treatment group.

TABLE 5
Interleukins in BAL of mice coexposed to ovalbumin and coarse and fine PM samples (9 mg/ml) compared to the ovalbumin-only group

		Interleukin-4		Interferon γ		Tumor necrosis factor α		Interleukin-5	
		Coarse	Fine	Coarse	Fine	Coarse	Fine	Coarse	Fine
Rome	Spring	0	0	0	0	=	=	=	=
	Summer	0	0	0	↓	=	=	↑	↑
	Winter	=	=	=	↓	=	=	=	=
Oslo	Spring	=	=	=	=	=	=	=	=
	Summer	0	0	=	↓	=	=	=	↑
	Winter	0	=	=	↓	=	=	=	=
Lodz	Spring	0	0	0	0	0	=	=	=
	Summer	0	0	0	0	0	=	=	=
	Winter	0	0	=	↓	↑	=	↑	=
Amsterdam	Spring	0	0	=	=	↑	=	↑	=
	Summer	0	=	=	↓	=	=	=	=
	Winter	0	0	=	↓	=	=	=	↑
De Zilk	—	0	0	=	↓	=	=	↑	↑
Total		0↑	0↑	0↓	8↓	2↑	0	4↑	4↑

Note. 0, Under the detection level; = similar response compared to the ovalbumin-only group; ↑ or ↓, significant ($p < .05$) increase or decrease relative to the ovalbumin-only group. An analysis of variance (ANOVA) was used to determine the statistical differences between groups. Student's two-sample t -test was used to derive the 95% confidence interval of each parameter for each treatment group.

Oslo, and Rome, the water-insoluble, coarse fraction showed increased immunoglobulin and pathological responses, while the water-soluble, fine fraction of the Lodz spring sample was active. Leukocytes and cytokines isolated by BAL showed no major differences.

DISCUSSION

In the present study, the adjuvant activity of EHC-93, coadministered with OVA, showed a dose-response relationship with immunoglobulins and histopathological lesions. However, other less allergy-specific parameters (cell differentiation and cytokines), did not show clear dose-dependent changes. More of the PM samples obtained from the five European sites evoked adjuvant activity when coexposed with OVA than did samples in a previous study with a concentration equal to one-third of ours

TABLE 7

Adjuvant activity of PM from De Zilk relative to other sites

Substance	Site	Particulate matter
Immunoglobulin E	<Lodz	Coarse > fine
Immunoglobulin G1	<Lodz	Coarse > fine
Immunoglobulin G2a	<Lodz	Coarse > fine
Reaction		
Peribronchial inflammation	NS	Coarse > fine
Perivascular inflammation	<Lodz	Coarse > fine
Hypertrophy of mucous cells	<Lodz	NS
Alveolitis	<Lodz	Coarse > fine
Eosinophil influx	>Lodz	Coarse > fine

Note. NS, not significant, or not significant between fine and coarse or between seasons; $p < .05$.

TABLE 6
Statistical evaluation of site, size, and season

	Site	Size	Season
Immunoglobulin E	Lodz > Rome = Oslo > Amsterdam	Fine > coarse	Spring = winter > summer
Immunoglobulin G1	Lodz > Oslo > Rome > Amsterdam	Fine > coarse	Spring = winter > summer
Immunoglobulin G2a	Lodz > Rome = Oslo > Amsterdam	Fine > coarse	Spring = winter > summer
Peribronchial inflammation	Lodz > Rome > Oslo > Amsterdam	NS	NS
Perivascular inflammation	Lodz > Rome = Oslo > Amsterdam	NS	NS
Hypertrophy of mucous cells	Lodz > Rome = Oslo > Amsterdam	NS	NS
Alveolitis	Lodz = Rome > Oslo > Amsterdam	NS	Spring > summer = winter
Eosinophil influx	Lodz > Rome > Oslo > Amsterdam	NS	Spring > summer = winter

Note. NS, not significant between fine and coarse or between seasons; $p < .05$.

TABLE 8

Immunoglobulins, pathological, and BAL results for mice coexposed to ovalbumin and crude, soluble and insoluble fractions of various coarse and fine PM samples (9 mg/ml) relative to the ovalbumin-only group

	Lodz, coarse particles, spring			Oslo, coarse particles, spring			Rome, coarse particles, spring			Lodz, fine particles, spring		
	Crude	Soluble	Insoluble	Crude	Soluble	Insoluble	Crude	Soluble	Insoluble	Crude	Soluble	Insoluble
Immunoglobulins												
IgE	↑	=	↑	=	=	↑	↑	=	↑	↑	↑	=
IgG1	↑	=	↑	=	=	↑	↑	=	↑	↑	↑	=
IgG2a	↑	=	↑	=	↑	↑	↑	=	↑	↑	↑	↑
Pathology												
Peribronchiolar infiltrate	↑	=	↑	=	=	↑	=	=	=	=	=	=
Perivascular infiltrate	↑	=	↑	↑	=	↑	↑	=	↑	↑	↑	=
Hypertrophy mucous cells		=	↑	↑	=	↑	↑	=	↑	↑	↑	=
Alveolitis	↑	=	↑	↑	=	↑	↑	=	↑	↑	=	↑
Eosinophil influx	=	=	↑	=	=	=	=	=	↑	↑	↑	↑
BAL cells												
Eosinophils	=	=	=	=	=	=	=	=	=	=	↑	=
Neutrophils	=	=	=	=	=	=	=	=	=	=	=	↑
Monocytes	=	=	=	=	=	=	=	=	=	=	↑	↑
Macrophages	=	=	=	=	=	=	=	=	=	=	=	↑
BAL cytokines												
Interleukin-4	=	=	=	=	=	=	=	=	=	↑	↑	=
Interferon γ	↑	↑	↑	=	=	↑	=	=	↑	=	↑	=
Tumor necrosis factor α	=	=	=	=	=	=	=	=	=	=	=	=
Interleukin-5	=	=	=	=	=	=	=	=	=	=	=	=

Note. Soluble, soluble fraction of particulate matter; insoluble, insoluble fraction of particulate matter; ↑, significant ($p < .05$) increase relative to the ovalbumin-only group; =, comparable levels compared to the ovalbumin-only group.

(Steerenberg et al., 2004). On the basis of the concentrations of 3 mg/ml and 9 mg/ml, related adjuvant activity was retrieved for all PM samples. Furthermore, for the coarse PM samples it is obvious that the insoluble fraction was responsible for the adjuvant activity of the sample.

We have shown that the adjuvant activity of PM is more or less dose dependent and that the optimal PM concentration is between 3 mg/ml and 9 mg/ml (150–450 μ g per mouse) in a mouse model for allergy. Our doses are much higher than the doses for exposure to concentrated air particles (CAP) (Goldsmith et al., 1999); however, no overload phenomenon was observed. Dust overload, typified as a progressive reduction of particle clearance from the deep lung, reflects an impaired clearance due to the loss of AM mobility (Morrow, 1988). The volume of particles phagocytized by AM is most critical in causing impairment of the clearance function (Oberdorster, 1995). Overloading is mainly used in connection with chronic exposure to high concentrations of relatively insoluble particles. (Morrow et al., 1996). Considering the dose of PM administered intratracheally, the facts that AMs are still present without phagocytizing particles and no free PM has been observed indicate that the phagocytizing capacity remained intact.

In a previous study (Steerenberg et al., 2004), we observed that the adjuvant activity can be ranked as follows: Lodz > Rome $\geq$ Amsterdam > Oslo. In Figures 5 and 6 we have incorporated the low dose used in the past (Steerenberg et al., 2004) and the high dose of 9 mg/ml in the present study to construct a dose-response relationship. These data were presented as the best fit of a dose-response curve (0 mg/ml, 3 mg/ml, and 9 mg/ml) for the concentration of IgE and the sum of scores for the pathological lesions. In general terms, the samples from the five sites resembled the kinetics of the EHC-93. In most cases, when the greatest response was reached, a plateau, or even a decrease, of the adjuvant activity was attained with higher concentrations. Such a decrease might be due to increased toxicity of the high dose for these particular samples, for which adjuvant activity was already great at a concentration of 3 mg/ml. We could rank PM samples on the basis of the arbitrary level of 20% of the positive reference standard. As Figure 5 shows, for a coarse particle in Lodz in the spring, for example, less (2 mg/ml), and for Oslo more (6 mg/ml), of the test material was needed to reach the cutoff point of 20% of the IgE antibodies against the OVA standard reference (Table 9). Regarding the IgE concentration and the sum of the pathological lesions, for the

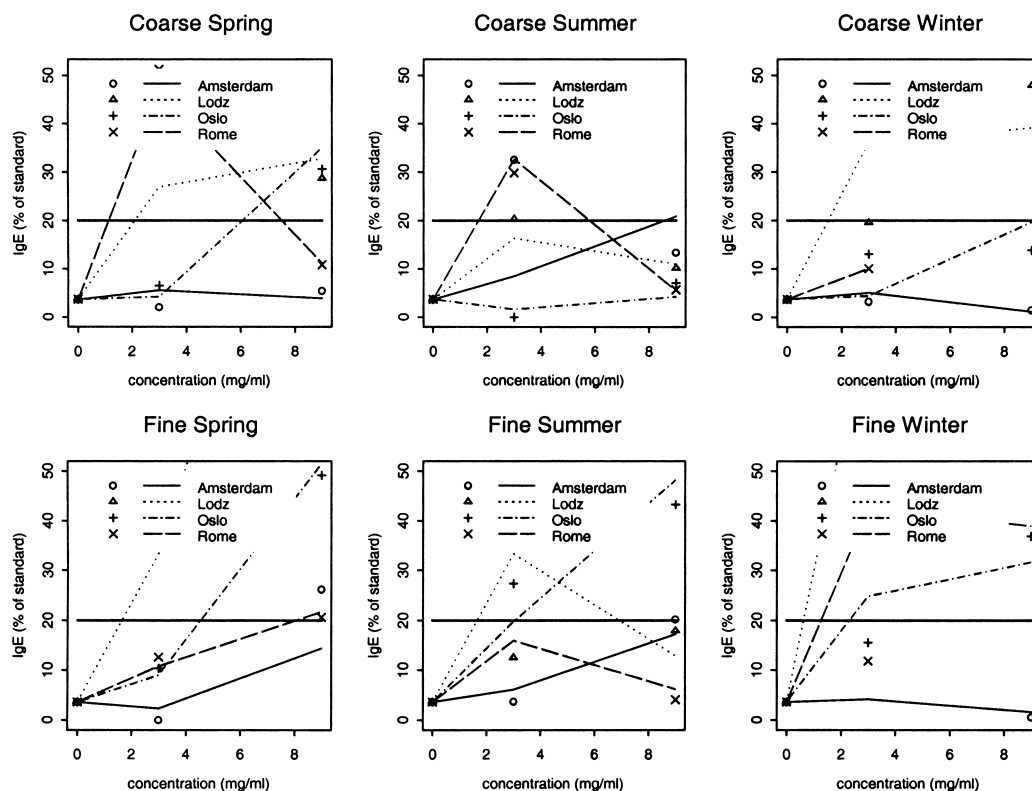


FIG. 5. Dose-response effect (fitted by regression function) on the antibody response to ovalbumin (IgE) by coarse and fine particulate matter from three seasons. The horizontal line is the cutoff point (the mean response in the group of ovalbumin alone + twice the standard variation) for a statistically significant response compared to the ovalbumin-alone group.

coarse fraction the sites were ranked as Lodz = Rome > Oslo = Amsterdam, and for the fine fraction, Lodz > Rome > Oslo > Amsterdam. Finally, it was surprising that particles from the De Zilk location showed a strong adjuvant activity comparable with that of Lodz and Rome. It was found that particles from De

Zilk and Amsterdam did not differ in composition except for Na and Cl concentrations. We concluded that a dose of 3 mg/ml is effective for screening samples with high activity, and 9 mg/ml for samples with low activity, in the ovalbumin-mouse model.

TABLE 9

Minimal concentration (mg/ml) of coarse and fine particles to evoke an adjuvant activity response after coexposure to PM and ovalbumin, which is comparable to the mean response in the group of ovalbumin alone + approximately twice the standard variation

	Coarse			Fine		
	Spring	Summer	Winter	Spring	Summer	Winter
IgE						
Rome	1.0	1.6	~	8.1	~	1.2
Oslo	6.0	~	9.0	4.5	3.0	2.3
Lodz	2.0	~	1.5	1.6	1.6	0.5
Amsterdam	~	8.6	~	~	~	~
Pathology						
Rome	1.3	1.5	2.7	~	~	3.1
Oslo	5.1	~	~	4.0	6.2	~
Lodz	1.0	0.9	1.1	1.1	2.0	1.3
Amsterdam	~	2.6	~	~	8.7	~

Note. ~, >9 mg/ml.

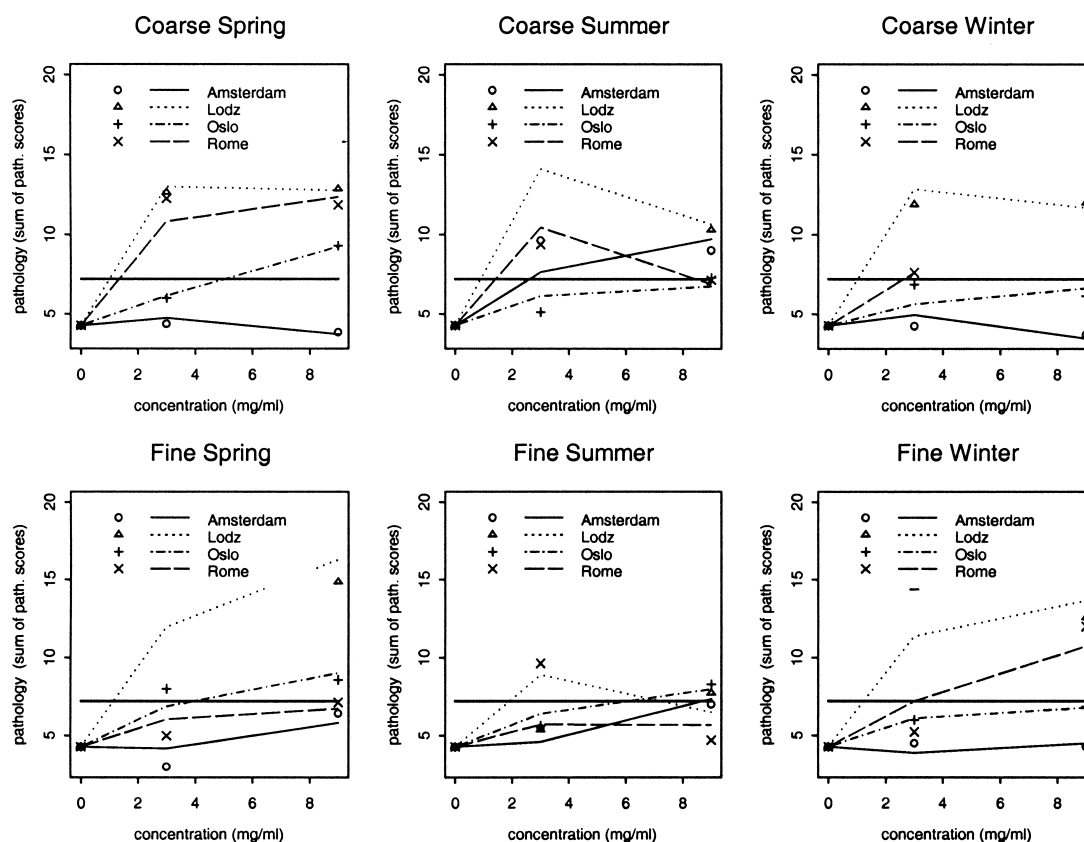


FIG. 6. Dose-response effect (fitted by regression function) on the sum of the scores of the histopathological lesions by coarse and fine particulate matter from three seasons. The horizontal line is the cutoff point (the mean response in the group of ovalbumin alone + twice the standard variation) for a statistically significant response compared to the ovalbumin-alone group.

Particles entering the lung will dissociate on the respiratory lining fluid in both water-soluble and water-insoluble constituents. In the present study, four samples were separated into a water-soluble fraction and a water-insoluble fraction. Differences were very clear-cut for individual samples. The water-insoluble fractions of all coarse samples were active comparable to the crude sample. However, only one of four samples (Lodz spring fine) showed an effect in the soluble fraction. It is possible that, due to insufficient centrifugation, a part of the fine PM was left in the soluble fraction, giving rise to the adjuvant activity. In vitro and in vivo studies have investigated which part is the most active (Brown et al., 2000; Ghio et al., 1999; Huang et al., 2003; Imrich et al., 2000; Lambert et al., 2000; Soukup et al., 2000, 2001). In vitro studies have shown that insoluble fractions of concentrated ambient particle (CAP) suspensions have mainly biological effects on AMs and certain particle adsorbed factors such as endotoxin (Ghio et al., 1999; Imrich et al., 2000; Soukup & Becker, 2001). However, soluble components of residual oil fly ash particles containing metal constituents have been shown to be active in vivo (Lambert et al., 2000). Moreover, Huang et al. (2003) found evidence that the soluble components have an effect on the pulmonary and hematological systems of humans. The question of the importance of soluble transient metals is still being debated, as

different studies came to different conclusions (Brown et al., 2000; Imrich et al., 2000).

It is tempting to analyze which constituent in the Lodz sample triggers this strong adjuvant activity. However, it is generally known that the constituents of particles are very closely linked. Although all PM samples were analyzed for their chemical composition, it is beyond the scope of this article to link this with the biological effects.

In conclusion, this model clearly shows the presence or absence of adjuvant activity after the intranasal application of particles (150–450 μg per mouse). The adjuvant activity of PM from the sites can be ranked in the order Lodz > Rome > Oslo > Amsterdam, and low-dose coarse PM and high-dose fine PM are closely associated with adjuvant activity (except for samples from De Zilk). With regard to season, spring and winter PM was more potent than summer PM. Although it appears that the water-insoluble fractions are more potent than the water-soluble fractions, what determines the differential potency of adjuvant effects of PM is yet to be elucidated.

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