

Adjuvant activity of ambient particulate matter of different sites, sizes, and seasons in a respiratory allergy mouse model

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Abstract

In the framework of an EU project entitled, “Respiratory Allergy and Inflammation due to Ambient Particles (RAIAP)”, various ambient particulate matter samples were tested for their adjuvant potency in an animal allergy model to ovalbumin. Coarse (2.5–10 μm) and fine (0.15–2.5 μm) particles were collected during the spring, summer, and winter in Rome, Oslo, Lodz, and Amsterdam. Coarse and fine particles were also collected near a seaside location in the Netherlands, where prevailing winds are westerly. These latter particles served as a control, with a minimum contribution by traffic. Ottawa dust (EHC-93) was used as a standard reference sample. Immunoglobulins (IgE, IgG₁, and IgG_{2a}), histopathological changes in the lung, cytokines, and the number of cells and their differentiation in lung lavages were used as effect parameters to study the adjuvant potency of these particles. The particles (3 mg/ml) were mixed with ovalbumin (0.4 mg/ml) and intranasally administered during the sensitization or the challenge phase. Intranasal administration of ovalbumin only induced very little antibody response, but introduced a minor inflammatory response in the lung or BAL during the sensitization and challenge phase. On the contrary, after coexposure to EHC-93 and ovalbumin, a major increase was found in immunoglobulin levels specific for ovalbumin, and a major inflammatory response in lung and BAL was induced. Coexposure to ovalbumin with 4 out of 12 collected PM samples (3 mg/ml) resulted in an increase of mainly IgE and IgG₁. The histopathological changes consisted of a small to severe peribronchial and perivascular inflammatory response, a hypertrophy of bronchiolar mucous cells and an increase in eosinophils and neutrophils in the BAL. Statistical evaluation of the above-mentioned parameters showed associations with PM_x (coarse and fine), site, season, season $\times$ PM_x, season $\times$ site and PM_x $\times$ site. In addition, adjuvant activity of the PM_x can be ranked as Lodz > Rome = Amsterdam > Oslo. When the different seasons were compared for IgE, PM from winter was found more active compared to PM from spring and summer. Only for the histopathological lesions, statistically significant difference in effects was found between coarse and fine (coarse > fine). No associations were found between the endotoxin content and the biological effects parameters, although endotoxin was much more confined to the coarse fraction.

In conclusion, PM, both coarse and fine, and from various geographic sites, was found to differ in adjuvant activity; furthermore, winter was found more active than spring and summer.

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Introduction

The prevalence of allergy has increased globally in the last few decades (Sly, 1999). Allergic rhinitis (“hay fever”)

Abbreviations: PM, particulate matter; BAL, bronchoalveolar lavage; DEP, diesel exhaust particles.

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and asthma are two of the most common allergic diseases. The prevalence of allergic disease varies considerably throughout the world (Anonymous, 1998; Arshad et al., 2001; Jarvis and Burney, 1998; Strachan et al., 1997). It has been widely accepted that atopy is for more than 40% inherited (Anonymous, 1997). However, it seems unlikely that genetic factors could contribute to the rising trend of allergy and asthma alone and lifestyle and environmental factors will likely play a crucial role in the increased prevalence of atopic diseases (Steerenberg et al., 1999). In the several hypotheses explaining the increased allergy/

asthma prevalence in the last 10 years attention was focused on ambient particulate matter (PM) derived both from industrial processes (Kim et al., 2001) and from traffic near the homes of subjects (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 originates from the combustion of carbon-containing fuels and beside carbonaceous compounds, also includes water-soluble metal salts (especially V, Ni and Fe sulfate) and metal complexes 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; it contains very little sulfate and metals (Westerholm and Egeback, 1994).

Epidemiological studies in large cities have consistently demonstrated associations between daily changes of ambient PM₁₀ concentrations, and morbidity and mortality, particularly among those with respiratory and cardiovascular diseases (Dockery et al., 1993; Schwartz and Dockery, 1992; Schwartz et al., 1996; Zanobetti et al., 2000).

What has emerged from several studies is that living in urban areas with a high incidence of traffic was positively associated with reported symptoms of allergic rhinitis and respiratory complaints (Ciccone et al., 1998; Duhme et al., 1998; Kramer et al., 2000; Weiland et al., 1994) where pollen sensitization is prevalent (Wyler et al., 2000). In other studies, traffic-related air pollution was also found to be associated with reported respiratory complaints, but not with an increase in the prevalence of allergic symptoms (Hirsch et al., 1999; Oosterlee et al., 1996; Wjst et al., 1993). However, there are uncertainties about the role of PM in the induction of traffic-related morbidity (Nicolai, 2002).

To study possible effects of PM on respiratory allergy, the ovalbumin model in the mouse has been used, as it mimics allergic reactions in the human airways (Hamada et al., 1999). Experimental studies in mice have shown adjuvant activity of DEP or airborne particles when co-exposed to ovalbumin (Fujimaki et al., 2001; Heo et al., 2001; Maejima et al., 1997; Nel et al., 1998; Steerenberg et al., 2003a; Takafuji et al., 1987, 1989; van Zijverden et al., 2000). Several studies have shown that PM influences cytokine production, enhances the IgE response or the allergen specific IgE, modulates the immunoglobulin isotype switching and increases inflammatory response in lung (Diaz-Sanchez et al., 1997, 2000; Maejima et al., 1997, 2001; Nel et al., 1998; Rusznak et al., 1997; Steerenberg et al., 2003a). The purpose of the present study was to screen the possible adjuvant activity of ambient PM for four European cities (sites) and study the impact of size (coarse and fine) and seasons (spring, summer and winter) using the ovalbumin mouse model. The sites were selected for the substantial contribution of traffic emissions to PM. This investigation was performed

in the framework of an European Union project entitled, “Respiratory Allergy and Inflammation due to Ambient Particles” (RAIAP).¹

Materials and methods

Animals. Six to eight-week-old male BALB/cByJ.ico mice, obtained from Charles River (L’Ardresle, France), were housed in Macrolon Type III cages. Food pellets and tap water were available ad libitum and all animals were allowed to adapt for at least 1 week before the experiments started. All experiments were performed according to international and institutional guidelines for animal care.

Sensitization and challenge allergens

Ovalbumin. The mice were randomly placed in groups ($n = 8$). For intranasal sensitization on days 0 and 14, the mice were slightly anaesthetized with halothane (Table 1). At the moment of waking, 0.4 mg/ml ovalbumin (grade V, Sigma, St. Louis, MO, USA) in a total of 50 μ l saline was administered into both nostrils. For the challenge mice were again intranasally exposed to ovalbumin at the same concentration on days 35, 38 and 41. 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. Animals were euthanized on day 42. According to our histopathological study, the quantity of a 150 μ g dose per mouse did not result in overloading of lung (Steerenberg et al., 2003a).

Particles

Particle collection. Ottawa dust (EHC-93) (Bouthillier et al., 1998), used as a standard reference, was a gift of Dr. R. Vincent (Environmental and Occupational Toxicology Division, Environmental Health Directorate, Health Canada, Ottawa, Ontario, Canada).

A high-volume cascade impactor with a multistage round slit nozzle impactor was used to collect PM fractions on methanol-cleaned polyurethane foam (PUF) by impaction (Demokritou et al., 2002; Kavouras, 2001). This instrument, developed at the Harvard School of Public Health, can be used to collect large amounts of fractionated PM for periods up to 1 week at a flow rate of 900 l per minute. The key feature of the sampler is the ability to collect particles in different size ranges using a selection of impactor stages with the appropriate size cutoffs. The selected stages for the RAIAP project have actual measured cut-points at 8.5, 2.35, and 0.12 μ m mass median aerodynamic diameter. The fractions between 8.5 and 2.35 and 2.35–0.12 μ m will be called coarse and fine, respectively.

¹ See <http://www.raiap.org> for additional information on this EU project.

Table 1
Experimental design

Group (N = 8)	Sensitization (days 0 and 12)	Challenge (days 35, 38, and 41)
1	NaCl	NaCl
2	ova + Ottawa dust (EHC-93)	ova
3	ova	ova
4	ova + coarse	ova
5	ova	ova + coarse
6	ova + fine	ova
7	ova	ova + fine

Autopsy was performed at day 42.

Sampling locations and seasons. The selection procedure of the five locations in Europe, as well as the sampling periods, has been extensively described earlier (Cassee et al., 2003).

The sampling sites in Rome, Oslo, Lodz, and Amsterdam are in the center of the cities with a high population, dominance of traffic and nearby roads (>10 and <100 m). With its prevailing southwesterly sea wind, very low traffic emissions, and natural allergens such as pollen, De Zilk was selected as a control site. The site is about 800 m away from the sea and surrounded with sand dunes and fields. The nearest traffic route is 500 m eastward.

Particle extraction from substrate. To perform chemical analysis and experiments, the PM was extracted from the PUF by methanol extraction based on a method described by Salonen et al. (2000). About 20 ml methanol was added to the tube containing the foam. This mixture was vortexed violently and sonicated for 30 min. The supernatant was decanted into a round-bottom flask and the above steps repeated twice. The suspension was then evaporated under vacuum by using a rotary evaporator at 25 °C for about 15 min, until about 1 ml suspension was left. This suspension was sonicated in the flask for several seconds to remove the PM from the wall of the flask. The suspension was transferred and divided over weighed and labeled Eppendorf tubes. The flask was washed with 1 ml fresh methanol and sonicated again to remove all PM from the wall of the flask; this suspension was also divided over the Eppendorf tubes. If the flask still contained PM, the wash step, as described above, was repeated. The Eppendorf tubes were transferred to a vacuum centrifuge and spun down for about 4 h. After this, the open tubes were put in an oven at 30 °C for 24 h. The next day, the dried PM samples were weighed and PM yield measured. Finally, these tubes were stored at –20 °C to prevent changes in the composition of PM due to evaporation or chemical reactions.

A Limulus amebocyte lysate (LAL) test (Heamachem Inc., St Louis, MO), as described by the manufacturer, was used to determine the endotoxin content of the 3 mg/ml PM suspensions; this included the BRP-3, which was used as a reference. The detection level was <0.125 ng endotoxin/ml. EHC-93 contained 50 ng endotoxin/ml.

Autopsy. At 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, Boxel, The Netherlands) and 0.65 ml of 20 mg/ml Rompun (Bayer, Leverkusen, Germany)]. Blood was taken by orbital puncture. A cannula was inserted into the trachea, and lung lavage was performed with 1 ml phosphate-buffered saline (PBS, pH 7.2, 37 °C). The lungs were lavaged three times with the same fluid and identical volumes, after which they were intratracheally fixed with formalin.

Serum samples. After collection, blood was kept at room temperature for about 2–4 h or overnight at 4 °C to allow for clotting. The blood samples were centrifuged for 5 min at 1200 × g at RT. Serum was removed and stored in small aliquots at –20 °C until use.

Bronchoalveolar lavage fluid (BAL). Fluid collected from the BAL of each animal was centrifuged at 400 × g, 4 °C, for 10 min. Supernatant from the lavage was used for cytokine analysis, including interleukin-4 (IL-4), IL-5, interferon-γ (IFNγ), and tumor necrosis factor (TNFα). Sediments were used for determination of total cell number [mixing 0.1 ml of the pelleted cells with 2.9 ml isoton (Beckman Coulter B.V., Mijdrecht, The Netherlands)] and for producing cytospin preparations on which differential cell counts were performed after staining with May-Grunwald Giemsa. Per cytospin preparation, 400 cells were counted.

Enzyme-linked immunosorbent assay (ELISA) for specific ovalbumin IgE. In short, enzyme-linked immunosorbent assay (ELISA) was performed with serum in flat-bottom 96-well polystyrene microtiter plates with a high binding capacity (MaxiSorp™, No 439454, Nunc, Denmark or No 655092, Greiner, Germany). All incubations were performed with 100-μl 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–dioxigenin (DIG) solution was prepared by labeling the ovalbumin with DIG using a reagent kit (see labeling DIG).

Microtiter plates were coated overnight at 4 °C with monoclonal rat antimouse IgE (Jackson, PA, USA) in Na₂CO₃ 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 (1:3 diluted) were incubated for 1 h at 37 °C in PBS. The IgE binding was measured by incubating the ovalbumin extract-DIG solution (0.01 μg/ml in blocking reagent) for 1 h at 37 °C, followed by an incubation of anti-DIG Fab fragments *poly* labeled with peroxidase (PO) (No. 1633716; Boehringer, Mannheim, Germany; 50 U/ml; 1:625) and an incubation of 3 min at 20 °C in the dark with tetramethylbenzidine/dimethylsulfoxide (TMB/DMSO) substrate. The enzyme reaction was stopped by adding 50 μl 2 M H₂SO₄, and 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, i.e., intraperitoneal sensitization by injection with 20 μ g ovalbumin emulsified in 2.25 mg aluminum hydrochloride in a total volume of 100 μ l on day 0 and challenged on days 35, 38, and 41 by exposure to a nebulized ovalbumin solution (aerosol: 1% in 7 ml PBS) using an ultrasonic nebulizer for 20 min. This was used as standard. Extinction values of samples were expressed as percentage of the standard.

ELISA for specific ovalbumin IgG₁ and IgG_{2a}. Microwells were coated overnight at 4 °C with 10 μ g ovalbumin/ml PBS (grade V, Sigma). Blocking reagent (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. Serum samples were diluted (IgG₁ 1:10⁶ and IgG_{2a} 1:50) in 0.5% bovine serum albumin (BSA/PBS) and were kept for 2 h at room temperature (RT) while shaking. After washing, the wells were incubated (shaking them for 1.5 h at RT) with biotinylated rat monoclonal antimouse IgG₁ (diluted 1:2000 in PBS) and IgG_{2a} (diluted 1:6000 in PBS) (Zymed, Laboratories, San Francisco CA, USA). The wells were washed and incubated with streptavidin-conjugated to poly-horseradish peroxidase (CLB, Amsterdam, The Netherlands) in a dose of 100 μ l 0.5% BSA/PBS for 45 min (shaking) at RT. This was followed by a washing of the wells and an incubation for 3 min at 20 °C in the dark with tetramethylbenzidine/dimethylsulfoxide (TMB/DMSO) substrate. The enzyme reaction was stopped by adding 50 μ l 2 M H₂SO₄, and the optical density read at 450 nm on a multichannel photometer.

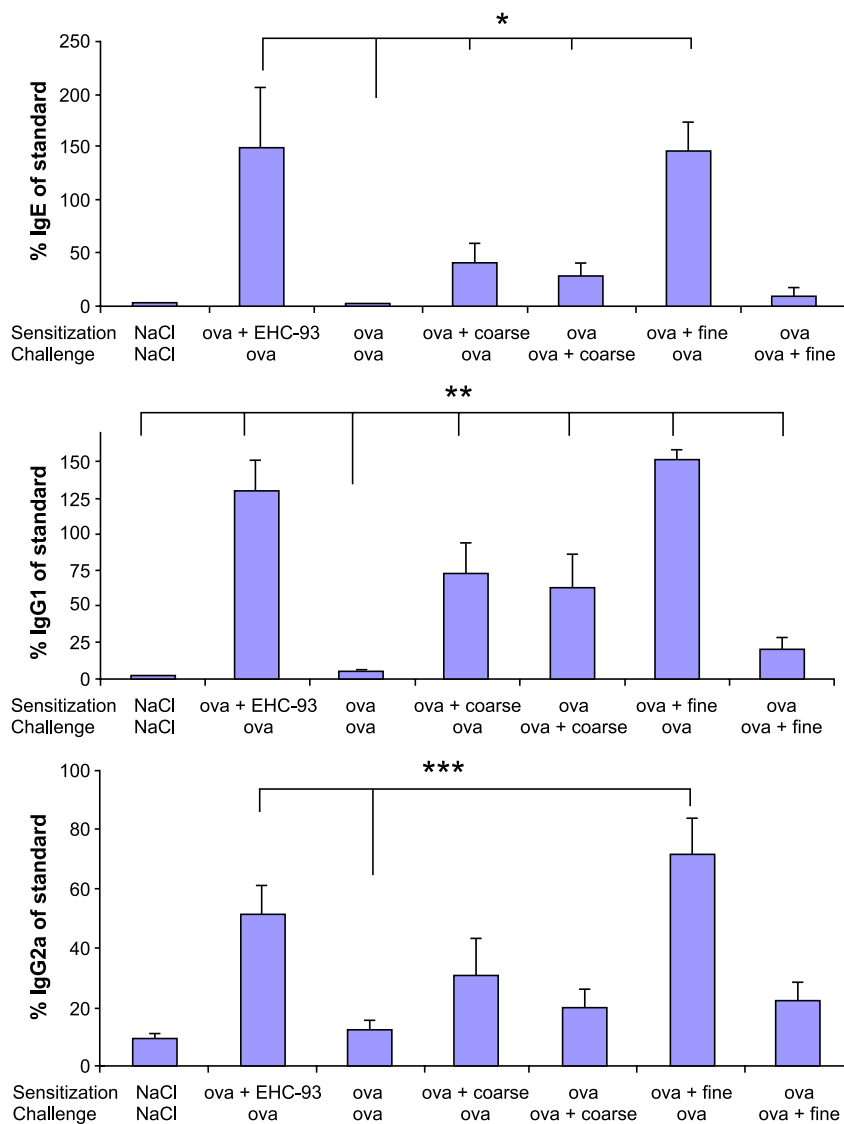


Fig. 1. (a, b, c) IgE, IgG₁, and IgG_{2a} responses of mice, sensitized and challenged to 20 μ g ovalbumin and coexposed during sensitization or challenge to coarse and fine PM fractions from Lodz spring. EHC-93 was used as a positive reference. IgE, IgG₁, and IgG_{2a} are expressed as percentage of a positive standard. Significant differences from OVA-exposed group: * P < 0.05, ** P < 0.01, *** P < 0.001.

Table 2

Summary of significantly increased responses of anti-IgE, IgG₁, and IgG_{2a} to ovalbumin after coadministration of various PM samples during the sensitization or challenge phase compared to the antibody response to ovalbumin only

Site	Season	Sensitization						Challenge					
		Coarse			Fine			Coarse			Fine		
		IgE	IgG ₁	IgG _{2a}	IgE	IgG ₁	IgG _{2a}	IgE	IgG ₁	IgG _{2a}	IgE	IgG ₁	IgG _{2a}
Rome	Spring	+	+	=	=	=	=	=	=	=	=	=	=
	Summer	=	=	=	+	=	=	=	=	=	=	=	=
	Winter	=	+	+	=	=	+	=	=	=	=	=	+
Oslo	Spring	=	=	=	=	=	=	=	=	=	=	=	=
	Summer	=	=	=	=	=	=	=	=	=	=	=	=
	Winter	=	=	=	=	=	=	=	=	=	=	=	=
Lodz	Spring	+	+	=	+	+	+	+	+	=	=	+	=
	Summer	=	=	=	=	=	=	=	=	=	=	=	=
	Winter	+	+	+	+	+	+	=	=	=	=	+	+
Amsterdam	Spring	=	=	=	=	=	=	=	+	=	=	+	=
	Summer	+	=	=	=	=	=	=	=	=	=	=	=
	Winter	=	=	=	+	+	=	=	=	=	=	=	=
De Zilk	–	=	=	=	=	=	=	=	=	=	=	=	=
Total		4	4	2	4	3	3	1	2	0	0	2	2

=: similar; +: statistically significant increased production.

Labeling proteins with DIG. Ovalbumin was labeled with digoxigenin-3-0-succinyl- ϵ -aminocaproic acid-*N*-hydroxy-succinimide ester (DIG-NHS) using the DIG antibody labeling kit (DIG Antibody Labeling Kit, No. 1367200, Boehringer). 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 determined. The solution had a protein concentration of 80 mg/ml (ovalbumin-dig) in PBS.

Cytokines. Kits for measuring the interleukins tumor necrosis factor (TNF α) and IL-5 were purchased from BioSource (Woerden, The Netherlands), and IL-4, and interferon gamma (IFN γ) kits were from R&D (ITK Diagnostics, Uithoorn, The Netherlands). The assays were performed according the manufacturer's instructions.

Histopathology. The excised lungs were inflated with 4% phosphate buffered formaldehyde (pH 7.2) by a syringe, inserted into the trachea. 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 were semiquantitatively and blindly scored from absent (0), minimal (1), slight (2), moderate (3), marked (4) to strong (5). The incidence as well as the severity of the lesions was incorporated into this score. Examples of the scoring values were illustrated in Steerenberg et al. (2003b).

Statistical analysis. Serum antibody titers and numbers of BAL cells were compared after sensitization and challenge with ovalbumin only and after ovalbumin in combination with PM. The statistical differences between groups were determined using an analysis of variance (ANOVA). The Student's two-sample *t* tests was used to derive the 95% confidence interval of each parameter for each treatment group. The histological parameters were statistically tested with the Wilcoxon test. The data were expressed as means $\pm$ SEM (standard error of the mean). The result was considered significant when a *P* value of <0.05 was found.

Table 4

Statistical evaluation of the production of immunoglobulins and histopathological lesions after coexposure to PM from different sites

	Rome	Oslo	Lodz	Amsterdam	Ova only
IgE	0.3688 ^a (2)	–0.0636 (3)	1.3044 (1)	0.2612 (2)	0.4677
IgG ₁	0.9023 (2)	0.6868 (2)	1.4405 (1)	0.6836 (2)	0.9282
IgG _{2a}	1.2487 (1)	0.6724 (2)	1.4583 (1)	0.7407 (2)	1.0301
Peribr.inf.	1.3401 ^a (2)	1.0684 (2)	2.5630 (1)	0.9868 (3)	1.4896
Periv.inf.	1.7595 (2)	1.2160 (3)	3.3628 (1)	1.1617 (3)	1.8750
Hypertrophy	2.3336 (2)	1.9532 (3)	3.4749 (1)	1.6271 (4)	2.3472
Alveolitis	0.4503 (3)	0.8851(2)	1.2927 (1)	–0.3920 (4)	0.5590
Eos-influx	0.7015 (2)	–0.2767 (4)	2.3320 (1)	0.0765 (3)	0.7083

() ranking from high (1) to low (4); ranking is based on the difference between the means of the log value (*P* < 0.05). Peribr.inf.: peribronchiolar infiltrate; periv.inf.: perivascular infiltrate; hypertrophy: hypertrophy of bronchiolar mucous cells; eos-influx: influx of eosinophilic granulocytes.

^a Mean of the log value.

Table 3

Statistical evaluation (*P* value) of variables on development of antibodies after coexposure to PM and ovalbumin compared to ovalbumin only

Variables	IgE	IgG ₁	IgG _{2a}
Site	0.0001	0.0941	0.0087
PM _x	0.1963	0.7047	0.7312
Season	0.0000	0.0000	0.0000
Site $\times$ PM _x	0.0689	0.0518	0.6995
Site $\times$ season	0.0007	0.0594	0.2710
PM _x $\times$ season	0.0164	0.1008	0.1242

Table 5

Statistical evaluation of the production of immunoglobulins and histopathological lesions compared to location of de Zilk after coexposure to PM from different sites

	Rome	Oslo	Lodz	Amsterdam
IgE	=	=	>	=
IgG ₁	>	>	>	>
IgG _{2a}	=	=	>	=
Peribr.inf.	>	=	>	=
Periv.inf.	>	=	>	=
Hypertrophy	>	=	>	=
Alveolitis	>	>	>	>
Eos-influx	>	>	>	=

Peribr.inf.: peribronchiolar infiltrate; periv.inf.: perivascular infiltrate; hypertrophy: hypertrophy of bronchiolar mucous cells; eos-influx: influx of eosinophilic granulocytes. (>) Statistically significantly ($P < 0.05$) stronger or (=) similar compared to location de Zilk.

Eight mice per group were used for each combination of site (Amsterdam, Lodz, Oslo, and Rome), season (spring, winter and summer), and test substance (coexposure to coarse PM and ovalbumin, to fine PM and ovalbumin, and ovalbumin only). Statistical analysis included visual inspection of the data with box-plots per season and site. A log 10 transformation (after adding the usual number 0.5 to prevent difficulties with zero) of the variables was chosen for use. The variables of pathology—although formally ordinal variables on a scale of 0 to 5—were considered to be linear; these were used as not transformed. The formal analysis has been done using an analysis of variance (ANOVA) with factors site, season, and test substance and by blocking the data according to the experiment number as an added control was used for each combination of site and season. A P value of less than 0.05 was considered to be significant.

The three-way interaction was included in the residual error (as the eight mice were each treated with one batch of each test substance in turn). A P value of less than 0.05 was considered to be significant. If a factor or interaction was found significant, further tests were performed using the Least-Significant Difference as test criterion (i.e., performing a Student's t test for each sublevel of the factor with threshold 0.05). Ranking was achieved when the difference between computed values was more than twice the standard error (SE) ($P < 0.05$).

Residuals were used to compute correlation matrices between all variables on the combined levels of site, season, and test substance (36 matrices in total). For each test substance, a combined correlation matrix was computed as an “average” over season and site using the Fisher's Z transformation with a bias correction. We computed a test on heterogeneity. If significant, it was not considered proper to perform further analysis using this correlation.

As the correlation is expected to exist a priori between the variables on the lowest level (site by season by test substance), we considered the difference between the correlation of test substances (coexposure with coarse against ovalbumin only, coexposure with fine against ovalbumin

only, and coexposure with coarse against coexposure with fine), once again using Fisher's Z test.

We noted here that a significant correlation does not mean that a (difference in) correlation is relevant, rather that a significant difference was considered relevant when larger than or equal to 0.30.

Results

From all the PM samples tested, both the coarse and fine fractions of the spring sample of Lodz showed the most prominent stimulation of the response to ovalbumin for all endpoints tested, when coexposed with PM during the sensitization or challenge phase. This is why we used, besides EHC-93, these PM results to exemplify the effects on the IgE, IgG₁, and the IgG_{2a}, the histopathological effects in the lung, the cytokines, and influx of inflammatory cells in the BAL.

Immunoglobulins

IgE

EHC-93 (standard reference PM) very strongly increased the IgE antibody response. The antibody response to ovalbumin was also significantly increased in mice coexposed to ovalbumin and coarse or fine particles from Lodz/spring during sensitization compared to the mice sensitized and challenged with ovalbumin only (Fig. 1). Coexposure with the coarse fraction during the challenge phase only evoked a significant IgE response to ovalbumin. Samples from Rome, Lodz, and Amsterdam showed respective adjuvant activity in spring/summer, spring/winter, and summer/winter. Samples from Oslo and the seaside (De Zilk) did not affect the responses. Table 2 showed the increase of IgE to be mostly confined to the sensitization phase (8 out of 26 vs. 1 out of 26 in the challenge phase).

IgG₁

EHC-93 (standard reference PM) strongly increased the IgG₁ antibody response in comparison with the standard reference serum. The antibody response to ovalbumin was significantly increased in mice when ovalbumin was coexposed with particles from Lodz/spring compared to the mice

Table 6

Statistical evaluation of the production of immunoglobulins after coexposure to PM from different seasons

	Spring	Summer	Winter	Ova only
IgE	1.1443 ^a (1)	0.8912 (2)	1.1030 (1)	0.6892
IgG ₁	1.5328 (2)	1.3103 (2)	1.7951 (1)	−0.3731
IgG _{2a}	0.9664 (3)	1.2461 (2)	1.7997 (1)	0.4153

() ranking from high (1) to low (2), ranking is based on the difference between the means of the log value ± 2 times the standard errors ($P < 0.05$).

^a Mean of the log value.

Table 7

Mean score $\pm$ SEM of histological parameters per group after administration during sensitization or challenge of coarse and fine PM from Lodz spring sample in the ovalbumin-mouse model

Sensitization	Saline	OVA + EHC-93	OVA	OVA + coarse	OVA	OVA + fine	OVA
Challenge	Saline	OVA	OVA	OVA	OVA + coarse	OVA	OVA + fine
Peribronchiolar infiltrate	0	2.9 $\pm$ 0.2 ^a	1.1 $\pm$ 0.1	2.4 $\pm$ 0.2 ^a	1.5 $\pm$ 0.3	3.0 $\pm$ 0.3 ^a	1.3 $\pm$ 0.2
Perivascular infiltrate	0.4 $\pm$ 0.2	4.1 $\pm$ 0.3 ^a	1.3 $\pm$ 0.2	3.3 $\pm$ 0.3 ^a	2.1 $\pm$ 0.2 ^a	4.3 $\pm$ 0.3 ^a	1.9 $\pm$ 0.4
Hypertrophy mucous cells	0	3.9 $\pm$ 0.2 ^a	1.6 $\pm$ 0.2	3.5 $\pm$ 0.4 ^a	2.9 $\pm$ 0.2 ^a	4.3 $\pm$ 0.2 ^a	2.4 $\pm$ 0.2
Alveolitis	0.5 $\pm$ 0.2	2.1 $\pm$ 0.4 ^a	0	1.6 $\pm$ 0.2 ^a	2.3 $\pm$ 0.4 ^a	1.1 $\pm$ 0.4 ^a	1.1 $\pm$ 0.2 ^a
Eosinophils influx	0	3.6 $\pm$ 0.2 ^a	0	1.9 $\pm$ 0.5 ^a	1.4 $\pm$ 0.5 ^a	3.1 $\pm$ 0.1 ^a	0.8 $\pm$ 0.5

^a Significantly different from group treated with OVA alone.

sensitized and challenged with ovalbumin only (Fig. 1). The coarse or the fine fractions coexposed with ovalbumin during the sensitization phase increased the IgG₁ response of the standard reference serum. Coexposure of ovalbumin with the coarse and fine fraction during the challenge phase evoked a significant IgG₁ response to ovalbumin.

Comparable results were found for coarse and fine fractions from Lodz/winter during sensitization, while Oslo and the Zilk were negative (Table 2). Coarse fractions from Rome/spring and winter (during sensitization) and Amsterdam/spring (during challenge) were also positive. Coexposure of ovalbumin and PM (coarse and fine) during the sensitization phase induced a higher incidence of increased responses than exposure during the challenge phase (7/26 vs. 4/26). As seen in Table 2, clearly the IgG₁ response runs mainly parallel with the presence of IgE antibodies.

IgG_{2a}

EHC-93 (standard reference PM) strongly increased the antibody response. The IgG_{2a} antibody response to ovalbumin was most significantly increased in mice when ovalbumin was coexposed with fine particles from Lodz/spring

during the sensitization phase compared to the mice sensitized and challenged with ovalbumin only (Fig. 1). From Table 2, it is shown that only Rome winter showed adjuvant activity comparable to Lodz winter.

Statistical consideration of effects on IgE, IgG₁, and IgG_{2a}

Statistical evaluation revealed that IgE, was correlated with the variables 'sites' 'season', 'site $\times$ season', PM_x $\times$ season (Table 3). IgG₁ was associated with season while IgG_{2a} was associated with site and season. Additional analysis based on the main antibody (IgE) showed that the sites could be ranked from high to low as follows: Lodz > Rome = Amsterdam > Oslo (Table 4). Ranking was based on the statistically significant difference between the means of the log value ($P < 0.05$). Separately, the antibody response after coexposure to PM from all sites was compared to the location de Zilk (Table 5). Lodz showed a significant increase of antibodies in comparison to de Zilk. With respect to season, the all-over antibody response was stronger ($P < 0.05$) by PM obtained in winter compared to spring and summer (Table 6).

Pathology

Administration of the coarse fraction from the Lodz spring sample showed a clear increased inflammatory response after administration during the sensitization and challenge phases (Table 7). With exception of the fine fraction after the challenge phase, the coarse and fine fractions of this location showed a significant increased histopathological response in comparison to the ovalbu-

Table 8

Summary of significantly pathological responses compared to the ovalbumin group after coadministration of various PM samples during the sensitization or challenge phase

Pathology		Sensitization		Challenge	
Site	Season	Coarse	Fine	Coarse	Fine
Rome	Spring	+	=	+	=
	Summer	=	=	+	=
	Winter	=	=	=	=
Oslo	Spring	=	=	=	=
	Summer	=	=	+	=
	Winter	+	=	=	=
Lodz	Spring	+	+	+	=
	Summer	+	=	+	=
	Winter	+	+	=	+
Amsterdam	Spring	=	=	=	=
	Summer	+	=	+	=
	Winter	=	=	=	=
De Zilk	—	=	=	=	=
Total		6	2	6	1

=: similar; +: statistically significant increase compared to ova treatment alone.

Table 9

Statistical evaluation (P value) of variables on development of histopathological lesions after coexposure to PM and ovalbumin compared to ovalbumin only

Variables	Peribr.inf.	Periv.inf.	Hypertrophy	Alveolitis	Eos-influx
Site	0.0000	0.0000	0.0000	0.0001	0.0000
PM _x	0.0090	0.0383	0.0006	0.0000	0.0515
Season	0.0000	0.0000	0.0000	0.0000	0.0000
Site $\times$ PM _x	0.1258	0.0039	0.0380	0.0011	0.0631
Site $\times$ season	0.0230	0.0108	0.0002	0.2613	0.0080
PM _x $\times$ season	0.0642	0.0001	0.1047	0.2353	0.0000

Peribr.inf.: peribronchiolar infiltrate; periv.inf.: perivascular infiltrate; hypertrophy: hypertrophy of bronchiolar mucous cells; eos-influx: influx of eosinophilic granulocytes.

Table 10

Statistical evaluation of effect of coarse and fine PM on histopathological lesions after coexposure with ovalbumin

	Coarse	Fine	Ova only
Peribr. inf.	1.6701 (1)	1.3090 (2)	1.4896
Periv. Inf.	2.0486 (1)	1.7014 (2)	1.8750
Hypertrophy	2.5694 (1)	2.1250 (2)	2.3472
Alveolitis	0.8090 (1)	0.3090 (2)	0.5590
Eos-influx	0.8547 (1)	0.5625 (1)	0.7083

Peribr.inf.: peribronchiolar infiltrate; periv.inf.: perivascular infiltrate; hypertrophy: hypertrophy of bronchiolar mucous cells; eos-influx: influx of eosinophilic granulocytes. mean of histopathological score; () ranking from high (1) to low (3), ranking is based on the difference between the means of the log value ± 2 times the standard errors ($P < 0.05$).

min-only group when administered during the sensitization or challenge phase. The saline group did not reveal any treatment-related responses, whereas the EHC-93 group showed a strong positive response as indicated by a strong inflammation and hypertrophy of bronchiolar mucous cells.

Six out of 13 coarse samples showed a strong adjuvant activity after administration during the sensitization phase (Rome spring, Oslo winter, Lodz spring/summer/winter, and Amsterdam summer) and 6 out of 13 samples after administration during the challenge phase (all sites in summer, and Rome and Lodz in spring) (Table 8). Two out of 13 fine samples (Lodz spring and winter) appeared to have strong adjuvant activity after administration during the sensitization phase and one sample (Lodz winter) out of 13 after administration during the challenge phase. The severity of lesions observed with all PM samples was comparable to the lesions observed with EHC-93.

Statistical consideration of effects on histopathological lesions

Statistical evaluation revealed that most of the histopathological lesions were correlated with 'site', 'PM_x', 'season',

'site * PM_x' and 'site $\times$ season' (Table 9). Additional analysis showed that the sites could be ranked from high to low as follows: Lodz > Rome > Oslo > Amsterdam (Table 4). In addition, Lodz and Rome showed significantly higher pathological responses than de Zilk (Table 5). Significant differences were found between coarse and fine; the coarse was found most severe (Table 10). No obvious ranking could be found between the spring, summer, and winter seasons, except for peribronchiolar infiltration and alveolitis of which spring/winter and spring, respectively, were more potent compared to the other seasons (not shown).

Bronchoalveolar lavage (BAL)

After coexposure with ovalbumin and EHC-93, a statistically significant increase was found for the number of eosinophils, neutrophils, and monocytes. The macrophages have been shown as the predominant cell type isolated by BAL. The results of coexposure to ovalbumin and all 13 PM samples is exemplified by the Lodz spring sample (Fig. 2). Coexposure to ovalbumin and the PM fraction (coarse and fine) from the Lodz spring sample during the sensitization period resulted in a statistically significant increase of eosinophils and neutrophils in comparison with ovalbumin only.

Coexposure to coarse PM with ovalbumin in the sensitization or challenge phase induced a statistically significant increase in eosinophils (4/13 and 5/13, respectively) and neutrophils (6/13 and 3/13, respectively) (Table 11). A remarkable increase in BAL neutrophils was observed after coexposure to ovalbumin and fine in both phases (11/13 and 8/13).

Statistical consideration on BAL cells

Statistical evaluation revealed the number of eosinophils from the BAL cells to be correlated with 'site', 'PM_x', 'season', 'site $\times$ season', and 'PM_x $\times$ season' (Table 12). The number of eosinophils correlated with many more

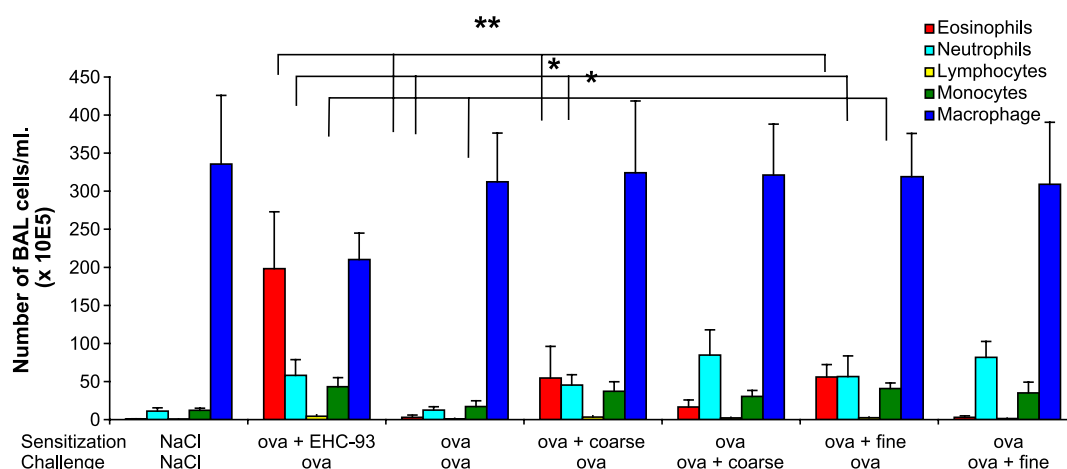


Fig. 2. BAL cell analysis of mice, sensitized and challenged with 20 μ g ovalbumin and coexposed during sensitization or challenge to coarse and fine PM fractions from Lodz spring. Statistically significant different (** $P < 0.01$) from OVA-exposed group.

Table 11

Effect of coarse and fine PM coadministered during sensitization or challenge with ovalbumin on the absolute counts of BAL cells

Site	Season	Sensitization								Challenge							
		Coarse				Fine				Coarse				Fine			
		Mφ	Eo	Neu	Mo	Mφ	Eo	Neu	Mo	Mφ	Eo	Neu	Mo	Mφ	Eo	Neu	Mo
Rome	Spring	=	+	=	=	=	=	=	=	+	=	=	=	=	=	=	=
	Summer	=	=	=	=	=	=	+	=	=	=	=	=	=	=	=	=
	Winter	+	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=
Oslo	Spring	=	+	=	+	=	=	+	+	=	+	=	=	=	=	+	=
	Summer	=	=	+	–	=	+	+	=	=	=	=	=	=	=	+	=
	Winter	=	=	=	=	=	=	+	=	=	=	=	=	=	=	+	=
Lodz	Spring	=	=	+	=	=	=	+	=	=	+	=	+	=	=	+	=
	Summer	–	+	+	+	–	=	+	+	=	=	=	=	–	=	=	=
	Winter	+	=	+	=	+	=	+	+	+	+	+	+	+	+	+	+
Amsterdam	Spring	=	=	+	=	=	=	+	=	=	=	+	=	=	=	+	=
	Summer	=	+	+	=	+	=	+	+	=	=	=	=	=	=	+	=
	Winter	=	=	=	=	+	+	+	=	=	+	=	=	=	=	+	=
De Zilk		=	=	=	=	+	=	+	+	=	+	+	+	=	=	=	=
Number of increases		2	4	6	2	4	2	11	3	2	5	3	3	1	1	8	1

Mφ: alveolar macrophages, Eo: eosinophils, Neu: neutrophils, Mo: monocytes; =: similar, –: statistically significant reduction; +: statistically significant increase compared to OVA treatment.

variables (4×) compared to the number of macrophages (1×), neutrophils (2×), and monocytes (2×). Further analysis revealed that particles from Lodz evoked more eosinophils and monocytes compared to particles from Amsterdam and Oslo and particles from the spring and winter season induced more eosinophils, neutrophils, and monocytes compared to spring (not shown).

Cytokines in BAL

The results of coexposure to Lodz spring is exemplified in Fig. 3. All coexposures are compared to ovalbumin only. The IL-4 content in the BAL showed a statistically significant increase for the fine fraction during both phases. The fine fraction during the challenge phase showed a statistically significant increase in IFNγ concentration of the BAL, whereas the coarse fraction (sensitization and challenge) and the fine fraction (sensitization) induced a nonstatistically significant decrease. TNFα and IL-5 concentrations showed mostly statistically significant increases during both phases. Table 13 presents all 13 fractions. In one-third of the cases, the concentration of IL-4 was under the detection level. In most of the BAL, the IL-4 concentration was found comparable to the ovalbumin-only group. IFNγ was found mainly comparable with the ovalbumin-only group or showed a statistically significant decrease. The TNFα concentration of the BAL was comparable to the ovalbumin-only group (39×) or was statistically significantly increased (7×) or decreased (6×). In most cases, the IL-5 concentration in BAL was comparable to the ovalbumin, but showed a significant increase 15 (coarse 8, fine 7) times and a decrease 5 (coarse 1, fine 4) times.

Statistical consideration of effects on cytokines in BAL

Statistical evaluation revealed the concentration of cytokines from the BAL to be correlated with site (IL4), PM_x (IFNγ), season (IFNγ), and site × season (IL4) (Table 14).

Additional analysis for IL4 revealed that Lodz was more potent than other sites and for IFNγ summer and winter were more potent than spring (not shown).

Endotoxin content of PM

In a large number of particle samples, endotoxin was encountered using the Limulus test (Fig. 4). The concentrations varied from 0 to 200 ng/ml. To ensure that all LPS contained within the particle fraction became available for detection, we have also ultrasonified the samples. No differences were found in LPS content, compared to particle fractions that were vigorously vortexed before use.

A statistically significant difference in endotoxin content was found between coarse and fine, and between seasons, but not between sites (not shown). The coarse fraction contained more endotoxin compared to the fine fraction, and winter was less compared to spring and summer (Table 15). Using the endotoxin level as a variable in the analysis of covariance, no statistical association was found for any of the parameters ($n = 16$) except for alveolitis and increased number of eosinophils, and IL-4 and TNFα in the BAL. However, adjusting for endotoxin

Table 12

Statistical evaluation (P value) of variables on the presence of leukocytes in BAL after coexposure to coarse and fine PM during sensitization challenge to ovalbumin

Variables	Macrophages	Eosinophils	Neutrophils	Monocytes
Site	0.7726	0.0000	0.2089	0.0047
PM _x	0.1020	0.1858	0.0017	0.1519
Season	0.0967	0.0000	0.0000	0.0006
Site × PM _x	0.5864	0.0671	0.3616	0.6776
Site × season	0.0302	0.0038	0.1177	0.5709
PM _x × season	0.5864	0.0001	0.3616	0.3199

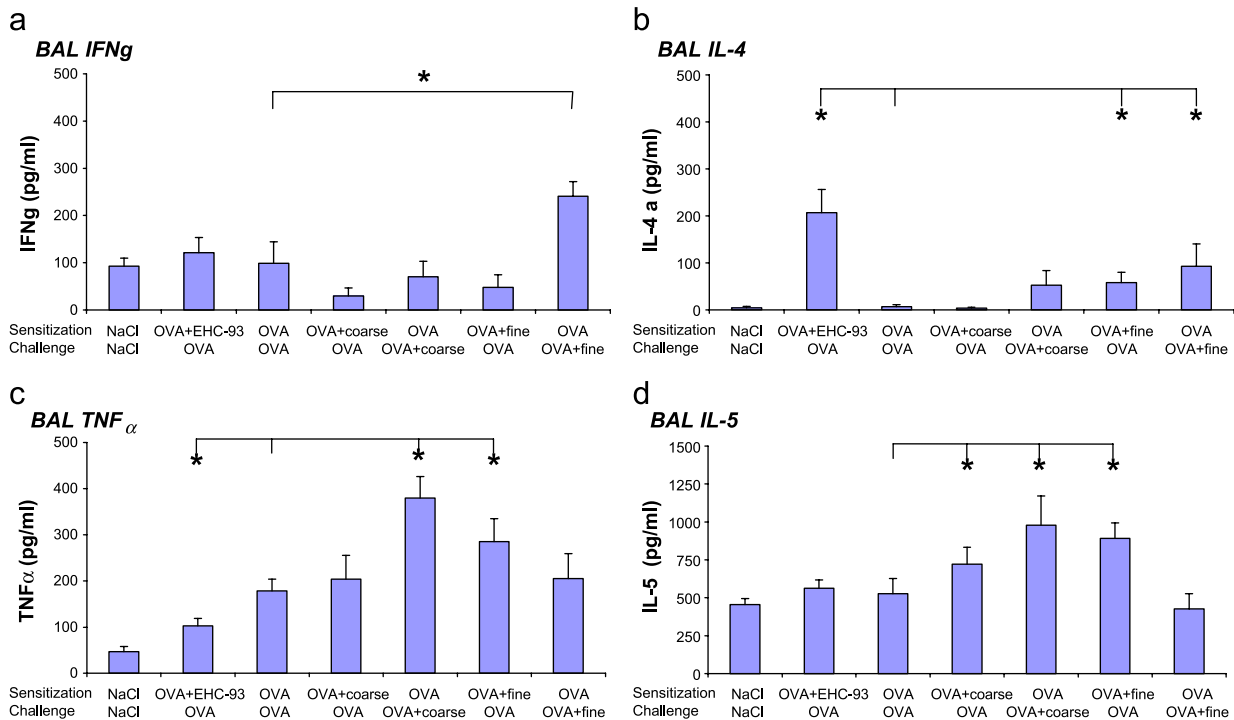


Fig. 3. (a–d) Cytokines (IFN γ , IL-4, TNF α , and IL-5) from BAL analysis of mice, sensitized, and challenged with 20 μ g ovalbumin and coexposed during sensitization or challenge to coarse and fine PM fractions from Lodz spring. Statistically significant differences (* $P < 0.05$, ** $P < 0.01$) from OVA-exposed group.

effects did not influence the statistical evaluations of the effects (Tables 3, 8, 12, and 14).

Discussion

Our investigation on the effect of adjuvant activity of PM on site, size, and season differences showed that (1) combined exposure to allergens (i.e., ovalbumin) and PM resulted

in a potentiation of allergic responses (antibody responses and histopathological lesions) and (2) that PM collected at different locations and different seasons has a different potential to act as an adjuvant. Among all the parameters studied, winter has more pronounced adjuvant activity than spring and summer with respect to the antibodies; for other parameters, there was no season dependency.

In this study, we have chosen for the application of PM by the intranasal route, based on our findings in the past

Table 13
Effect of coarse and fine PM on the amount of cytokines in BAL

Site	Season	Sensitization								Challenge							
		Coarse				Fine				Coarse				Fine			
		IL4	IFN γ	TNF α	IL5	IL4	IFN γ	TNF α	IL5	IL4	IFN γ	TNF α	IL5	IL4	IFN γ	TNF α	IL5
Rome	Spring	=	=	=	+	=	–	=	+	+	–	+	=	=	=	=	=
	Summer	=	=	=	=	=	–	–	=	=	–	–	=	=	–	–	=
	Winter	0	=	=	=	0	=	=	=	0	=	=	=	0	+	=	=
Oslo	Spring	=	–	=	=	=	–	=	=	=	–	=	=	=	–	=	=
	Summer	0	=	=	=	0	–	=	=	0	–	+	=	0	–	=	–
	Winter	=	=	–	=	=	=	–	=	=	=	=	=	=	=	–	–
Lodz	Spring	=	=	=	+	+	=	+	+	=	=	+	+	+	+	=	=
	Summer	0	=	=	=	0	=	=	=	0	=	+	+	0	–	=	=
	Winter	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	+
Amsterdam	Spring	0	=	=	+	0	–	=	=	0	–	=	=	0	=	=	+
	Summer	=	=	=	+	=	=	=	+	=	=	=	=	=	=	=	=
	Winter	=	=	=	=	=	–	=	–	=	–	=	–	=	–	=	=
De Zilk		=	=	=	+	=	=	+	+	=	=	+	+	=	+	=	+

=: similar; –: statistically significant reduction; +: statistically significant production compared to ova treatment alone; 0: below detection level.

Table 14

Statistical evaluation (*P* value) of variables on interleukins isolated from BAL after coexposure to PM during sensitization and challenge to ovalbumin

Variables	IL4	IFN γ	TNF α	IL5
Site	0.0086	0.6335	0.2159	0.2891
PM _x	0.7828	0.0001	0.4547	0.9566
Season	0.5657	0.0000	0.7754	0.2489
Site $\times$ PM _x	0.0571	0.1952	0.3786	0.4701
Site $\times$ season	0.0023	0.8308	0.1490	0.4976
PM _x $\times$ season	0.4787	0.1952	0.3786	0.4946

(Steerenberg et al., 2003b). As four collaborative laboratories with their own toxicological parameters were involved in this RAIAP project, whole-body inhalation was not achievable by the restricted availability of PM. However, the mouse is an obligatory nose inhaler, which favors the intranasal application because the whole route of respiratory tract is involved.

We have analyzed the influence of three variables on many critical parameters: (1) site, (2) PM size fraction, and (3) season. With respect to site, the adjuvant activity of PM from different sites can be ranked based on increased IgE/IgG₁ production: Lodz > Rome = Amsterdam > Oslo. Coexposure to 4 out of 13 samples of the collected PM (coarse and fine) resulted in a statistically significant increase of IgE. The concentration IgG₁ in serum, which is 10⁶ times higher than IgE, showed an almost similar sensitivity. The response was confined to a Th2-mediated response, while the IgG_{2a} response was almost absent, with exception of PM from Lodz winter, which showed adjuvant activity in all measured immunoglobulins (IgE, IgG₁ and IgG_{2a}). This was also found for EHC-93, where, in 10 out of 13 experiments, IgG_{2a} was increased after coexposure. Therefore, it is concluded that the adjuvant activity is confined to the allergic response, and also that PM can trigger the Th1 response depending on its potency. The

Table 15

Statistical evaluation between endotoxin content and PM_x (coarse and fine) or season (spring, summer, and winter)

	Coarse	Fine	se ^a	Spring	Summer	Winter	se
Endotoxin	1.7321 ^b	0.8321	0.1394	1.3330	1.5391	0.9742	0.1708

^a Se: standard error for differences of means.

^b Mean of the log value.

histopathological changes in the lung reflected small to severe peribronchiolar and perivascular inflammatory responses during both the sensitization and the challenge phase. Based on the difference in response during the challenge phase, it seems that the histopathological changes occurred before the development of the specific production of the IgE response. This holds true, in particular, for the coarse fraction during the challenge phase. The adjuvant activity of PM from different sites can be shown to be ranked as Lodz > Rome > Oslo > Amsterdam. The Zilk, which served as a control, with a minimum contribution by traffic, was comparable to Amsterdam and to a lesser degree comparable to Oslo.

After coexposure during the sensitization phase, eosinophils were found as the prominent cell type in the BAL that increased in number. This is in line with the increased responses of IgE and IgG₁ antibodies, and the presence of an eosinophil influx in the lung tissue. In contrast, neutrophils were predominantly present in the fine fraction, suggesting that coarse and fine PM might differ in their mechanisms of action due to differences in size or composition.

Cytokines found in the BAL, however, were generally not predictive for the antibody production and the histopathological observations in the lung. Cytokines such as IL-4, IL-5, and TNF α enhance IgE synthesis, while IFN γ inhibits IgE production (Purkerson and Isakson, 1992). Furthermore, the IgE production critically depends on a high ratio of IL-4 and IFN γ (Belardelli, 1995; So et al., 2000). In our study, a correlation between this ratio and IgE

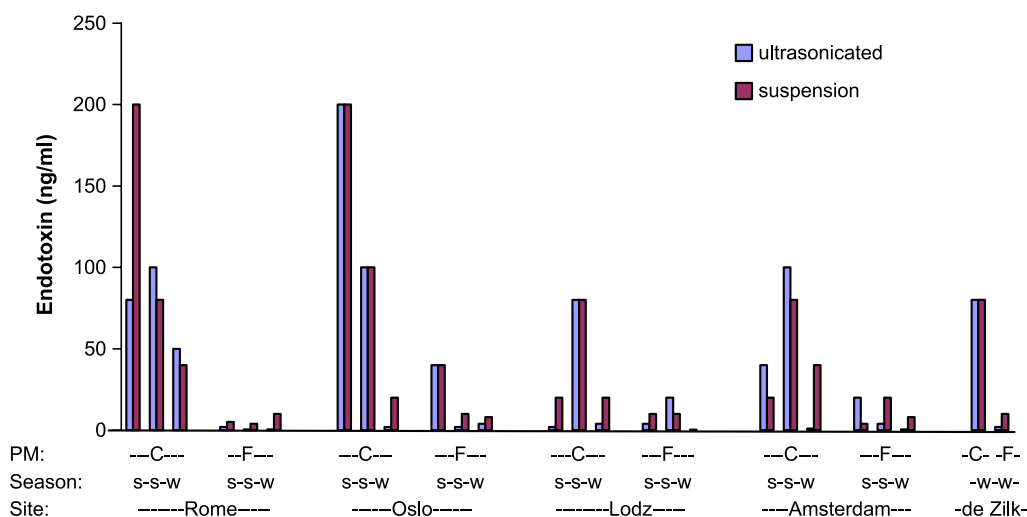


Fig. 4. Endotoxin concentrations in coarse and fine fractions from five sites in three seasons. C = coarse; F = fine; s-s-w = spring-summer-winter.

stituents of coarse and fine fractions will differ, and determine the toxicity (Becker et al., 2003; Kleinman et al., 2003) and its potency to provoke adjuvant activity. This subject will be addressed and discussed in a next paper.

For variable 3, season, seasonal differences were expected to influence the composition and the physicochemical characteristics of PM (Diociaiuti et al., 2001; Paoletti et al., 2002). Our results regarding the influence of season, however, did not reveal much differences. Only for the antibody responses, winter was more active compared to spring and summer.

Thus, a clear ranking was obtained for the four sites based on the antibody response and the histopathological observations. With respect to size, the coarse fraction resulted in more pronounced histopathological lesions than the fine fraction.

Because there is a physiological coherence between the chosen parameters, e.g., cytokines, triggering the Th2 pathway, resulting in inflammatory responses by predominantly eosinophils, IgE, and histopathological lesions, correlations between these parameters are evident. Table 16 shows a correlation for the sensitization phase between antibodies and the parameters of the histopathological lesions except for alveolitis. Moreover, in the BAL, the number of eosinophils and monocytes were also associated with the pulmonary lesions. In contrast, the interleukin production did not correlate to antibodies, lung lesions, and the number of cells in the BAL. There was, however, a correlation between IL-4 and IFN γ . These data suggests that for monitoring purposes, a restricted number of parameters can be chosen: in our study, concentrations of antibodies and histopathological observations were the most discriminatory parameters in comparison to BAL, cytokines, and inflammatory cells data.

Endotoxin is known to play an important role in allergy and asthma. Nowadays, data are present that endotoxin can protect children in the initial phase from allergy and asthma, while at a later age endotoxin may exacerbate atopic

[illegible]

diseases (Holla et al., 2002; Litonjua et al., 2002; von Mutius et al., 2000). Ambient air particles have been shown to contain endotoxin. We have measured this with the Limulus amoebocyte lysate test, as done by others (Holla et al., 2002; Huang et al., 2002; Ning et al., 2000; von Mutius et al., 2000). This test reacts with LPS, but the proclotting enzyme can also be activated with glucans, chemicals, proteins, and other agents (Hollander et al., 1993). Studies with macrophage cell lines show an association of cytokine products with the endotoxin content of PM to which the cells were exposed (Huang et al., 2002; Ning et al., 2000). Inactivation of endotoxin by polymyxin B abrogated almost all the TNF α induction by concentrated air particle suspensions and to a lesser extent the MIP-2 production (Huang et al., 2002; Ning et al., 2000). In the present study, we found no difference in endotoxin content, comparing the different sites. However, the coarse fraction contained relatively higher concentrations of endotoxin compared to the accompanying fine fraction, albeit that in the fine fractions endotoxin is still detected. This is in agreement with Huang et al. (2002) and Becker et al. (2003), who found the microbial products preferentially in the coarse fraction of PM. In our experiments, endotoxin is less evident in winter than in seasons with a higher temperature. Wan et al. (2000) demonstrated that coexposure with ovalbumin and endotoxin (40 ng/ml) potentiates allergen-specific airway inflammation and IgE responses. Although the immunization scheme of Wan et al. (2000) was different from ours, the adjuvant activity of PM based on the endotoxin content in the sample cannot be excluded. However, it is not expected that endotoxin is responsible for the increase in IgE and other biomarkers after coexposure to PM and ovalbumin, as demonstrated by the absence of correlation in 12 out of 16 parameters. Moreover, the four parameters with a positive correlation did not influence the statistical evaluation of the tested variables (site, PM, season, etc.).

In summary, we have shown PM collected at different geographic sites in Europe to evoke an adjuvant activity differing in severity. It remains to be determined if the chemical composition can explain the differences. No differences were found between coarse and fine; however, when histopathological lesions in the lung were considered, the coarse fraction induced more severe reactions there. The time of the year, at which PM has been collected, did not seem to affect the measured responses.

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References

- Anonymous, 1997. Genes for asthma? An analysis of the European community respiratory health survey. *Am. J. Respir. Crit Care Med.* 156, 1773–1780.
- Anonymous, 1998. Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC). *Eur. Respir. J.* 12, 315–335.
- Arshad, S.H., Karmaus, W., Matthews, S., Mealy, B., Dean, T., Frischer, T., Tsitoura, S., Bojarskas, J., Kuehr, J., Forster, J., 2001. Association of allergy-related symptoms with sensitisation to common allergens in an adult European population. *J. Invest. Allergol. Clin. Immunol.* 11, 94–102.
- Becker, S., Soukup, J.M., Sioutas, C., Cassee, F.R., 2003. Response of human alveolar macrophages to ultrafine, fine, and coarse urban air pollution particles. *Exp. Lung Res.* 29, 29–44.
- Belardelli, F., 1995. Role of interferons and other cytokines in the regulation of the immune response. *APMIS* 103, 161–179.
- Bouthillier, L., Vincent, R., Goegan, P., Adamson, I.Y., Bjarnason, S., Stewart, M., Guenette, J., Potvin, M., Kumarathasan, P., 1998. Acute effects of inhaled urban particles and ozone: lung morphology, macrophage activity, and plasma endothelin-1. *Am. J. Pathol.* 153, 1873–1884.
- Cassee, F.R., Fokkens, P.H.B., Leseman, D.L.A.C., Bloemen, H.J.Th., Boere, A.J.F., 2003. Respiratory allergy and inflammation due to ambient particles (RAIAP). Collection and characterisation of particulate matter samples from 5 European sites. RIVM, Report, 863001001.
- Ciccone, G., Forastiere, F., Agabiti, N., Biggeri, A., Bisanti, L., Chellini, E., Corbo, G., Dell'Orco, V., Dalmasso, P., Volante, T.F., Galassi, C., Piffer, S., Renzoni, E., Rusconi, F., Sestini, P., Viegi, G., 1998. Road traffic and adverse respiratory effects in children. SIDRIA Collaborative Group. *Occup. Environ. Med.* 55, 771–778.
- Demokritou, P., Gupta, T., Ferguson, S., Koutrakis, P., 2002. Development and laboratory performance evaluation of a personal cascade impactor. *J. Air Waste Manage. Assoc.* 52, 1230–1237.
- Diaz-Sanchez, D., Tsien, A., Fleming, J., Saxon, A., 1997. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. *J. Immunol.* 158, 2406–2413.
- Diaz-Sanchez, D., Jyräla, M., Ng, D., Nel, A., Saxon, A., 2000. In vivo nasal challenge with diesel exhaust particles enhances expression of the CC chemokines RANTES, MIP-1 α , and MCP-3 in humans. *Clin. Immunol.* 97, 140–145.
- Diociaiuti, M., Balduzzi, M., De Berardis, B., Cattani, G., Stacchini, G., Ziemacki, G., Marconi, A., Paoletti, L., 2001. The two PM (2.5) (fine) and PM (2.5–10) (coarse) fractions: evidence of different biological activity. *Environ. Res.* 86, 254–262.
- Dockery, D.W., Pope, C.A., Xu, X., Spengler, J.D., Ware, J.H., Fay, M.E., Ferris, B.-G.J., Speizer, F.E., 1993. An association between air pollution and mortality in six U.S. cities. *N. Engl. J. Med.* 329, 1753–1759.
- Dreher, K.L., Jaskot, R.H., Lehmann, J.R., Richards, J.H., McGee, J.K., Ghio, A.J., Costa, D.L., 1997. Soluble transition metals mediate residual oil fly ash induced acute lung injury. *J. Toxicol. Environ. Health* 50, 285–305.
- Duhme, H., Weiland, S.K., Rudolph, P., Wienke, A., Kramer, A., Keil, U., 1998. Asthma and allergies among children in West and East Germany: a comparison between Munster and Greifswald using the ISAAC phase I protocol. *International Study of Asthma and Allergies in Childhood. Eur. Respir. J.* 11, 840–847.
- Edwards, J., Walters, S., Griffiths, R.K., 1994. Hospital admissions for asthma in preschool children: relationship to major roads in Birmingham, United Kingdom. *Arch. Environ. Health* 49, 223–227.

- Fujimaki, H., Ushio, H., Nohara, K., Ui, N., 2001. Induction of the imbalance of helper T-cell functions in mice exposed to diesel exhaust. *Sci. Total Environ.* 270, 113–121.
- Gavett, S.H., Madison, S.L., Dreher, K.L., Winsett, D.W., McGee, J.K., Costa, D.L., 1997. Metal and sulfate composition of residual oil fly ash determines airway hyperreactivity and lung injury in rats. *Environ. Res.* 72, 162–172.
- Hamada, K., Goldsmith, C.A., Kobzik, L., 1999. Increased airway hyper-responsiveness and inflammation in a juvenile mouse model of asthma exposed to air-pollutant aerosol. *J. Toxicol. Environ. Health, Part A* 58, 129–143.
- Heo, Y., Saxon, A., Hankinson, O., 2001. Effect of diesel exhaust particles and their components on the allergen-specific IgE and IgG1 response in mice. *Toxicology* 159, 143–158.
- Hirsch, T., Weiland, S.K., von Mutius, E., Safeca, A.F., Grafe, H., Csaplovics, E., Duhme, H., Keil, U., Leupold, W., 1999. Inner city air pollution and respiratory health and atopy in children. *Eur. Respir. J.* 14, 669–677.
- Holla, A.D., Roy, S.R., Liu, A.H., 2002. Endotoxin, atopy and asthma. *Curr. Opin. Allergy Clin. Immunol.* 2, 141–145.
- Hollander, A., Heederik, D., Versloot, P., Douwes, J., 1993. Inhibition and enhancement in the analysis of airborne endotoxin levels in various occupational environments. *Am. Ind. Hyg. Assoc.* 54, 647–653.
- Huang, S.L., Cheng, W.L., Lee, C.T., Huang, H.C., Chan, C.C., 2002. Contribution of endotoxin in macrophage cytokine response to ambient particles in vitro. *J. Toxicol. Environ. Health, Part A* 65, 1261–1272.
- Jarvis, D., Burney, P., 1998. ABC of allergies. The epidemiology of allergic disease. *BMJ* 316, 607–610.
- Kavouras, I.G.K.P., 2001. Use of polyurethane foam as impaction substrate/collection medium in conventional inertial impactors. *Aerosol Sci. Technol.* 34, 46–56.
- Kim, Y.K., Baek, D., Koh, Y.I., Cho, S.H., Choi, I.S., Min, K.U., Kim, Y.Y., 2001. Outdoor air pollutants derived from industrial processes may be causally related to the development of asthma in children. *Ann. Allergy Asthma Immunol.* 86, 456–460.
- Kleinman, M.T., Sioutas, C., Chang, M.C., Boere, A.J., Cassee, F.R., 2003. Ambient fine and coarse particle suppression of alveolar macrophage functions. *Toxicol. Lett.* 137, 151–158.
- Kramer, U., Koch, T., Ranft, U., Ring, J., Behrendt, H., 2000. Traffic-related air pollution is associated with atopy in children living in urban areas. *Epidemiology* 11, 64–70.
- Litonjua, A.A., Milton, D.K., Celedon, J.C., Ryan, L., Weiss, S.T., Gold, D.R., 2002. A longitudinal analysis of wheezing in young children: the independent effects of early life exposure to house dust endotoxin, allergens, and pets. *J. Allergy Clin. Immunol.* 110, 736–742.
- Maejima, K., Tamura, K., Taniguchi, Y., Nagase, S., Tanaka, H., 1997. Comparison of the effects of various fine particles on IgE antibody production in mice inhaling Japanese cedar pollen allergens. *J. Toxicol. Environ. Health* 52, 231–248.
- Maejima, K., Tamura, K., Nakajima, T., Taniguchi, Y., Saito, S., Takenaka, H., 2001. Effects of the inhalation of diesel exhaust, Kanto loam dust, or diesel exhaust without particles on immune responses in mice exposed to Japanese cedar (*Cryptomeria japonica*) pollen. *Inhalation Toxicol.* 13, 1047–1063.
- Nel, A.E., Diaz-Sanchez, D., Ng, D., Hiura, T., Saxon, A., 1998. Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. *J. Allergy Clin. Immunol.* 102, 539–554.
- Nicolai, T., 2002. Pollution, environmental factors and childhood respiratory allergic disease. *Toxicology* 181–182, 317–321.
- Ning, Y., Imrich, A., Goldsmith, C.A., Qin, G., Kobzik, L., 2000. Alveolar macrophage cytokine production in response to air particles in vitro: role of endotoxin. *J. Toxicol. Environ. Health, Part A* 59, 165–180.
- Nitta, H., Sato, T., Nakai, S., Maeda, K., Aoki, S., Ono, M., 1993. Respiratory health associated with exposure to automobile exhaust. I. Results of cross-sectional studies in 1979, 1982, and 1983. *Arch. Environ. Health* 48, 53–58.
- Oosterlee, A., Drijver, M., Lebre, E., Brunekreef, B., 1996. Chronic respiratory symptoms in children and adults living along streets with high traffic density. *Occup. Environ. Med.* 53, 241–247.
- Paoletti, L., De Berardis, B., Diociaiuti, M., 2002. Physico-chemical characterisation of the inhalable particulate matter (PM10) in an urban area: an analysis of the seasonal trend. *Sci. Total Environ.* 292, 265–275.
- Purkerson, J., Isakson, P., 1992. A two-signal model for regulation of immunoglobulin isotype switching. *FASEB J.* 6, 3245–3252.
- Rusznak, C., Bayram, H., Devalia, J.L., Davies, R.J., 1997. Impact of the environment on allergic lung diseases. *Clin. Exp. Allergy* 27 (Suppl. 1), 26–35.
- Salonen, R.O., Pennanen, A.S., Halinen, A.I., Hirvonen, M.R., Sillanpaa, M., Hillamo, R., Karlsson, V., Koskentalo, T., Aarnio, P., Ferguson, S., Koutrakis, P., 2000. A chemical and toxicological comparison of urban air PM10 collected during winter and spring in Finland. *Inhalation Toxicol.* 12, 103 (Suppl.).
- Schwartz, J., Dockery, D.W., 1992. Particulate air pollution and daily mortality in Steubenville, Ohio. *Am. J. Epidemiol.* 135, 12–19.
- Schwartz, J., Dockery, D.W., Neas, L.M., 1996. Is daily mortality associated specifically with fine particles? *J. Air Waste Manage. Assoc.* 46, 927–939.
- Sly, R.M., 1999. Changing prevalence of allergic rhinitis and asthma. *Ann. Allergy Asthma Immunol.* 82, 233–248.
- So, E.Y., Park, H.H., Lee, C.E., 2000. IFN-gamma and IFN-alpha post-transcriptionally down-regulate the IL-4-induced IL-4 receptor gene expression. *J. Immunol.* 165, 5472–5479.
- Steerenberg, P.A., van Amsterdam, J.G., Vandebriel, R.J., Vos, J.G., Van Bree, L., van Loveren, H., 1999. Environmental and lifestyle factors may act in concert to increase the prevalence of respiratory allergy including asthma. *Clin. Exp. Allergy* 29, 1303–1308.
- Steerenberg, P.A., Withagen, C.E., Dormans, J.A., van Dalen, W.J., van Loveren, H., Cassee, F.R., 2003a. Adjuvant activity of various diesel exhaust and ambient particles in two allergic models. *J. Toxicol. Environ. Health, Part A* 66, 1421–1439.
- Steerenberg, P.A., van Dalen, W.J., Withagen, C.E., Dormans, J.A., van Loveren, H., 2003b. Optimization of route of administration for coexposure to ovalbumin and particulate matter to induce adjuvant activity in respiratory allergy in the mouse. *Inhalation Toxicol.* 15, 1309–1325.
- Strachan, D., Sibbald, B., Weiland, S., Ait-Khaled, N., Anabwani, G., Anderson, H.R., Asher, M.I., Beasley, R., Bjorksten, B., Burr, M., Clayton, T., Crane, J., Ellwood, P., Keil, U., Lai, C., Mallol, J., Martinez, F., Mitchell, E., Montefort, S., Pearce, N., Robertson, C., Shah, J., Stewart, A., von Mutius, E., Williams, H., 1997. Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC). *Pediatr. Allergy Immunol.* 8, 161–176.
- Takafuji, S., Suzuki, S., Koizumi, K., Tadokoro, K., Miyamoto, T., Ike-mori, R., Muranaka, M., 1987. Diesel-exhaust particulates inoculated by the intranasal route have an adjuvant activity for IgE production in mice. *J. Allergy Clin. Immunol.* 79, 639–645.
- Takafuji, S., Suzuki, S., Koizumi, K., Tadokoro, K., Ohashi, H., Muranaka, M., Miyamoto, T., 1989. Enhancing effect of suspended particulate matter on the IgE antibody production in mice. *Int. Arch. Allergy Appl. Immunol.* 90, 1–7.
- van Vliet, P., Knape, M., de Hartog, J., Janssen, N., Harssema, H., Brunekreef, B., 1997. Motor vehicle exhaust and chronic respiratory symptoms in children living near freeways. *Environ. Res.* 74, 122–132.
- van Zijverden, M., van der Pijl, A., Bol, M., van Pinxteren, F.A., de Haar, C., Penninks, A.H., van Loveren, H., Pieters, R., 2000. Diesel exhaust, carbon black, and silica particles display distinct Th1/Th2 modulating activity. *Toxicol. Appl. Pharmacol.* 168, 131–139.
- von Mutius, E., Braun-Fahrlander, C., Schierl, R., Riedler, J., Ehlermann, S., Maisch, S., Waser, M., Nowak, D., 2000. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin. Exp. Allergy* 30, 1230–1234.
- Wan, G.H., Li, C.S., Lin, R.H., 2000. Airborne endotoxin exposure and the

- development of airway antigen-specific allergic responses. *Clin. Exp. Allergy* 30, 426–432.
- Weiland, S.K., Mundt, K.A., Ruckmann, A., Keil, U., 1994. Self-reported wheezing and allergic rhinitis in children and traffic density on street of residence. *Ann. Epidemiol.* 4, 243–247.
- Westerholm, R., Egeback, K.E., 1994. Exhaust emissions from light- and heavy-duty vehicles: chemical composition, impact of exhaust after treatment, and fuel parameters. *Environ. Health Perspect.* 102 (Suppl. 4), 13–23.
- Wjst, M., Reitmeir, P., Dold, S., Wulff, A., Nicolai, T., Loeffelholz-Colberg, E.F., von Mutius, E., 1993. Road traffic and adverse effects on respiratory health in children. *BMJ* 307, 596–600.
- Wyler, C., Braun-Fahrlander, C., Kunzli, N., Schindler, C., Ackermann-Lieblich, U., Perruchoud, A.P., Leuenberger, P., Wuthrich, B., 2000. Exposure to motor vehicle traffic and allergic sensitization. The Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) Team. *Epidemiology* 11, 450–456.
- Zanobetti, A., Schwartz, J., Dockery, D.W., 2000. Airborne particles are a risk factor for hospital admissions for heart and lung disease. *Environ. Health Perspect.* 108, 1071–1077.