Respiratory Allergy and Inflammation Due to Ambient Particles – A European-wide Assessment (RAIAP)

Final Report

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1. Introduction

Three broad classes of exogenous factors, in addition to allergens, have been proposed as underlying causative or regulating factors for the induction and elicitation of respiratory allergies, namely dietary factors, early childhood microbial exposure and air pollutants. The RAIAP research project has focused on one specific type of air pollutant, namely ambient suspended particles. Given that there are widely different prevalence rates of respiratory allergies and asthma among the countries of Europe and that exposure to ambient particles is substantial in urban environments throughout Europe, the RAIAP project has addressed the following research question: may qualitative differences in particulate air pollution at different locations in part explain differences in prevalence or severity of respiratory allergies throughout Europe?

The overall objective of the RAIAP research project has been to assess the role of ambient suspended particles (PM$_{10}$ and PM$_{2.5}$) in causing local inflammation in the respiratory tract and induction and elicitation of respiratory allergies, in order to understand the underlying mechanisms for involvement of particles in the development of these diseases.

The specific objectives of this research project have been:

- To collect representative ambient particulate matter in 4 major European cities (one western (Amsterdam), one eastern (Lodz), one northern (Oslo) and one southern city (Rome)), as well from a sea-side background site (De Zilk, The Netherlands), using identical sampling methodology
- To characterise the particulate samples by physical-chemical parameters as well as to determine binding of allergens, endotoxins and β-glucans to the particles
- To screen all the collected samples for IgE-mediated allergic potential using the rodent popliteal lymph node assay and for inflammatory cytokine production in cultured epithelial lung cells and macrophages
- To verify the respiratory allergic, adjuvant and/or inflammatory potential by examining samples from the screens in rodent in vivo respiratory allergy and inflammation models
- To study the mechanisms underlying modulation of molecular and cellular functions of the immune system by particles
- To study the mechanisms underlying modulation of signalling pathways by particles in rat and human epithelial lung cell cultures
- To study the mechanisms underlying the modulating effects of particles on in vivo respiratory immune responses
- To summarise the scientific knowledge on the role of ambient particles for respiratory allergies and the implications of this knowledge for regulators, the general public and industry.
The RAIAP project has consisted of the following workpackages:

1. European-wide collection of particulate samples.
2. Physical-chemical and immunological characterisation of particulate samples.
3. Allergy screening.
4. Inflammation screening.
5. Inhalation allergy verification.
6. Inflammation verification.

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2. Materials and methods

2.1 European-wide collection of particle samples

Representative ambient particulate matter samples (PM2.5-10 and PM0.1-2.5) were collected in 4 major European cities (Amsterdam, Lodz, Oslo and Rome) at locations dominated by traffic emissions. In addition, one seaside location (De Zilk) was used as a control situation.

2.1.1 Description of sampling framework and the precise locations in 4 cities and a seaside site

Five locations in Europe were selected to sample coarse (10-2.5 µm) and fine (0.15 – 2.5 µm) particulate matter.

- The sampling site in Oslo was located along a throughway called Ring 2 (section Kirkeveien/Griffenfeldts gate) and placed approximately 20 meters from the kerb side of the road.

- The monitoring site in Lodz was situated in downtown (Srodmiescie) Lodz (15, Wieckowskiego street) near the theatre (Teatr Nowy). The sampling site was 30 meters from Zachodnia street with heavy traffic, however without a significant contribution of heavy trucks but with a lot of busses and near a traffic light.

- The monitoring site in Rome was situated at Viale Regina Elena nr 299, Northeast of the Termini railway station and the centre of Rome. The proprietor of the sampling site is the Instituto Superiore di Sanita (ISS). The ISS site was located in an area where various public buildings are present (hospitals, university, administrative centre of the National Research Council, and other public or private office buildings. Viale Regina Elena is a large street and the monitor is positioned at a distance of about 8/10-meters from the vehicle flux.

- The monitoring site in Amsterdam was situated nearby the Olympic, south of the centre and north-east of a busy motorway (Ring South). In addition, a busy city street (Amstelveense weg) is located 100 meters eastward of the site.

- With a prevailing westerly wind, De Zilk was selected as a control site with low traffic emissions and natural allergens such as pollen. The site is only about 800 meters away from the sea and surrounded with sand dunes and fields. The nearest traffic route is 500 meter eastward.

2.1.2 Sampling seasons

Within the RAIAP project there will be three periods of sampling for each city location that contrast in levels of pollen or temperature (associated with differences in combustion processes).

1. Pollen are present from March throughout September, but the composition changes during the time course. The project team had identified birch pollen was the pollen of interest. This type of pollen is released from early April until late May. Due to geographical and meteorological differences between the sampling cities it was possible to select consecutive sampling periods in Lodz and Oslo for the first of the two samplers (sampler A) that were used in the project. The shift in start of pollen period between Lodz and Oslo is approximately a month. This means that the sampling period cannot be longer then 4 weeks (+ 1 week for
transportation). Sampler B was used to sample in Amsterdam during April, which is the “birch” period in the Netherlands. The presence of birch pollen is seldom in Rome. However, the olivea pollen, to which a high number of people can develop allergic symptoms, is ubiquitous.

2. The “summer” period coincided for all sampling cities with another pollen period, the grass pollen. This sampling period lasts from the beginning of June until late August, and is distinguished from Period 1 by temperature and type of pollen. Post-sampling pollen analyses would have to reveal the dominant type of grass pollen. Since the PM levels in Oslo were expected to be much lower than 40 µg/m³, a prolonged sampling period during the summer period in Oslo was proposed.

3. The “winter” period is the period with low amounts of pollen for all sampling cities. On the other hand this is a period with high PM10 levels for some cities (especially Oslo and Lodz). These sampling period lasted from the middle of November until the end of February. The “De Zilk” location, used as a background control, was not expected to be influenced by pollen, in particular not in the suggested time of the year.

![Fig. 1. Map of Europe showing the sampling cities](image-url)
2.1.3 Particle sampling

High volume sampling

A high-volume cascade impactor with a multi-stage round slit nozzle impactor has been used to collect PM fractions on polyurethane foam (PUF) by impaction (Kavouras et al., 2001; Demokritou, 2002). This instrument, developed by the Environmental Chemistry Laboratory at the Harvard School of Public Health, can be used to collect samples for periods up to one week or longer (depending on how much particle pollution exists in the ambient atmosphere) at a flow rate of 900 liters per minute. The impactor cut-points used are for the range 10, 2.5 and 0.1 µm.

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 cut-offs. For the RAIAP project the selected stages have actual measured cutpoints at 9.9, 2.52, and 0.12 µm. There is also the option for a final stage, which uses a PUF filter to collect ultrafine particles (below the lowest used impactor cutpoint). This final stage has not been used in the RAIAP project.

The design features round slit acceleration jets, with corresponding PUF rings for impaction substrates. The jets are mounted in modular cylindrical housings. The housings are stacked in sequence, with the selected stages in proper order (by descending size cut-offs). A removable rain cover can be attached to the top stage.
Fig. 3. RAIAP PM collection device with the HVCI on top of a box with the pump to pull the air through the impactor (right panel) and the inside of the HVCI showing the pink PUF with collected PM (black) of the fine mode stage (left panel).

Low-volume sampling

Low volume sampling (LVS) has been carried out to provide specimens for transmission electron microscopical (TEM) characterisation of PM2.5/PM10 and for immunogold labelling techniques in the scanning electron microscope (SEM). The high volume sampler (HVS) was scheduled to run for 4 to 5 weeks. To prevent the TEM- and SEM-specimens from overloading, it was decided that the LVS should be operated with low flow (< 10 litres per minute), only for a restricted period (2 weeks) at each sampling site and run intermittently at pre-set intervals. LV sampling covered the same sites as the HVS. The HV- and LV-sampling pumps were normally switched on at the same time at the beginning of each sampling period; it was sampled only once at each site. The LV pump was run at 6 litres per minute resulting in approximately 2 litres per minute through each impactor.

The LV-sampling equipment was assembled in Oslo from commercially available parts and later integrated into the HVS housing in Bilthoven. Figure 4A shows the pumping unit connected to the “manifold” containing 3 impactor units, 2 for PM10 and one for PM2.5. Figure 4B gives a view of the Timer and Flow Controller. The impactors, which were purchased from URG Corp. (USA), contain a 25 mmØ filter pack with a 0.4 μm polycarbonate filter as collecting medium.
2.2 Physical-chemical and immunological characterisation of particulate samples

From each sampling site the PM$_{2.5}$ and one half of the PM$_{10}$ low-volume filters were vacuum-coated with a layer of approximately 10 nm carbon or germanium in a vacuum evaporator (JEE-4X, Jeol Ltd, Japan). Carbon replicas for examination in TEM were made according to a standardised ISO-procedure (103 12). The carbon extraction replicas were imaged and analysed in a JEM 100CX TEM (Jeol Ltd., Japan) fitted with an ISIS-XRMA detector (Link Ltd., UK). Particles >0.5 um were counted and classified to examine differences in particle type between the four European cities. The ISIS-analyser recorded elements from fluorine and above. To analyse carbon containing soot, germanium replicas were used and analysed in a Philips CM-12 connected to an EDAX XRMA detecting from beryllium and above. The amount of sulphur and oxygen was calculated with carbon as reference (1=100%). To examine any possible difference between the four cities and three seasons the relative intensity ratios (RIR) of carbon, sulphur and oxygen were compared (carbon divided by sulphur/oxygen = RIR).

The uncoated second half of the low volume PM$_{10}$ filter was immunolabelled directly on the filters by incubating with primary antibodies to pollen allergens, latex and β-glucans followed by secondary antibodies conjugated with gold spheres of 30 nm in size. The gold spheres were visualised as white spots in the backscatter imaging mode of a scanning electron microscope, SEM (840, Jeol Ltd., Japan). The appropriate positive and negative controls were included for each antibody. The density of labelling was recorded semi-quantitatively and ranged from no labelling (-), to weak (+), moderate (++) and strong labelling (+++). The density of labelling was judged during microscopy of at least 10 different areas on each specimen.

Antibodies used

Most antibodies were kindly given to us, and the donator’s names are stated accordingly: A monoclonal (moAb) antibody to latex, anti-Hev b 3, produced from non-ammoniated natural rubber latex (NRL) (Kurup, V.P., Wisconsin, USA) was applied on all filters. Three moAbs to birch were used, anti-Bet v 1and BIP 1 (Jensen-Jarulim, E., Wienna, Austria) and mP16 (Wiche, R., Langen, Germany), and one polyclonal antibody (poAb) rabbit anti-Bet v (purchased from Pharmacia Upjohn, Sweden) were applied to all filters from Oslo and Lodz and the spring filter from Amsterdam. Anti-timothy, Phl p 1, (Petersen, A., Borstel, Germany) was applied to all filters from Oslo, Lodz and Amsterdam. MoAbs to pellitory-on-the-wall,
Par j 1, and olive, Ole e 1, (Duffort, O., ALK-Abello, Spain) were used on all filters from Rome. A poAb to β-glucans (Doekes, G., Utrecht, Netherlands) was used on all filters from all seasons and cities.

The gold probes used were goat anti-mouse IgG-30 nm and goat anti-rabbit IgG-30nm (Bio-Cell Research Laboratories, Cardiff, UK). As positive controls latex glove (Safeskin Corp. Boca Raton, Florida, USA) and the respective pollen allergens (ALK, Copenhagen, Denmark) were used. Positive control for the β-glucans was Macrogard® Sol 2% BioTec, Tromsø, Norway). The negative controls were buffer replacing the primary antibody and isotype controls from mouse and normal rabbit serum (IgG).

2.3 Chemical characterisation of particulate samples

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). Samples were stored at –20 ºC to prevent changes in the composition of PM due to evaporation or chemical reactions.

Elemental composition

The supplied methanol sample containing 0.1 mg was evaporated to dryness. Initially an existing multi-element method with HR-ICPMS was used as a screening method, which was subsequently optimized and applied. This method included the following elements: Li, Be, B, Na, Mg, Al, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Sn, Sb, Ba, Ce, Nd, Sm, Au, Hg, Tl, Pb, Bi and U. Hg was not analysed due to losses during digestion caused by its volatility. Si was determined in all samples although the background Si (using glass containers) might be large. The calibration of Si was carried out as a one-point-calibration. Rh was used as an overall internal standard. The samples were quantified with external 2-point calibration. For quality control of the whole procedure and for controlling the completeness of digestion a standard reference material (0.01 g NIST 2710 “Montana soil”) was treated in the same way. Each digestion run included a procedural blank, which was used for the calculation of detection limits. The analyses were performed under the QC/QA system of the laboratory.

Secondary aerosol

Two of the supplied methanol samples, each containing 1 mg were evaporated to dryness, dissolved in water by sonication, filtered to remove remaining particles and analysed using ion-chromatography (Cl, NO₃ and SO₄) or photometry (NH₄). The anions were analysed using a Dionex guard column (AG-4A), separation column (Dionex AS-4A) and pulsed electrochemical detector (Dionex-PED). Detection limits were 0.003, 0.002 and 0.001 mmol/l for Cl, NO₃ and SO₄ respectively

Polycyclic aromatic hydrocarbons (PAH) and traffic tracers

The supplied methanol sample containing approximately 6 mg was evaporated to dryness. An aliquot (50 µl) of internal standards (6 deuterated PAHs and d2-C29-aaa(20R)-ethylcholestane) and 50 ml dichloromethane/isohexane (1:1) was added and the compounds were released by ultrasonic extraction. After filtration the extract was concentrated by evaporation to nearly dryness and mixed with 0.5 ml standard solution of 2,4-dichlorobenzyltetradecylether, which was used to correct for the variation of the injected volume. 1 µl was inject (splitless mode) at 290 ºC on a 30 m 0.25 mmWCOT DB-5MS column (film 0.25 µm) using a column temperature programmed from 90 – 160 – 290 ºC in a Fisons 8000 series
gas chromatograph equipped with an Interscience MD800 mass spectrometer with EI in SIR mode. Detection limits are approximately 0.1 ng/extract.

Endotoxins

Endotoxin concentrations were determined using a Limulus amebocyte lysate (LAL) test (Limusate, Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) as described by the manufacturer, with a detection level (<0.125 ng endotoxin/ml). The LAL test may be substituted for the U.S. Pharmacopeia (USP) Endotoxin Pyrogen Test (EPT) and is recommended for the quantitation of endotoxin in raw materials. The USP Bacterial Endotoxins Test is the official LAL test referenced in specific USP monographs (Bacterial Endotoxins Test, p. 1696-1697. USP 23 NF 18. 1994. U.S. Pharmacopeial Convention, Inc., Rockville, MD) and has been applied to PM samples used in the RAIAP in vitro studies. The method is based on cleavage by endotoxin of pyrochrome into 4-nitroaniline which absorbs at 540 nm and can be read in an ELISA-reader using microplates. It is more quantitative than the LAL-test. For more information:


Table 1. Overview of all elements and chemicals determined in high-volume PM samples

<table>
<thead>
<tr>
<th>Elements (total)</th>
<th>Light PAHs</th>
<th>Heavy PAHs</th>
<th>Traffic tracers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li</td>
<td>Al</td>
<td>NH4</td>
<td>Naphthalene</td>
</tr>
<tr>
<td>Be</td>
<td>Si</td>
<td>Cl</td>
<td>1-Methyl-naphtalene</td>
</tr>
<tr>
<td>Sr</td>
<td>Ca</td>
<td>NO3</td>
<td>Bifeny</td>
</tr>
<tr>
<td>Mo</td>
<td>Sc</td>
<td>SO4</td>
<td>2,5-Dimethyl-naphtalene</td>
</tr>
<tr>
<td>Cd</td>
<td>Ti</td>
<td>Br</td>
<td>Acenaphthylene</td>
</tr>
<tr>
<td>Sb</td>
<td>V</td>
<td></td>
<td>Acenaphthen</td>
</tr>
<tr>
<td>Ba</td>
<td>Cr</td>
<td></td>
<td>2,3,5-Trimethyl-naphtalene</td>
</tr>
<tr>
<td>La</td>
<td>Mn</td>
<td></td>
<td>Fluorene</td>
</tr>
<tr>
<td>Ce</td>
<td>Fe</td>
<td></td>
<td>Fenantrene</td>
</tr>
<tr>
<td>Nd</td>
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<td>Hg</td>
<td>Zn</td>
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<td>Dibenzo[a,h]antracene</td>
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<td>Ti</td>
<td>Na</td>
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<td>Benzo[g,h,i]peryline</td>
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<tr>
<td>U</td>
<td>As</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>Se</td>
<td></td>
<td>Lipopolysaccharide (LPS)</td>
</tr>
</tbody>
</table>

2.4 Allergy screening plus mechanisms

Animals

Specific pathogen-free 6-week-old female Balb/cA mice were obtained from Bomholtgård/-Taconic M&B A/S, Ry, Denmark. Mice were randomly distributed in groups of eight animals per cage for the allergy screening experiments and 5-8 mice for the mechanism studies. The mice were housed on Beekay GLP Bedding (B & K Universal AS, Nittedal, Norway) in type III macroron cages in filter cabinets (Scantainers). The animal room had a 12/12-hour light/dark cycle (30-60 lux in cages), and temperature and moisture were within recommended limits. The animals were given pelleted food (RM1, SDS, Essex, UK) and tap water ad libitum. The mice were rested for about one week before entering the experiments. The experiments were performed in conformity with the laws and regulations controlling experiments with live animals in Norway, and were approved by the local officer of the Experimental Animal Board under the Ministry of Agriculture in Norway.
Particulate material

Ambient air particles were collected in Oslo, Rome, Lodz, and Amsterdam during the spring, summer, and winter 2001/2002. In addition, particles were collected in de Zilk, a seaside background location in the Netherlands. Coarse and fine fractions of dry particle samples, extracted with methanol from the polyurethane foams, were provided after the successful particulate matter sampling campaign described under 2.1. Standard Reference Material 1650 was obtained from the National Institute of Standard Technology (Gaithersburg, Maryland, USA), whereas Ottawa dust (EHC-93) was kindly provided by Dr. Renaud Vincent.

Before injection, particles were suspended in Hank’s balanced salt solution (HBSS) and stirred at 18 °C over night using a magnetic stirrer. When appropriate, allergen was added to the suspension simultaneously with particles.

Selection of particulate matter for mechanisms studies

Based on the allergy screening results, we chose for the mechanism studies to use Diesel Exhaust Particles (DEP), the fine particle fraction from Rome (spring) and the fine particle fraction from Oslo (summer, in the following called Oslo1). These three particle suspensions had been found to give a qualitatively different adjuvant effect with regard to an allergy- or non-allergy (Th2 or Th1) biased antibody response, and were therefore in the mechanism studies compared as to how they modulated the primary cellular response in the lymph node.

In the allergy screening experiments, the fine particle fractions (0.1-2.5µm) had been found to have a stronger adjuvant activity than the coarse fractions (2.5-10 µm) on the antibody response to OVA. Therefore, we wanted to study if selected cellular parameters are more influenced by the fine particle fractions than the coarse fractions. We compared the cellular responses after injection of OVA together with the fine or coarse particle fractions from Amsterdam (spring), Lodz (summer) and Oslo (spring, in the following called Oslo2). The coarse and fine fractions of these three particle suspensions had shown a statistically significant (except Oslo) difference in their levels of OVA-specific IgE, IgE and IgG1, and IgG2a, respectively.

Allergen

As allergen, ovalbumin (Gal d1, OVA; chicken egg albumin, grade VII, Sigma, St. Louis, MO, USA) was used. The allergen was given in a dose of 50 µg per injection, whereas 100 µg of particles were given per injection, as in the allergy screening experiments. Particles were suspended in Hank’s balanced salt solution (HBSS; PAA Laboratories GmbH, Linz, Austria) with or without OVA and stirred at 18°C over night using a magnetic stirrer.

The popliteal lymph node assay

Mice were given a footpad inoculation with 20 µl of a suspension of particulate material in HBSS with or without the model allergen ovalbumin, or alternatively with allergen alone. The injection was done in a heel-to-toe direction using a 100 µl Hamilton syringe (Hamilton Bonaduz AG, Switzerland) with a 30 G needle. Six days later under CO2 anaesthesia, mice were killed by cervical dislocation, and popliteal lymph nodes from both hind legs were dissected out and kept on ice in HBSS. Further preparation of the lymph nodes was carried out essentially as described earlier. In short, after removal of excess fat and connective tissue, the lymph nodes were weighed. Each lymph node was then teased into a single cell suspension by means of two 18 G needles, and popliteal lymph nodes from both hind legs were dissected out and kept on ice in HBSS. Further preparation of the lymph nodes was carried out essentially as described earlier. In short, after removal of excess fat and connective tissue, the lymph nodes were weighed. Each lymph node was then teased into a single cell suspension by means of two 18 G needles, and the number of cells was determined in a Coulter Counter. Cell suspensions (1×10^6 cells per ml), 200 µl in triplicates, were put onto 96-well microtiter plates, before 25 µl ^3H-labelled thymidine (1.25 µCi) was added to each well. Incubation at 37 °C were carried out for 18-20 hours before harvesting and counting of
incorporated $^3$H-labelled thymidine in a scintillation counter. Weight, cellular and proliferative indices were calculated by dividing the value of the right (inoculated) lymph node with that of the left (non-inoculated). An index above one signifies reactivity.

**Serum samples**

For measurements of allergen specific antibodies, mice were sensitised by footpad inoculation with 20 µl of a suspension of OVA in the presence or absence of particulate material, exactly in the same way as for the popliteal lymph node assay. Untreated and HBSS-treated mice were included as controls. The mice were given a second injection of OVA twenty days after the first one, and five days later the mice were anaesthetised with CO$_2$ and exsanguinated by heart puncture. Sera were stored at −20 °C until analysed.

**ELISA for allergen specific antibodies**

The ELISA assay for allergen specific IgE has been described previously. In short, microtitre plates coated with rat anti-mouse IgE were allowed to capture IgE in mouse sera diluted 1:20. Capture was followed by sequential incubation with biotinylated OVA, preformed complexes of streptavidin and biotinylated alkaline phosphatase, and finally with $p$-nitrophenyl phosphate diluted in diethanolamine buffer. Detection of IgG1 and IgG2a anti-OVA, however, was done in microtitre plates coated with OVA in concentration 0.5 and 2.0 µg/ml, respectively. OVA was diluted in 0.05 M carbonate/bicarbonate buffer pH 9.6, and 100 µl was added per well and incubated at room temperature for one hour, followed by incubation at 4 °C over night. IgG1 and IgG2a anti-OVA was captured from sera diluted 1:3200 and 1:10, respectively, in 0.05 M Tris/HCl buffer pH 7.4 containing 0.05% Tween 20 (Tris/Tw) and 1% bovine serum albumin (BSA/Tris/Tw). Before capturing of IgG2a anti-OVA, the plates were blocked in BSA/Tr is/Tw for one hour at room temperature. Further detection was done as described in the IgE anti-OVA ELISA assay, except that biotinylated OVA was substituted with biotinylated rat anti-mouse IgG1 diluted 1:2000 or IgG2a diluted 1:500 (BD Biosciences, San Jose, California, USA) in Tris/Tw. The microtitre plates were washed five times in Tris/Tw between each incubation step. A serum pool from Balb/cA mice, immunised intraperitoneally with OVA and Al(OH)$_3$, was used for making a standard curve on each IgE and IgG1 plate. A standard curve was made on each IgG2a plate as well, using a serum pool from Balb/cA mice immunised subcutaneously at the base of the tail with OVA and CpG. Optical density was measured between 1.5 and 2.0 at 405 nm on a MRX Microplate Reader (Dynatech Laboratories, Chantilly, Virginia, USA). The amount of antibodies in arbitrary units was calculated by means of BioLinx software (Dynatech Laboratories, Chantilly, Virginia, USA).

**Cytokine ELISA**

The levels of IL-4, IL-10 and IFN-$\gamma$ in cell culture supernatants were determined by sandwich ELISA (Mouse DuoSets, R&D Systems Inc., MN, USA) according to the protocol provided by the manufacturer. Optical density (OD) values were measured on a MRX Microplate Reader (Dynatech Laboratories, Chantilly, VA, USA) connected to a PC using Revelation™ software (Thermo Labsystems, VA, USA). The optimal incubation time for the measurement of IL-4, IL-10 and IFN-$\gamma$ was found to be 24, 24 and 48 hours, respectively. Therefore, these time points are used in the presentation of the data.

**Lymphocyte preparation**

The popliteal lymph node (PLN) was excised and brought to the laboratory on ice in HBSS with 2% foetal calf serum (FCS; used to improve cell viability; GibcoBRL, Aukland, New Zealand). A single cell suspension was prepared from each lymph node by placing the node in
200 µl HBSS with 10% FCS on a Petri dish and teasing it into a single cell suspension using two 18-G needles. This cell suspension was aspirated three times in a 300 µl pipette to break up clumps, and transferred to glass tubes containing 800 µl HBSS with 10% FCS. The cell suspensions from the two PLN from each animal were pooled, and the cell concentration was measured at a Coulter Counter Z1 (Beckman Coulter Incorporated, FL, USA), and presented as the total cell number (10⁶) per PLN. The cells were centrifuged for 5 minutes on 200 x g, and resuspended as appropriate.

Cytokine production in vitro

Lymph node cells were prepared as described above and resuspended to 5 x 10⁶ cells/ml in culture medium. 5 x 10⁶ (1 ml) cells were added per well on 24 well cell culture plates (Corning Incorporated, NY, USA). ConA (Concanavalin A, Sigma, MO, USA) was added to a final concentration of 5 µg/ml. The cells were cultured at 37°C and 5% CO₂ for 24 and 48 hours. The plates were centrifuged at 200 x g for 10 minutes, and the supernatants were collected and stored at –70 °C until cytokine ELISA analyses.

Flow cytometry

Lymph node cells were prepared as described above, and resuspended to a concentration of 5 x 10⁶ cells/ml in phosphate buffered saline (PBS, pH=7.4) with 10% FCS. 100 µl cell suspension was transferred to centrifuge tubes, and a second wash was performed in 0.5 ml of PBS with 10% FCS. The cells were resuspended for staining in 100 µl PBS with 10% FCS containing the optimal (pre-diluted) concentration of FITC- or PE-conjugated monoclonal antibodies against CD3 (T lymphocyte marker), MHC class II (both from Southern Biotech, Birmingham, AL, USA) and CD19 (B lymphocyte marker), CD4 (T-helper cell marker), CD86 (costimulatory molecule) and CD23 (low affinity IgE receptor) (all from BD PharMingen, San Diego, CA, US) or the appropriate isotype controls. The cell samples were incubated with the antibodies at 4 °C for 30 minutes in the dark, thereafter washed twice in 1 ml PBS with 10% FCS. The cells were resuspended in 500 µl PBS with 10% FCS, and kept at 4 °C in the dark until flow cytometry analysis. A minimum of 10,000 lymphocytes were analysed.

The samples were analysed on an EPICS XL instrument (Coulter, FL, USA) connected to a PC using EXPO32™ Software (Applied Cytometry Systems, Sheffield, UK). Expression of surface markers were determined in two ways, 1) the relative number of positive cells 2) the relative amount of epitopes per cell measured as median fluorescence intensity. Positive cells were generally defined as the cell population (%) with fluorescence intensity stronger than 99% of the isotype control-stained cells. However, CD19⁺, CD3⁺ and MHC class II⁺ lymphocytes gave well-defined positive peaks, and for these surface markers the positive cells were defined as % cells in the positive peak. The expression of various surface molecules within the B and T cell populations was analysed by double staining for the specific marker with either anti-CD19 or anti-CD3. The results are then presented as the relative number of positive cells of either B or T lymphocytes, or the median fluorescence channel (i.e. the relative amount) of the detected molecule per B or T lymphocyte. Dead cells constituted a separate population on the scatter dot plot as verified by propidium iodide staining. The dead cell population was excluded from analyses by restricted gating on live cells. The loss of cells was proportional among lymphocyte populations.

Experimental design for mechanisms studies

To reduce the number of animals used, and still obtain a sufficient number of cells, in the mechanisms we injected the RAIAP particle suspensions into both hind footpads. In an introductory method experiment, injection of OVA+polystyrene particles of relevant size (0,1
µm) into one or two footpads were compared. Injection into one or both footpads did not induce different lymph node cell numbers on day 5 or different antibody responses to OVA on day 26 (data not shown). 20 µl of the different solutions were injected subcutaneously (s.c.) into both hind footpads (heel-toe direction) using a 100 µl Hamilton syringe (Hamilton Bonaduz AG, Switzerland) with a 30 G sterile needle (BD Medical Systems, Ireland). In the experiments comparing coarse and fine fractions, particles alone were not given. The primary cellular response in the popliteal lymph node (PLN) has earlier been shown to peak on day 5 after injection with OVA and polystyrene particles. Therefore, on day 5 after injection, the animals were killed by cervical dislocation under CO₂ anaesthesia, and both popliteal lymph nodes (PLN) were excised. The cells from the two PLN from each animal were pooled, and the total cell number per PLN was determined (Coulter Counter). The cellular response was studied by flow cytometric measurements of surface markers or quantification of cytokines in cell culture supernatants after ConA-stimulation. In the cytokine experiments, the PLN cells from 3 and 3 animals were pooled, to obtain sufficient cell numbers for stimulation with ConA for 24 and 48 hours. The levels of IL-4, IL-10 and IFN-γ in the cell supernatants were determined by sandwich ELISAs (see below).

All experiments were performed twice (limited by the available amount of collected particles) with largely similar results.

Statistical analysis

Statistical analysis was performed with SigmaStat ® Statistical Analysis System for Windows Version 2.03 (Jandel Scientific, Erkrath, Germany).

For the allergy screening data, the Student’s t-test was used for pairwise comparisons when normality and equal variance tests were passed. When normality or equal variance tests failed, or some of the test samples were outside the detection range, the Mann-Whitney Rank Sum Test was used. The following comparisons were made: All “particle and OVA groups” versus OVA. In the popliteal lymph node assay additional comparisons of the “particle alone” group versus DEP, and versus the respective “particle and OVA group” were done as well. Antibody arbitrary values outside the detection range was set to a value above the highest one measured in each group. It should be noticed that SigmaStat does not adjust for ties. The Kruskal-Wallis one-way analysis of variance on ranks was used for multiple comparisons of locations. Limit for statistical significance was set at P=0.05.

For the mechanism data, when normality and equal variance tests were passed, a one way ANOVA was run including data from all eight groups. When the ANOVA stated a statistically significant difference between the groups (P≤0.01) a parametric Tukey’s post test was performed. If the normality and/or equal variance tests failed, an ANOVA on Ranks was run, and the pairwise comparisons were performed by non-parametric Tukey’s post test. Tukey’s post test does perform all possible pair-wise comparisons, however for the mechanism data the following comparisons were considered: all “OVA+particles” groups versus OVA, HBSS or the respective “particle alone”, “particles alone” versus HBSS, OVA alone versus HBSS and comparisons between all three “particles alone” or all three “OVA+particles”. In the experiment comparing coarse and fine fractions, the comparisons of “OVA+particle” versus HBSS or OVA, as well as between all “OVA+particle” groups were considered. Limit for statistical significant differences between groups was set at P≤0.05.

The allergy screening experiments could for practical reasons be performed only once. In the mechanism studies, repeat experiments were performed, and differences are presented as statistically significant if they were so in both experiments, unless otherwise is noted.
2.5 Inflammation screening plus mechanisms

Reagents

Culture medium, Nutrient Mixture F-12 Ham (Cat. No N-3520), deferoxamine mesylate, 2,7-dichlorofluorescin diacetate (DCFH-DA), N-acetyl-L-cysteine (NAC), lipopolysaccharide (LPS), polymyxin B sulphate, horseradish peroxidase-conjugated goat anti-rabbit IgG and propidium iodide (PI) were obtained from Sigma Chemical Company, St. Louis, MO, USA. Foetal bovine serum (FBS) was obtained from Gibco BRL, Paisley, Scotland. The antibiotics ampicillin and fungizone were purchased from Bristol-Myers Squibb AB, Denmark, penicillin/streptomycin and the culture medium Williams’ E were from BIO Whittaker, Walkersville, MD, USA). The ERK-inhibitor 2-amino-3-methoxyflavone (PD98059), the p38 inhibitors 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316) and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190), the EGF-receptor kinase inhibitor 4-(3-chloroanilino)-6,7-dimethoxyquinazolone (AG1478) and the inhibitor of the Src family of protein tyrosine kinases 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP2) were obtained from Calbiochem (Nottingham, UK). Monoclonal antibodies raised against p-ERK and total ERK-2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibodies against p-p38 and p-JNK were from Cell Signaling (Beverly, MA, USA). The Cytoscreen enzyme-linked immunosorbent assays (ELISA) for analysis of IL-6, IL-8 and MIP-2 were obtained from Biosource International, Camarillo, CA, USA. Rat TNF-α and IL-6 ELISA assays were obtained from R&D Systems Europe, Oxon, UK. The Trans-AM™ transcription factor assay kit for the NF-κB subunit p50 was obtained from Active Motif (Rixensart, Belgium). The standard reference material urban PM (SRM 1648), collected in St. Louis, MO, USA, and diesel particulate matter (SRM 2975) were purchased from National Institute of Standards & Technology, Gaithersburg, MD, USA. Ottawa dust (EHC-93) was kindly provided by Dr. Renauld Vincent, Health Canada, Ottawa, Ontario, Canada. All other chemicals used were purchased from commercial sources at the highest purity available.

Particle preparation

Ambient air particles were collected in the cities Amsterdam, Lodz, Rome and Oslo during spring, summer and winter 2001/2002. Particles representing the control situation were collected at the seaside site DeZilk. Coarse (PM\textsubscript{10-2.5}) and fine (PM\textsubscript{2.5}) fractions of dry particle samples extracted from the polyurethane foams (PUF) were provided after the successful PM sampling campaign described in Deliverable 1 and 2 (Cassee, F.R. and A.J.F. Boere, 2001; Cassee et al., 2002). Based on the results obtained in the in vitro inflammation screening studies, as well as the characterisation of the particulate matter samples described in Deliverable 5 (Cassee et al., 2003), particle samples were selected for the mechanistic studies.

Particle suspensions

Particles were suspended in 0.9% NaCl to a concentration of 20 mg/ml and stirred over night. Required amounts of the 20 mg/ml suspension was further diluted in culture medium to obtain a stock solution of 2 mg/ml. Before use in the exposure studies, these stock solutions were stirred on a magnetic stirrer overnight. Dry particle samples, as well as particle suspensions, were stored at - 20 °C.

Particle extracts

For extraction of water-soluble components, stock solutions of particles (2 mg/ml in culture medium) were centrifuged (2500 x g) for 20 minutes to remove the particles.
Cell cultures

Different types of epithelial cells from rat and human lung as well as rat alveolar macrophages were used to investigate if the collected PM samples varied in their potential to induce markers of inflammatory processes.

Primary rat lung cells

Male rats (Crl/Wky) were purchased from Harlan, UK. Alveolar macrophages were collected by airway lavage. Alveolar type 2 cells and Clara cells were isolated as previously described (Låg et al., 1996). In brief, the lungs were perfused via the pulmonary artery with a calcium- and magnesium-supplemented phosphate buffer before removal. Subsequently, the lungs were digested with protease and minced and filtered to give a single cell suspension. This was followed by cell separation by size, using centrifugal elutriation. Cell viability was assayed by trypan blue exclusion and exceeded 90% for all cell populations. Purity of the type 2 cells and the alveolar macrophage populations exceeded 90%, purity of the Clara cell populations exceeded 60%. The type 2 cell and Clara cell populations were suspended in Williams E medium and purified by differential attachment. The purified cell populations were added to 35 mm 6 well culture dishes and cultured in medium with 5% heat-inactivated FBS for two days before exposure. Alveolar macrophages were suspended in RPMI medium and added to 35 mm 6 well culture dishes. Non-attached cells were removed after 1 hour, whereas the attached macrophages were used for exposure.

Human epithelial lung cells

The human alveolar cell line A549 (American Tissue Type Culture Collection, Rockville, MD, USA) and Small Airway Epithelial Cells (SAEC, BioWhittaker, Inc., Walkersville, MD, USA) isolated from human tissue, were used as model systems. The A549 cells were maintained in Nutrient Mixture F12 HAM (F12K) medium with 10% heat-inactivated FBS, whereas the SAEC cells were maintained in Small Airway Epithelial Cell Growth Medium BulletKit® (SAGM™ BulletKit®) with 1% BSA-FAF. Cells were grown in 35mm 6-well culture dishes.

Exposure of cell cultures to particles

Previous studies with exposure of lung cells to different types of particles revealed that an FBS-free environment at the time of exposure, and the subsequent hours, resulted in a more sensitive exposure system compared to a system with continuous presence of FBS. Hence, FBS-supplemented culture medium was replaced by culture medium without FBS before exposure. At exposure, particle suspensions, or corresponding volumes of particle extracts, were added to the various cell cultures. FBS was then added 6 hours after start of exposure. The different cell types used in this study were exposed to the various particle samples for up to 20 hours (and up to 40 hours with the A549 cells) in a total of 1 ml/ well of medium (1.5 ml/well for the SAEC cells). The culture medium was subsequently collected and centrifuged to remove cells (250 x g) and to remove particles (2500 x g). Supernatants were stored at –70 °C until further analysis of inflammatory cytokines. Responses after exposure to coarse and fine fractions collected within each season were studied in the same experiment and repeated at least three times. The reference particles Ottawa dust and diesel (SRM 2975) were included in each setup, whereas the coarse and fine fraction from De Zilk were studied in the same experiments as the winter samples. When inhibitors were used in experiments, cell cultures were pre-incubated for 1 h before particle exposure.
Cytokine assays

Analysis of the inflammatory cytokines was performed using enzyme-linked immunosorbent assay according to the manufacturer’s manual. The increase in colour intensity was quantified using a plate reader with software (TECAN Sunrise with Magellan V 1.10).

Cell survival

Cell suspensions were stained with PI (5 µg/µl). Uptake of PI by damaged cells was analysed using fluorescence microscopy (250-300 cells from controls and exposed cell cultures were counted).

Immunoblotting (Western technique)

After incubation with 100 µg/ml (A549 cells) or 25 µg/ml (type 2 cells) for 30 or 240 min, PBS supplemented with 1 mmol/L phenyl-methylsulfonyl fluoride was added to the cells. The cells were then scraped off the dishes and diluted in sample buffer (2% SDS, 3.75% β-mercaptoethanol, 10% glycerol, 0.002% bromphenol blue and 0.06 mol/L Tris-HCl, pH 6.8). After ultra-sonication (5 x 1 sec.) and boiling, particles were removed from the samples by centrifugation (2500 x g for 10 min.). Subsequently, 12.5 µg protein was subjected to 10% SDS-PAGE (Låg et al., 1996). Molecular weight markers were included. The proteins were electro-transferred to nitrocellulose membranes and the blots were stained for proteins with Ponceau S. Blots with equal loading of protein were probed with monoclonal phospho-ERK MAP kinase antibody, phospho-p38 MAP kinase antibody (Thr 180/Tyr 182) or phospho-SAPK/-JNK MAP kinase antibody (Thr 183/Tyr 185) in 5% BSA overnight at 4 ºC, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:2,000). The Western blots were developed using the ECL chemiluminescence system according to the manufacturer’s instructions (Super Signal West Dura, Pierce Biotechnology, Rockford, IL, USA).

Flow cytometry

Generation of reactive oxygen species (ROS) was analysed using EPICS XL flow cytometer (Coulter, FL, USA) equipped with an argon laser (488 nm emission) and EXPO32™ Software (Applied Cytometry Systems, Sheffield, UK). A549 cells were cultured in 6-well plates until confluence. The cell cultures were pre-incubated for 30 min at 37 °C with DCFH (20 µM) before addition of particle samples. Control and exposed cells were collected 90 min after exposure to particles and stored on ice until flow cytometric analysis. From each sample 10,000 events were analysed with forward light scatter gain set in the linear mode and fluorescence gain set in the logarithmic mode. Green fluorescence from DCFH was measured in the FL1 channel, and the results are expressed as the median fluorescence intensity.

NF-κB subunit specific assay

The NF-κB subunit p50 (NF-κB1) was quantified using the Trans-AM™ transcription factor assay kit. This is an ELISA-based assay in which the NF-κB binding site is immobilised onto the 96-well plate. Nuclear cell extracts were added to the wells and assayed for p50 according to the manufacturers instructions. Optical density was determined on a spectrometer complete with software at 450 nm (TECAN Sunrise with Magellan V1.10).

2.6 Inhalation allergy verification plus mechanisms

The mice (BALB/cByJ.ico) were randomly allocated to a group (n=7-8). For intranasal sensitization at day 0 and 14 the mice were slightly anaesthetised with halothane. At the moment of awakening, 0.4 mg/ml ovalbumin (grade V, Sigma Chemical, St. Louis, MO.
USA) in a total of 50 µl saline was administered into both nostrils. For the challenge on day 35, 38 and 41, mice were again intranasally exposed to OVA at the same concentration. To study the effects of particles, for sensitisation the suspension of particles and OVA were mixed at a final concentration of 0.4 mg/ml OVA and 3 or 9 mg/ml particles, which resulted in a dose of 20 µg ovalbumin/mouse and 150-450 µg PM/mouse. Animals were sacrificed at day 42. Blood and bronchoalveolar lavage fluid (BAL) were collected and cytokine analysis including interleukin-4 (IL-4), IL-5, Interferon-γ (IFNγ) and TNF-α. Sediments were used for determination of total cell number and for producing cytospin preparations on which differential cell counts were performed after staining with May-Grunwald Giemsa. The ELISA was performed for specific ovalbumin IgE, IgG1 and IgG2a. The histopathological lesions in the lung were semi-quantitatively and blindly scored. For the more mechanisms studies male Balb/cJ, Balb/cIl4<tm2Nnt> (deficient for IL-4), C57BL/6 and B6.129P2-Nos2tmLau (deficient for iNOS) mice were obtained from Jackson Laboratory, Bar Harbor, ME, USA. Balb/c/AnPt and C.D2-Vil6 (reduced macrophage activation) mice were obtained from RIVM, Bilthoven, The Netherlands. EHC-93 (Ottawa dust), which was used as the standard reference material, contained 50 ng endotoxin/ml (in a 3 mg/ml PM suspension). The statistical differences between groups were determined using an analysis of variance (ANOVA).

2.7 Inflammation verification

Young (6-8 weeks, 185-220g) male Wistar rats were obtained from the Nofer Institute of Occupational Medicine, Poland breeding colony. The animals were kept under conventional conditions with free access to standard rodent pelleted diet and tap water. All experiments were performed according to international and institutional guidelines for animal care. Rats divided into groups of 8 animals each, were anaesthetized using halothane ~2.0% v/v and were instilled via a transoral tube with 1.0 and 2.5 mg PM. Control rats received intratracheally saline (negative control) or 1.0 and 2.5 mg of EHC-93 dust (positive control). Twentyfour hours after instillation the rats were killed under ketamine/xylazine anaesthesia and the right lung was lavaged in situ twice through the trachea with PBS (2.5 ml per lavage). In BAL fluid the following measurements were performed: total protein, albumin, Clara cell protein, LDH, MIP-2, TNFα and, total cells and cell differentials, by means of generally used methods. Albumin was determined by latex immunoassay with antibodies from ICN Biomedicals (Costa Mesa, CA, USA). Clara cell protein as a marker of brionchiolar epithelium was determined by latex immunoassay (Bernard 1992; Halatek and Jakubowski 1991). Specific rabbit antibodies against CC16 and a standard for CC16 based on the purified protein were obtained as described earlier (Halatek et al.1998). The analysis of the inflammatory cytokines MIP-2, TNFα BAL levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits from BioSource Int., (Camarillo, CA, USA). Histopathological analyses were also performed. The left lung was inflated with 10% neutral buffered formalin to obtain the proper dilatation of alveoli. In addition to lung, liver, kidneys, spleen, large and small intestine were fixed in 10 % neutral buffered formalin. Specimen from above organs and tissues were embedded in paraffin, sectioned to a thickness of 4-6 µm and stained with hematoxylin and eosin according to standard protocols. Covariance analysis and cluster analysis was used for statistical analysis.
3. Results

3.1 European-wide collection of particle samples

High-volume sampling particle collection

<table>
<thead>
<tr>
<th>Sampler City</th>
<th>Spring</th>
<th>Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRU-1 Oslo</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>MRU-2 Lodz</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>MRU-3 Amsterdam</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>MRU-4 De Zilk</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Fig. 5. Schedule of the RAIAP sampling campaign. By using two identical samplers two parallel campaigns were set up. The boxes marked with X were used within RAIAP

The sampling campaign was performed according to the schedule presented in Figure 5. Slight deviations from the intended schedule were caused by delays during transport (e.g. due to Foot and Mouth disease epidemic in the Netherlands), but were not expected to affect the objectives of the project.

The collected masses of the particle collection campaign are listed in Table 2. In general, the masses in Oslo were lower than expected. Furthermore, the “pollen” peak was also missed during the first Spring period. These two facts were the reason for sampling twice in Oslo during the Spring. Collected masses of the Amsterdam samples were also slightly lower compared to Rome and Lodz. The explanation could be the distance to the nearest street: a busy motorway dominates the location in Amsterdam and the distance is approximately 200 m and the nearest busy street was approximately 100 meters away mostly downwind of the sampler. Dilution of the motorway emissions has probably caused the lower collected masses. Collected masses were usually higher in Winter compared to the other two seasons, which might be caused by contributions of combustion processes for heating purposes as well as the generally more intense inversion layers during the Winter season.
Table 2. Summary of PM collection campaign. Oslo was sampled twice due to low collected masses and apparent lack of pollen in the air.

<table>
<thead>
<tr>
<th>Site</th>
<th>Season</th>
<th>Fraction</th>
<th>Spring</th>
<th>Amsterdam</th>
<th>Summer</th>
<th>Winter</th>
<th>De Zilk</th>
<th>-</th>
<th>Coarse</th>
<th>-</th>
<th>Fine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Coarse</td>
<td>Coarse</td>
<td>Fine</td>
<td>Coarse</td>
<td>Fine</td>
<td></td>
<td>Coarse</td>
<td></td>
<td>Fine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mass (mg)</td>
<td>229</td>
<td>277</td>
<td>204</td>
<td>301</td>
<td></td>
<td>881</td>
<td></td>
<td>527</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mg/week)</td>
<td>57</td>
<td>69</td>
<td>51</td>
<td>75</td>
<td></td>
<td>88</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>Site</td>
<td>Season</td>
<td>Fraction</td>
<td>Spring</td>
<td>Lodz</td>
<td>Summer</td>
<td>Winter</td>
<td>Oslo</td>
<td></td>
<td>Spring</td>
<td></td>
<td>Coarse</td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Coarse</td>
<td>Coarse</td>
<td>Fine</td>
<td>Coarse</td>
<td>Fine</td>
<td></td>
<td>Coarse</td>
<td></td>
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<td></td>
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<td></td>
<td>Mass (mg)</td>
<td>399</td>
<td>560</td>
<td>334</td>
<td>401</td>
<td></td>
<td>432</td>
<td></td>
<td>1,476</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mg/week)</td>
<td>100</td>
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<td>86</td>
<td></td>
<td>295</td>
</tr>
<tr>
<td>Site</td>
<td>Season</td>
<td>Fraction</td>
<td>Spring</td>
<td>Rome</td>
<td>Summer</td>
<td>Winter</td>
<td>Oslo</td>
<td></td>
<td></td>
<td></td>
<td>Coarse</td>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Coarse</td>
<td>Coarse</td>
<td>Fine</td>
<td>Coarse</td>
<td>Fine</td>
<td></td>
<td>Coarse</td>
<td></td>
<td>Winter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mass (mg)</td>
<td>442</td>
<td>508</td>
<td>368</td>
<td>397</td>
<td></td>
<td>455</td>
<td></td>
<td>989</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(mg/week)</td>
<td>111</td>
<td>127</td>
<td>92</td>
<td>99</td>
<td></td>
<td>91</td>
<td></td>
<td>198</td>
</tr>
</tbody>
</table>

Low Volume-sampling

The results from the LV-sampling is summarised in Table 3 with regard to location, season, fractions, masses and concentrations. With the impactors used here the Mass- and Concentration-values represent the PM10 and PM2.5 fractions, rather than the threshold values of 2.5 and 10 μmØ, which for the HV-sampling is referred to as Fine- and Coarse-fractions.

One would have expected similar mass values for the two PM10 samples collected at each sampling site. As seen from Table 3 the deviation is, in certain instances, rather significant; for instance the two summer specimens in Lodz, the mass values are 114 and 74 μg. Apart from different flow rates through the samplers, it is difficult to think of any particular reason for this deviation. The way sampling was organised it was not possible to monitor the flow rate through each impactor on location. After checking the total flow rate to 6 litres per minute, it was assumed that the flow through each impactor was close to 2 l/min minute. Deviations may have occurred if there was different flow resistance among the filter packs.

In Table 3 there is another odd observation if one looks at the PM2.5-values from Amsterdam (winter), and De Zilk (October and November): these values are higher than those of the corresponding PM10. This should hardly be expected unless the majority of ambient particles present at the time of sampling were heavily dominated by PM2.5. The results presented in WP 1: Deliverable 1 (HV-sampling) would probably tell more about that.

Yet another strange observation is that mass values of the two parallel series carried out at De Zilk in October should have resulted in close to similar values; this was not the case and it is difficult to explain the reason for this.
<table>
<thead>
<tr>
<th>Location</th>
<th>Season</th>
<th>Type**</th>
<th>Start date*</th>
<th>End date*</th>
<th>Mass* (µg)</th>
<th>C.(µg/m³)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lodz</td>
<td>Spring</td>
<td>PM10</td>
<td>10.04.01</td>
<td>24.04.01</td>
<td>51,00</td>
<td>22,9</td>
</tr>
<tr>
<td>Lodz</td>
<td>Spring</td>
<td>PM2.5</td>
<td>10.04.01</td>
<td>24.04.01</td>
<td>47,00</td>
<td>21,1</td>
</tr>
<tr>
<td>Lodz</td>
<td>Spring</td>
<td>PM10 (Ge)</td>
<td>10.04.01</td>
<td>24.04.01</td>
<td>26,00</td>
<td>11,7 a)</td>
</tr>
<tr>
<td>A'dam</td>
<td>Spring</td>
<td>PM10</td>
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Explanation to the asterisks *, ** and ***, please see next page.
a) Filter collapsed due to rain; Mass underestimated. b) only PM10(Ge) did not collapse.
c) Impactor “manifold” and impactor housing redesigned.

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*) Based on locally estimated PM loads for the different seasons (Table 2), low volume sampling (LVS) was carried out for 2 weeks with ON (min) and OFF (hrs) intervals ranging between 4 and 9 min and 1 and 2 hrs, respectively. The individual settings should have resulted in filter loads below 100 µg; higher loads normally obscure structural details when imaged in the transmission electron microscope (TEM).

**) Particle sizes up to PM10 and PM2.5, respectively. PM10(Ge) is a sampling filter which has been vacuum coated with an approx. 10 nm layer of Germanium to facilitate analytical TEM.

***) Considering the effective interval pumping time, the average PM concentration, C (µg m⁻³), is calculated for each site.
3.2 Physical-chemical and immunological characterisation of particulate samples

Morphology and elemental composition

Examination of the morphological and elemental composition of PM$_{10}$ and PM$_{2.5}$ in carbon and germanium extraction replicas showed that the collected samples did not differ significantly among the four European cities during the three seasons. In both PM$_{10}$ and PM$_{2.5}$ specimens, the majority (>90%) of the ambient particles consisted of combustion particles that aggregate into grape-like clusters (soot aggregates) and silicates of pure or of complex nature containing Mg, Al, K, Ca, and Fe. Silicates have their origin in dust resuspended from soil and roads. In addition all samples, except for Amsterdam in spring, small spherical particles were present. Most of the sulphur dioxide is released from industrial sources. Particles containing sulphur and calcium were also present in most samples. Since these particles were non-fibrous, they may have been formed through chemical reactions between calcium in concrete and acidic aerosols such as sulphur dioxide gas present in ambient air. The pure iron particles found in the specimens were either irregular in shape and size, typical for particles made through abrasion or grinding processes, or spherical typically produced by melting at high temperatures. An overview of the morphology and elemental composition of these particles are shown in Figure 6. The relative intensity ratios (RIR) of the elements C, S and O were highest in spring specimens collected in Lodz, Rome and Amsterdam. The specimens collected in Oslo revealed only minor seasonal differences (Figure 7).

![Fig. 6. Transmission electron micrographs and their corresponding XRMA spectra of different kind of particles found in the ambient air samples a) quartz (Si) and sulphur (S) (arrows), b) complex silicate (arrow), c) pure iron (Fe) particles (arrows); XRMA spectrum of spherical iron alloy d) pure calcium (Ca) and gypsum (CaS) particles; Spectre of the CaS particle only, e) plaster (gypsum) fibre (CaS), f) the small, rectangular structures (arrowheads) present in the winter specimen from Amsterdam only, are probably organic since no XRMA spectre was identified.](image-url)
Fig. 7. Based on calculations from the XRMA spectra, the relative intensity ratio (RIR) for the elements carbon (C), sulphur (S) and oxygen (O) reveals higher levels from all spring specimens and sampling sites, except from Oslo where there are only small seasonal differences. (No winter specimens were investigated from Rome due to an overload of particles on the filters.)

Immunogold labelling

The mass concentrations and the semi-quantitative results from the immunolabelling of PM$_{10}$ filters from all four cities and all three seasons are summarised in Table 4. The semi-quantitative results are given for the combustion particles and for the organic material where found (marked with asterisk). The labelling showed that the antibody to latex (Hev b 3) weakly labelled the combustion particles, of all sizes, on all filters. The anti-Timothy (Phl p I) labelled the combustion particles on filters from all three seasons indicating a cross-reactive epitope, while antibodies to Birch (Bet v I, MP16, BIP I and rabbit anti Bet) were all negative. Plant materials from all seasons were strongly labelled with anti-β-glucan. The two pollen antibodies to the allergens pellitory-on-the-wall and olive, labelled both plant material and combustion particles on all filters from Rome. Although combustion particles dominated on all filters, silicates, sulphur and iron were recognised, but never labelled. All positive controls were moderately labelled, but no labelling was observed on the negative controls. The results show that airborne antigens corresponding to several allergens are adsorbed to and carried by combustion particles and micronic particles of vegetal origin in the polluted air (see examples in Figure 8).
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a) heavy rain during sampling, * labelling density of organic material/plants, □ very little material on the filter, nd not done
Fig. 8. Scanning electron microscopy of LV filters immunolabelled with antibodies against pollen allergens and β-glucans. The gold labelling are visualised as white spots in BEI mode (upper micrographs in each pair). The same micrograph in SEI mode, show the morphology of the carbon particles and aggregates on the same collection filter (lower micrographs). Note that the gold particles label carbon particles and aggregates only and not the filter background.
3.3 Chemical characterisation of particulate samples

Overall composition

The overall chemical composition of each main location is presented in Figure 9 for the spring season. More detailed results can be found in Deliverables Nos. 3 and 5. Although the values are projected as percentages, substantial differences were found among the locations in the spring, whereas this was less evident for the summer and the winter. In general more secondary aerosol was measured in the fine fraction compared to the coarse fraction. The distribution of these four fractions across the three seasons seems rather stable for Amsterdam, highly variable for Rome whereas the other two locations (Lodz, Oslo) show moderate variability.

Elemental composition

Since a lot of emphasis is put on transition metals as (part of) the causal factors a selection of these constituents is presented in Figure 13. In general concentrations of metals were high in Rome with the exception of zinc. The location in Amsterdam is characterised by relatively high magnesium (Mg) and vanadium (V) levels in the coarse fraction during all three seasons. Lead (Pb) and zinc (Zn) were relatively high in the fine mode fraction in Lodz with the highest levels in the Winter. Iron (Fe), manganese (Mn), aluminium (Al), chromium (Cr) and copper (Cu) were usually higher in the coarse fraction, whereas Zn, Pb, nickel (Ni), vanadium (V) are higher in the fine mode of PM. The former set of elements is typical for crustal material. The latter suite is often related to combustion processes such as traffic. There is no clear contrast in composition among the three seasons.

Inorganic constituents

Nitrate, sulphate, ammonia, chloride, sodium and potassium are the major constituents of the non-organic part of the collected PM (Figure 10). The processing of the samples might cause losses of ammonium nitrate and as such the found levels might be low estimates. Sea-spray aerosol, containing sodium chloride, is a substantial part of the samples collected at the sites in Amsterdam and De Zilk. Depending on the atmospheric aging of the particulate matter causing the depletion of chloride due to reaction with acids in the atmosphere the ratio of sodium and chloride might shift to higher values than expected based on the composition of sea spray. Ammonia and sulphate are predominantly found in the fine mode fraction, irrespective the sampling site or season. Nitrate is measured in both the fine and coarse mode fraction of PM, and the highest levels were observed for the Dutch locations. This observation complies with experiences in other studies. High levels of potassium were measured at the location in Rome.

Organic constituents

The amounts of light and heavy PAHs, as well as hopanes and steranes, are presented in Figure 11. The highest yield of PAHs are derived from the Lodz location, in particular during the Winter season. The PAHs are found in both size fractions. The PAHs may reflect the type of fossil fuel used for heating in Lodz, namely coal instead of natural gas or oil. A more diverse pattern is found for the traffic markers hopanes and steranes. Although generally higher amounts are found in the fine fraction, relatively high amounts of steranes were observed for both Winter and Summer samples of the location in Oslo, as well as the Winter samples from Rome and the Summer samples from Lodz. This pattern is not reflected in the hopane levels, which seem to be more dominant is both the Spring and Winter from Lodz, Oslo and Rome.
Fig. 9. Overall composition of PM in spring samples
Fig. 10. Inorganic contents of PM of Spring, Winter and Summer PM samples from Amsterdam, Lodz, Oslo and Rome. The pie charts are for De Zilk.

Fig. 11. Examples of seasonal size fraction variation in light and heavy polycyclic hydrocarbon among 5 locations in Europe used in the RAIAP project.
Endotoxins

The levels of endotoxins were measured using a pyrochrome assay. Clearly, coarse PM contains more endotoxin per mg PM compared to the fine fraction. The difference is 5-6 times for the Summer and Winter period and 13 times in the Spring. The levels of endotoxin in the coarse fraction are relatively low in the more polluted cities (Rome, Lodz) as well as in the seaside location (De Zilk) compared to the other cities in Spring. This pattern changes with season.

3.4 Allergy screening

The popliteal lymph node assay

In the popliteal lymph node assay it was, for practical reasons, impossible to screen all ambient fractions from one season in the same experiment. To be able to compare the different sampling sites, the coarse and fine fractions were therefore examined in separate experiments. In all experiments DEP was included as a positive control. DEP and ambient
particles were given in doses of 50 and 100 µg per mouse, respectively. The model allergen OVA was given in doses of 50 µg per mouse. These doses were based on dose-response experiments using DEP and Ottawa dust (data not shown).

The popliteal lymph node response to the coarse and fine fractions of ambient particulate matter collected in the four European cities of Oslo, Rome, Lodz, and Amsterdam, in the spring, summer, and winter season, respectively, was examined. No, or only weak weight, cellular, and proliferative responses to OVA was obtained. In the presence of particles, however, the OVA response showed a statistically significantly increase. Particles, with a few exceptions, elicited only weak popliteal responses in the absence of OVA. Following a pairwise comparison of particles from each location a significantly stronger weight and cell number response in the presence than in the absence of OVA was observed for a majority of the fractions. An exception was the coarse summer samples, as well as the coarse spring sample from Rome, which seemed to have stronger irritant activity than the remaining ambient fractions. Beyond that, no clear seasonal differences were observed. Although some statistically significant differences between locations were observed (e.g. Lodz winter coarse fraction sample showed comparatively strong adjuvant activity), few marked differences were apparent. It is noticeable that both the coarse and fine sample from the rural background location de Zilk did not stand out from the others. Taken together, both coarse and fine fractions from all locations, collected in all three seasons, seem to significantly increase the popliteal lymph node response to OVA, measured as weight, cellular, and to some extent proliferative responses. Thus, the results indicate that ambient particulate matter has an adjuvant activity in general. As an example, in Figure 14 are shown the popliteal lymph node responses for coarse particles collected in spring.
Fig. 14. Weight (upper panel), cell number (middle panel), and proliferation indices (lower panel) in the poplitcal lymph node 6 days after footpad inoculation of Balb/cA mice with coarse ambient particles collected in the spring season. An index above 1 signifies reactivity. Individual results (symbols) and medians (columns) for groups of eight mice are shown. The following comparisons were made: all “particle alone” groups versus DEP (* under “particle alone” columns). All “particle and OVA groups” versus OVA (* under “particle and OVA” columns), and versus the respective “particle alone” group (+). Brackets denote statistically significant differences between similar preparations from different cities.
Allergen specific antibody responses

To further examine the adjuvant activity of the ambient particulate matter, its ability to elicit an allergen specific IgE and IgG1 response was analysed, indicating an allergy-related Th2-immune response. In addition, the production of allergen specific IgG2a was measured, which indicates a non-allergic Th1-immune response. Coarse and fine particulate matter collected in the same season were examined in one experiment. Again, DEP were included as a positive control, whereas ambient dust collected in Ottawa (EHC-93) was included as a commercially available reference particle. DEP and ambient particles were given in doses of 50 (30 in the spring experiment) and 200 µg per mouse, respectively. The model allergen OVA was given in doses of 50 µg per mouse. These doses were based on dose-response experiments using DEP and Ottawa dust.

For all seasons, the antibody response to OVA was usually significantly stronger as compared to the control groups, including untreated and HBSS-treated mice. Moreover, all the European ambient particle fractions, from all seasons, significantly increased the IgG1 response to OVA. With exception of a few coarse ones, all fractions also significantly increased the IgE anti-OVA response. As an example, the results for the summer season are shown in Figure 15.

To find out whether a lower particle dose would discriminate better between particles from the different locations than the relatively high dose of ambient particles used (200 µg per mouse), the experiment with ambient particles collected in the spring season was repeated using the same dose of OVA (50 µg per mouse), but reducing the dose of ambient particles to 50 µg per mouse. The lower dose of ambient particles resulted in weaker allergen specific IgE responses, and for some fractions the statistical significance of the adjuvant activity was lost. Consequently, to ensure that all fractions with an innate adjuvant activity were detected, the allergy screening of the summer and winter particulate matter was carried out with doses of 200 µg per mouse.

All fine fractions, and some of the coarse ones, gave a significant increase in the IgG2a response to OVA as well. Thus, it seemed as the ambient particles not only are able to stimulate an allergic Th2-, but also a non-allergic Th1-immune response.

Although not observed for all locations, it seemed as the fine particles have a stronger adjuvant activity than the coarse ones following a pairwise comparison of the coarse and fine particles from the same location. This observation is confirmed following a pairwise comparison of allergen specific IgE, IgG1, and IgG2a from all locations, both for the coarse and fine particulate matter. Taken together, few marked differences in the antibody response were observed neither between the locations, nor between the seasons. The most marked difference in allergy adjuvant activity was between fine and coarse particles.
Fig. 15. The upper, middle and lower panels show the levels of IgE, IgG1, and IgG2a anti-OVA, respectively, in Balb/cA mice 5 days after footpad inoculation with OVA, and 25 days after inoculation into the same footpad as specified in the figure above. Data for summer particles are shown. Individual (points) and medians (columns) for groups of eight mice are shown. Stars indicate a statistically significant stronger response compared to OVA. Brackets denote statistically significant differences between similar preparations with particles from different cities, and between coarse and fine fractions from the same location.
3.5 Inflammation screening

Macrophages

Different levels of TNF-α release were observed after exposure of macrophages to particulate matter (PM) collected at the various sampling sites. The coarse fractions collected in Lodz during pollen and summer seasons demonstrated the highest potency to induce TNF-α (Figure 16, lane A). Furthermore, the coarse particles collected in Rome during pollen and summer season seemed to induce higher levels of TNF-α compared to the samples from Oslo and Amsterdam. Regarding the increase in cytokine release observed with the winter samples, no marked differences in potency were observed among the coarse fractions. The most obvious seasonal variation in potency was demonstrated among the coarse fractions collected in Lodz. Compared to the pollen and summer samples, a much lower potency to induce TNF-α release was observed with the winter sample. With respect to the fine fractions collected in the four cities, no significant increase in TNF-α was observed in any season (Figure 16, lane B). The coarse fraction of PM sampled at De Zilk induced a marked increase in TNF-α release. Results after exposure to the coarse and fine fraction of PM sampled at this seaside background site are included in the figures showing results from the winter samples. The reference particles Ottawa dust and diesel are included through all seasons.
Fig. 16. Release of TNF-α from alveolar macrophages exposed to coarse (panel A) or fine fraction (panel B) of ambient PM collected in different European cities during pollen (upper), summer (middle) and winter season (lower). Results are mean ± SEM, n = 3.

A relatively similar order of potency between the coarse fractions was found with respect to IL-6 (Figure 17, lane A). The pollen and summer samples from Lodz were most potent, and the coarse fractions from Rome induced higher levels of IL-6 than the samples from Oslo and Amsterdam. With respect to the samples from Oslo, however, the results indicate a higher potency of the summer sample compared to the pollen sample. These results show that the coarse fractions of particles collected during summer may be equally or more potent than particles collected during pollen season. Among the coarse fractions collected during winter, the order of potency seemed different from the results seen in the pollen and summer season. The samples from Rome and Amsterdam induced higher levels of IL-6 than the samples from Lodz and Oslo. With respect to the fine fractions, a small increase seemed to be induced, at least by the summer particles (Figure 17, lane B). As seen with TNF-α, the coarse fraction from De Zilk elicited a marked increase in IL-6 release.
Fig. 17. Release of IL-6 from alveolar macrophages exposed to coarse (panel A) or fine fraction (panel B) of ambient PM collected in different European cities during pollen (upper), summer (middle) and winter season (lower). Results are mean ± SEM, n = 3.

Taken together, both size fractions, sampling site and sampling season seem to be of significance for the potency of PM to induce cytokines in macrophages. With respect to PM collected in the four cities, the coarse fractions seemed more potent than the fine. Furthermore, marked differences in potency were demonstrated between samples collected during pollen and summer season in the different cities. The samples from Lodz demonstrated the highest potency, whereas samples from Oslo and Amsterdam seemed least potent. A corresponding site-specific variation in potency was not observed between the samples collected during winter. Regarding seasonal variations at each sampling site, the coarse fractions collected in Lodz demonstrated the most obvious variation in potency. The coarse fractions collected in Rome, however, seemed equally potent during all seasons. With respect to the samples collected at the seaside site, a similar situation, with the coarse fraction being more potent than the fine, was observed.
Type 2 cells

When type 2 cells were exposed to the various PM samples, different levels of MIP-2 release were induced (Figure 18). As with macrophages, the coarse fractions were more potent than the fine. With respect to the coarse fractions collected in the pollen season, the Lodz and Rome samples induced higher levels of MIP-2 than the samples from Oslo and Amsterdam (Figure 18, lane A). Among the summer samples, the coarse fraction from Lodz demonstrated the highest potency, whereas no marked differences in potency were observed between the coarse fractions from Rome, Oslo and Amsterdam. The observed decrease in MIP-2 at exposure above 25 µg/ml of the coarse particles from Amsterdam may be explained by a decrease in cell survival (Figure 20, lane A). Regarding the winter samples, coarse fractions from Lodz, Oslo and Amsterdam were less potent compared to the respective pollen and summer samples. The most obvious differences in seasonal variation in potency to induce MIP-2 were observed within the samples from Lodz, whereas no marked seasonal variation was revealed between the coarse fractions from Rome. As described earlier, a similar observation was made with the macrophages. As compared to the macrophages, the coarse fraction from the seaside elicited a marked increase in cytokine release in the type 2 cells. The potency was at the same level as the coarse fractions collected in Rome. Only minor responses were observed by the fine fractions from the urban sites.

With respect to IL-6, the spontaneous release from control cells was approximately ten times higher in type 2 cells than in macrophages. This is in agreement with previous findings (Becher et al., 2001). Apart from that, the type 2 cells demonstrated a relatively similar pattern for particle-induced IL-6 release compared to the macrophages (Figure 19). Regarding the responses after exposure to the coarse fractions collected during pollen and summer season, however, the relative differences in potency between the particle samples seemed less in the type 2 cells compared to macrophages. With respect to seasonal variation, the coarse fractions of the pollen and summer samples from Lodz induced high levels of IL-6, whereas the winter sample was less potent (Figure 19, lane A). A corresponding variation was not observed within the coarse samples from the other cities. None of the fine fractions collected in the cities induced any marked increase in IL-6 release in the type 2 cells (Figure 19, lane B). Compared to the other fine fractions, however, the fine fraction collected in Rome during pollen season seemed to be more toxic (Figure 20, lane B). This probably explains the decrease in IL-6 release observed at exposures above 25 µg/ml (Figure 19, lane B). The coarse fraction collected at De Zilk was equally potent to induce IL-6 as the coarse fractions from Rome and Amsterdam.

The results from the type 2 studies are in agreement with the results obtained with the macrophages. Corresponding differences in potency among PM samples collected at different sites were demonstrated. With respect to PM from the urban sites, the coarse fractions were more potent than the fine fractions. Furthermore, seasonal variations were demonstrated. Similar to the urban samples, the coarse fraction of the sample collected at the seaside site was more potent than the fine.
<table>
<thead>
<tr>
<th>Season</th>
<th>Coarse fraction of PM (µg/ml)</th>
<th>Fine fraction of PM (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oslo (C)</td>
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<td></td>
</tr>
<tr>
<td>Rome (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lodz (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A’dam (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ottawa dust</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diesel (SRM 2975)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollen</td>
<td></td>
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<td>Oslo (F)</td>
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<tr>
<td>Rome (F)</td>
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<tr>
<td>Lodz (F)</td>
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<tr>
<td>A’dam (F)</td>
<td></td>
<td></td>
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<tr>
<td>Ottawa dust</td>
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<td></td>
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<tr>
<td>Diesel (SRM 2975)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oslo (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rome (C)</td>
<td></td>
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<tr>
<td>Lodz (C)</td>
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<tr>
<td>A’dam (C)</td>
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<tr>
<td>De Zilk (C)</td>
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<tr>
<td>Ottawa dust</td>
<td></td>
<td></td>
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<tr>
<td>Diesel (SRM 2975)</td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 18. Release of MIP-2 from type 2 cells exposed to coarse (panel A) or fine fraction (panel B) of ambient PM collected in different European cities during pollen (upper), summer (middle) and winter season (lower). Results are mean ± SEM, n = 3.
Fig. 19. Release of IL-6 from type 2 cells exposed to coarse (panel A) or fine fraction (panel B) of ambient PM collected in different European cities during pollen (upper), summer (middle) and winter season (lower). Results are mean ± SEM, n = 3.
Clara cells

The coarse and fine fractions of the summer samples were chosen to study cytokine responses in the Clara cells. This was based upon the findings that PM collected during summer season represented the most potent samples with respect to induction of cytokine release in macrophages and type 2 cells. Furthermore, observed differences in potency between particles from the four cities were most obvious for particles collected during summer. The different coarse fractions induced a dose-dependent increase in MIP-2 release in the Clara cells (Figure 21A). However, marked differences in the potency between samples from the different cities, as observed with macrophages and type 2 cells, were not observed in this cell type. With respect to the fine fractions, only a minor increase in MIP-2 was observed (Figure 21B). In contrast to the results demonstrated in macrophages and type 2 cells, only the coarse fraction from Lodz was able to induce a slight increased in IL-6 release in the Clara cells (Figure 22A). No increase was observed after exposure to the fine fractions (Figure 22B). The different pattern of responses to the coarse fractions between the epithelial type 2 cells and the Clara cells may be due to differences in their secretion of proteins important in the defence against toxic substances. Several surfactant proteins, as well as Clara cell specific proteins (CCSP), may be of importance for the observed differences in responses between the two cell types.
A549 cells

The human alveolar cell line A549 was chosen as a model system for human type 2 cells. With respect to the coarse particles, a dose-dependent increase in IL-8 release was observed at all seasons (Figure 23, lane A). In contrast to observations after exposure of the primary rat lung cells to the pollen samples, where the sample from Rome was equally or more potent than the other samples, the sample from Rome was significantly less potent than the other samples to A549 cells after 20 h of exposure. This was also the case after 40 h of exposure (Figure 24A). The relatively low levels of IL-8 induced by the sample from Rome was not due to a higher toxicity of the sample, since no decrease in cell survival was observed (Figure 25A). With respect to toxicity of the pollen samples, however, the samples from Lodz and Oslo seemed to exert a more toxic effect compared to the samples from Rome and Amsterdam. The coarse fractions collected during summer season tended to be more potent to induce IL-8 in A549 cells than the coarse fractions collected during pollen season, and the sample from Oslo seemed most potent. With respect to the winter samples, no marked differences in potency between samples from the different cities were observed. Furthermore, the potencies seemed lower compared to the summer samples. With respect to the fine fraction of the collected PM samples, a clear dose-dependent increase in IL-8 was observed at all seasons (Figure 23, lane B). This is in contrast to what was demonstrated in the
macrophages, type 2 cells and Clara cells. With respect to the fine fractions from the pollen season, the potency to induce IL-8 seemed to be higher by the particles from Lodz and Rome compared to particles from Oslo and Amsterdam at 20 h. This difference was also demonstrated after 40 h of exposure (Figure 24B). At this time point, a dose-dependent increase in toxicity of the fine particles from Lodz and Rome may explain the marked decrease in IL-8 release (Figure 25B). Among the fine fractions collected during winter, the fine sample from Oslo was most potent. And in contrast to the other results, the fine fraction appeared more potent than the corresponding coarse. Thus, the seasonal variations in potency of samples within one city showed a somewhat different picture in the A549 cells compared to macrophages and type 2 cells. Together with the fine samples from Oslo, the most obvious variations were observed within the coarse samples from Rome. Furthermore, no higher potency of the coarse back-ground particles from De Zilk, as compared to the fine fraction, was observed in the A549 cells. The marked decrease in IL-8 release after exposure to the reference particle SRM2975 (diesel) may be due to binding of the protein IL-8 to diesel particles. This has previously been confirmed by others (Paul Borm, Janet Lightbody, personal communication).
Fig. 23. Release of IL-8 from A549 cells after 20h of exposure to coarse (panel A) or fine fraction (panel B) of ambient PM collected in different European cities during pollen (upper), summer (middle) and winter season (lower). Results are mean ± SEM, n = 3.
Fig. 24. Release of IL-8 from A549 cells after 40h of exposure to coarse (A) or fine fraction (B) of ambient PM collected in different European cities during pollen season. Results are mean ± SEM, n = 3.

Fig. 25. Cell survival in A549 cells after exposure to coarse (panel A) or fine fraction (panel B) of ambient PM collected in different European cities during pollen (upper), summer (middle) and winter season (lower). Cell cultures were exposed for 40h (pollen season) or 20h (summer and winter season). Results are mean ± SEM, n = 3.
Small Airway Epithelial Cells (SAEC)

The small airways contain ciliated and non-ciliated epithelial cells including the Clara cells. Characteristics of the SAEC cell cultures used in this study are therefore assumed to be influenced by the presence of Clara cells. As with the Clara cells isolated from rat lung, the SAEC cell cultures were exposed to the coarse or fine fractions of the summer samples. Among the coarse fractions, the Lodz and Oslo samples seemed most potent to induce IL-8 release in this cell type, whereas the Amsterdam sample was least potent (Figure 26A). With respect to the fine fractions, the sample from Lodz showed the highest potency to induce increased release of IL-8 (Figure 26B). Compared to the rat Clara cells, more obvious differences between PM from different cities were demonstrated in these human small airway epithelial cell cultures.

Fig. 26. Release of IL-8 from Small Airway Epithelial Cells (SAEC) after 20h of exposure to coarse (A) or fine fraction (B) of ambient PM collected in different European cities during summer season. Results are mean SEM, n = 5.

Importance of soluble components

Based on the same criteria as for the experiments with Clara cells and SAEC cells, particles collected during summer season were chosen for the experiments aimed to study if soluble components of the particles were responsible for the observed effects. A549 cells were exposed to particle suspensions, as in the previous experiments, or corresponding volumes of particle-free extracts, for 20 h. As shown in Figure 27, no marked increase or site-specific variation in IL-8 release was observed. These results showed that the particle-induced cytokine release demonstrated in this study was not primarily caused by water-soluble components. Consequently, the particles themselves, or particle-bound components, constitute the main characteristics determining the potency of ambient air particles to induce inflammatory markers in the present in vitro systems.
3.6 Inhalation allergy verification

We have analysed the influence of three variables on a number of critical parameters in an *in vitro* respiratory allergy model: 1) site, 2) PM size fraction and 3) season. With respect to site, results of the low (3 mg/ml) and high doses (9 mg/ml) particles were used to construct a dose response relationship. These data were presented as the best fit of a doses response curve (0, 3 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 5 sites resembled the kinetics of the standard reference material EHC-93. In most cases when the highest response was reached, a plateau or even decrease of the adjuvant activity was attained with higher concentrations. This decrease might be due to increased toxicity of the high dose for these particular samples, which adjuvant activity was already high at 3 mg concentration. In this way PM samples could be ranked based on the chosen comparison level of 20% of the positive reference standard. As Table 5 shows, for e.g. Lodz coarse spring less (2 mg/ml) and for Oslo more (6 mg/ml) sample was needed to reach the cut off point of 20% of the IgE antibodies against OVA of the standard reference. Regarding the IgE concentration and the sum of the pathological lesions, for the coarse fraction the sites were ranked as followed: 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 Lodz and Rome. It was found that ‘De Zilk’ and Amsterdam did not differ in composition except for Na and Cl concentration. It was concluded that a dose of 3 mg/ml is effective to screen samples with high activity and 9 mg/ml for samples with low activity in the ovalbumin mice model.

Table 5. Minimal concentration (mg/ml) of coarse and fine particles to evoke an adjuvant activity-response after co-exposure to PM and ovalbumin which is comparable to the mean response in the group of ovalbumin alone + approximately 2 times the standard variation. ∼ > 9 mg/ml

<table>
<thead>
<tr>
<th>IgE</th>
<th>Coarse</th>
<th>Fine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
<td>Summer</td>
</tr>
<tr>
<td>Rome</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Oslo</td>
<td>~</td>
<td>6.0</td>
</tr>
<tr>
<td>Lodz</td>
<td>~</td>
<td>2.0</td>
</tr>
<tr>
<td>Amsterdam</td>
<td>~</td>
<td>8.6</td>
</tr>
</tbody>
</table>

**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 | ~ |
With respect to size (variable 2), particulates were categorized into a coarse (> 2.5 µm) and fine (0.1 - 2.5 µm) fraction. Both coarse and fine PM fractions can stimulate an allergic response mediated by IgE and IgG1, when ovalbumin is co-exposed with PM during the sensitization phase. Taking all results together (Table 6) it was found that the fine fraction was more active than the coarse. Histopathological examination revealed changes in the lung which were more pronounced due to the coarse fraction exposures both during the sensitisation and challenge phase at the 3 mg PM concentration, when compared to the fine fraction, but when all data were taken together no significant difference were present between fine and coarse. Neutrophils were more strongly induced by the coarse than by the fine fraction at a 9 mg/ml concentration.

The particle size defined by coarse and fine fraction is based on the collection method rather than on the size instilled. In our study, fractions are collected on foams. After collecting and eluting the material, the particles will not be identical to their original size, although from scanning electron microscopical examination of clumps of material, one can distinguish an architecture build up from coarse or fine particles (not shown). For variable 3, season, seasonal differences were expected to influence the composition and the physico-chemical 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 and spring was more active compared to summer.

Table 6. Statistical evaluation of site, size and season

<table>
<thead>
<tr>
<th></th>
<th>site</th>
<th>size</th>
<th>season</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>fine&gt;coarse</td>
<td>spring=winter&gt;summer</td>
</tr>
<tr>
<td>IgG1</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>fine&gt;coarse</td>
<td>spring=winter&gt;summer</td>
</tr>
<tr>
<td>IgG2a</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>fine&gt;coarse</td>
<td>spring=winter&gt;summer</td>
</tr>
<tr>
<td>Peribronchial inflammation</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Perivascular inflammation</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Hypertrophy of mucous cells</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Alveolitis</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>ns</td>
<td>spring=summer=winter</td>
</tr>
<tr>
<td>Eosinophils influx</td>
<td>Lodz&gt;Rome=Oslo&gt;A’dam</td>
<td>ns</td>
<td>spring=summer=winter</td>
</tr>
</tbody>
</table>

For statistical analysis see M&M,

ns = not significant between fine and coarse or between seasons; >: p<0.05; ns: not significant

In the study, 4 samples were separated into a water soluble and non-soluble fraction. Differences were very clear cut on individual samples, being active in the water soluble or in the insoluble fraction. However, only one out of 4 samples (Lodz spring fine) showed an effect in the soluble fraction. It is not excluded that due to insufficient centrifugation a part of the fine PM was retained in the soluble fraction giving rise to the observed adjuvant activity. A number of in vitro and in vivo studies have been carried out to investigate which part is the most active. In vitro studies have shown that insoluble fractions of concentrated ambient particle suspensions have most of the biological effects on AMs and certain particle adsorbed factors such as endotoxin. However, soluble components of residual oil fly ash particles containing metal constituents have been shown to be active in vivo. Moreover, others have presented evidence that in human the soluble components have an effect on the pulmonary and haematological systems.
3.7 Inflammation verification

Epithelial cells and alveolar macrophages participate in the modulation of inflammatory responses by producing a number of biologically active substances including a broad range of cytokines that can exert effects on inflammatory cells. All particle samples after intratracheal instillation induced cellular response such as increased number of alveolar macrophages and granulocytes. Both histopathological examination and BALF analysis confirmed the inflammatory response in the lung. In general, the highest response was noted for the coarse fraction at the higher dose level. The degree of response depended on the dose of particle sample.

In histopathology study dust particles surrounded by numerous macrophages and/or granulocytes or granuloma-like structures (conglomerates of macrophages and dust particles) were observed (Table 7). Coarse particles collected in all towns particularly in spring (pollen) season caused the more prominent cellular response. Fine PM samples induced ambiguous responses in lung of rats. This relation is non-specific. For example, the metal-rich particle sample from Rome and the PAH-containing sample from Lodz evoked inflammatory reactions with a similar degree of response.
Table 7. The degree of reactive response in the lungs after intratracheal administration of dust samples

<table>
<thead>
<tr>
<th>Town</th>
<th>Season</th>
<th>Dust fraction</th>
<th>Degree of response after instillation of dust samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 mg</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amsterdam</td>
<td>Pollen</td>
<td>Coarse</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>2.5 (0-3)</td>
</tr>
<tr>
<td>Łódź</td>
<td>Pollen</td>
<td>Coarse</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>Coarse</td>
<td>1.5 (1-3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>3 (3)</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>Coarse</td>
<td>2.5 (1-3)</td>
</tr>
<tr>
<td>Oslo</td>
<td>Summer</td>
<td>Fine</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>Coarse</td>
<td>2.8 (1-4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>1.2 (1-3)</td>
</tr>
<tr>
<td>Rome</td>
<td>Pollen</td>
<td>Coarse</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>Coarse</td>
<td>3.0 (1 – 2)</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>Coarse</td>
<td>1.3 (1-3)</td>
</tr>
<tr>
<td>Ottawa</td>
<td>-</td>
<td>-</td>
<td>1.8 (1-3)</td>
</tr>
</tbody>
</table>

Classification of degree of response to the particles in lungs:
0 – free particles in lumen of alveoli (without cellular response)
1 - particles surrounded by some macrophages and/or granulocytes
2 - particles surrounded by a numerous of macrophages and/or granulocytes
3 - solitary granuloma-like structures (agglomerates of macrophages and dust particles)
4 - numerous granuloma-like structures within the lung tissue
In brackets the range of scores is given.

With BALF respect to measurements the highest response was noted for the coarse fraction at the higher dose level. No marked inflammatory effects were demonstrated since there were only significantly higher responses for two the nine inflammation markers investigated (MIP-2 and TNF-α). No marked seasonal differences in inflammatory responses were noted. From covariance analysis by city, the most potent PM with respect to inflammation was found for particulate matter collected in the city of Lodz. In Table 8 results of random effects linear regression modelling in relation to Season, City, Coarse and Fine PM form and doses 1.0 and 2.5 mg PM after in vivo instillation are shown. When taking into account the impact of all these factors, a dose-effect relationship was documented. However, for CC16 and TNF-α a strong relationship was found with respect to size form (coarse, fine) and localisation (city). With respect to seasonal variations, a strong relationship was found for MIP-2 only.
When data from cluster analysis (Cassee 2003) was used, a strong relationship between components indicative of combustion sources (industry combustion/incinerators) and cytokines MIP-2 and TNF-α were found (Table 9). Epithelial cell deterioration indicated by CC16 appeared to be strongly associated with components from traffic sources (traffic-hopanes, long range transport elements).
Table 9. Results of random effects linear regression modelling between studying of in vivo parameters in relation to representative compound of cluster

<table>
<thead>
<tr>
<th>Cluster compound</th>
<th>MIP-2</th>
<th>TNF-α</th>
<th>Protein</th>
<th>Alb</th>
<th>CCL3</th>
<th>Macrophage</th>
<th>Neutrophil</th>
<th>Leucocyte</th>
<th>Viability</th>
<th>LDH</th>
<th>Body Weight</th>
<th>Lung Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crustal material (Si, Al)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Long range transport elements (NH₄, NO₃, SO₄)</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Wood smoke (biphenyle, acenaphthene)</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(+)</td>
</tr>
<tr>
<td>Industry combustion/ incinerators (fluorene, Anthracene)</td>
<td></td>
<td>+++</td>
<td>+++</td>
<td></td>
<td>+</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Traffic (17a(H)-22.29.30-trisnorhopane, (abb-20)R-cholestone, 5a-cholestanone)</td>
<td></td>
<td>+++</td>
<td>++</td>
<td></td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Sea salt/endotoxin (ETP, LAL ultr, LAL susp.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
</tbody>
</table>

(+)=p<0.01,  + = p<0.05,  ++ = p<0.01,    +++ = p<0.001

3.8 Mechanisms underlying modulation of molecular and cellular functions of the immune system by particles

Cellular changes during the primary immune response to allergen with diesel exhaust particles (DEP), Rome fine (spring) and Oslo fine (summer) particle fractions

On day 5 after injection of allergen (OVA), particles alone, OVA+particles or buffer alone, the cellular response in the draining lymph node was determined. The particles used in these experiments were diesel exhaust particles (DEP), Rome fine (spring) and Oslo fine (summer, hereafter called Oslo1) samples.

Cell numbers, cell types, and cell surface markers in the lymph node

The total cell numbers in the popliteal lymph node were higher in the OVA+particle groups than in the groups given HBSS, OVA or the corresponding particle alone. Further, in three of six experiments, the total cell numbers in the Oslo1 group were statistically significant higher than in the HBSS group. Also, after injection of the Rome faction, the total cell numbers seemed to be increased, although less so than for the Oslo1 fraction. Thus, a certain trend for total cell numbers to be increased by particles alone was observed.

The relative numbers of B lymphocytes (CD19⁺ cells) in the PLN were significantly higher (about 40-60% of the cells) in the OVA+particle groups than in the groups given HBSS, OVA or the corresponding particle alone (about 20%, figure 28B). Correspondingly, the relative numbers of T lymphocytes (CD3⁺ cells) were significantly lower (about 40-60% of the cells)
in the OVA+particle groups than in the groups given HBSS, OVA or the corresponding particle alone (about 70%, figure 28C). Thus, the qualitative cellular composition in the lymph node was strongly altered by OVA with particles. As the relative number of B and T cells are not independent parameters but rather reciprocal, the reduction in the relative T cell number are most probably caused by a stronger increase in the absolute B cell number, as shown for polystyrene particles (manuscript to be published). Also, the change in the relative numbers of B and T lymphocytes were significantly larger in the OVA+DEP group than in the OVA+Rome group (for T cells also compared to the OVA+Oslo1 group). No differences between the relative number of B or T lymphocytes in the OVA+Rome and OVA+Oslo1 groups were observed. Particles alone did not alter the relative number of B or T lymphocytes compared to the HBSS group. The relative number of CD4\(^+\) T lymphocytes (CD4\(^+\) CD3\(^+\) cells, T helper cells) did not differ between the groups (figure 28D).

The expression of MHC class II on B lymphocytes showed a statistically significant increase in the OVA+particle groups compared to the groups given HBSS, OVA or the corresponding particle alone (data not shown). The expression of MHC class II on B lymphocytes also was significantly higher in the OVA+DEP group than in the OVA+Rome and OVA+Oslo1 groups. No differences were observed between the OVA+Rome and OVA+Oslo1 groups. The MHC class II expression caused by Rome particles alone, the Oslo1 particles alone and OVA alone were marginally (statistically significant in one of two experiments) higher than in the HBSS group.

The relative number of CD86\(^+\) B lymphocytes was markedly and significantly higher in the OVA+DEP group than in the groups given HBSS, OVA and DEP alone, as well as in the OVA+Rome and OVA+Oslo1 groups (data not shown). In one of two experiments the relative number of CD86\(^+\) B lymphocytes was increased moderately but significantly in the OVA+Oslo1 group compared to the groups given OVA alone, Oslo1 particles alone or OVA+Rome particles. OVA+Rome particles did not increase the relative number of CD86\(^+\) B lymphocytes compared to the control groups, neither did particles alone or OVA alone.

The relative number of CD23\(^+\) B cells was high (more than 90% positive B cells in all groups), but the relative number was repeatedly significantly increased for the OVA+particle groups (about 97% positive B cells) compared to the control groups (data not shown). The expression of CD23 molecules per B lymphocyte was significantly higher in the OVA+DEP group than in the groups given HBSS, OVA and DEP alone, as well as in the OVA+Rome and OVA+Oslo1 groups (data not shown). Also, the expression of CD23 on B lymphocytes in the OVA+Oslo1 group was significantly higher than in the HBSS and OVA groups, and significantly higher in the OVA+Rome than in the HBSS group. Particles alone or OVA alone did not alter the expression of CD23 on B lymphocytes compared to the HBSS group.

Taken together, OVA+particles do most often, but to a varying degree, increase both the cell numbers and the expression of the surface molecules compared to the OVA group. Overall, DEP increased the expression of the surface markers more than the two RAIAP particles tested, whereas this difference was not seen for the total cell number. The potency of the Rome and Oslo1 particles to increase the measured parameters in the primary response to OVA did not differ significantly. Particles alone did not induce significant changes in the surface marker expression, however trends in the total cell number and MHC class II expression on B cells were observed.
Fig. 28. Total cell numbers and cell subtypes in the draining lymph node 5 days after a single injection into both hind footpads of HBSS, 50 µg OVA, 100 µg DEP, Rome or Oslo1 particles alone or 50 µg OVA + 100 µg DEP, Rome or Oslo1 particles. Total cell numbers (A) were measured on a Coulter cell counter. Single cell suspensions were stained with antibodies against CD19 (B), CD3 (C), and CD3 and CD4 (D), and the relative number of positive cells were determined by flow cytometry. Values for individual mice (circles) and median values (columns) for groups of nine (A) or five mice are shown. #, * and + indicate a statistically significant difference (p<0.05) compared to HBSS, OVA and the corresponding particle, respectively. All groups significantly changed relative OVA were also significantly changed relative HBSS. Brackets indicate a statistically significant difference (p<0.05) between similar preparations with different particles. Data are representative for two independent experiments.

In vitro cytokines

After 24 and 48 hours of culture of the PLN cells with the mitogen ConA, the supernatant cytokine concentrations were determined by sandwich ELISA. IL-4 and IL-10 production after 24 hours followed a similar pattern as the cell surface markers, as the cytokine levels in the OVA+particle groups tended to be higher than in the control groups (figures 29A and 4B). However, as the levels of IL-4 and IL-10 in the OVA alone group tended to be increased compared to the HBSS group (significant in one of two experiments), only the level of IL-4 in the OVA+Oslo1 group repeatedly showed a statistically significant higher level than OVA alone. However, the levels of IL-4 and IL-10 in all OVA+particle groups were significantly higher than in the groups given HBSS or the respective particle alone. Although not
statistically significant, the Rome and Oslo1 fractions alone repeatedly seemed to increase the levels of IL-4 or IL-10 compared to the HBSS group.

IFN-γ production from the PLN cells after 48 hours did not differ between the groups.

Taken together, on day 5 after injection, the levels of the Th2 associated cytokines IL-4 and IL-10 were most often increased after injection of OVA+particles compared to HBSS, OVA alone and the respective particle alone. The levels of the Th1 associated cytokine IFN-γ were not altered. The RAIAP particles alone showed a non-significant tendency to increase the production of the IL-4 and IL-10 production.

![Graphs of cytokine production](image)

Fig. 29. In vitro cytokine production from lymph node cells on day 5 after a single injection into both hind footpads of HBSS, 50 µg OVA, 100 µg DEP, Rome or Oslo1 particles alone or 50 µg OVA + 100 µg DEP, Rome or Oslo1 particles. Single cell suspensions were prepared, and cells from 3 animals were pooled and cultured with ConA (5 µg/ml) for 24 and 48 hours. IL-4 (A), IL-10 (B) and IFN-γ (C) in supernatants were measured by ELISA, and data from optimal time points are shown (24, 24 and 48 hours for IL-4, IL-10 and IFN-γ, respectively). Each point represents a pool of three animals and columns represent the group median value. * and + indicate a statistically significant difference (p<0.05) compared to OVA and the corresponding particle, respectively. All groups significantly changed relative OVA were also significantly changed relative HBSS. Brackets indicate a statistically significant difference (p<0.05) between groups. Data are representative for two independent experiments.
Do the coarse and fine fractions induce different changes in the primary cellular response in the draining lymph node?

On day 5 after injection of OVA + coarse or fine particle fractions, OVA alone or buffer alone, the cellular response in the draining lymph node was determined. The particles used in these experiments were the coarse and fine fractions of the Amsterdam (spring), Lodz (summer) and Oslo (spring, hereafter called Oslo2) fractions.

Coarse and fine particles: cell numbers, cell types and cell surface markers in the lymph node

The total cell numbers were increased in the OVA+particle groups versus the HBSS and OVA groups, however not statistically significant for the Amsterdam coarse and fine fractions. Similarly, the relative number of B lymphocytes were higher in all OVA+particle groups than in the HBSS (all statistically significant) and OVA groups (OVA+Amsterdam coarse and OVA+Lodz fine not significantly). Moreover, the levels of B lymphocytes in the OVA+Lodz fine group was statistically significantly higher than in the OVA+Lodz coarse and OVA+Amsterdam fine groups.

Expression of MHC class II on B lymphocytes was significantly increased for the OVA+particle groups compared to the HBSS group and, except for the two Amsterdam fractions, also compared to the OVA group. The MHC class II expression in the OVA+Lodz fine group was significantly higher than in the OVA+Amsterdam coarse and fine fractions. The relative numbers of CD86+ B cells were raised compared to the HBSS and OVA groups for all OVA+particle groups except OVA+Lodz coarse and OVA+Oslo2 fine. The relative numbers of CD23+ B lymphocytes were significantly increased for all OVA+particle groups compared to HBSS and OVA. Although marginally and not statistically significant, the levels were consistently higher in the fine than in the coarse fractions. The expression of CD23 molecules per B lymphocyte was higher in most OVA+particle groups compared to the HBSS and OVA groups, but not significantly so in the OVA+Amsterdam fine and OVA+Lodz coarse groups (data not shown).

Overall, the total cell number, the relative number of B lymphocytes and expression of the measured surface markers were to varying degrees (not always statistically significant) increased in all OVA+particle groups compared to the HBSS and OVA groups. No systematic differences between the coarse and fine fractions were seen, although expression of MHC class II seemed to be marginally, but consistently, higher for the fine fraction. The Lodz fine fraction with OVA induced the marginally largest changes in all parameters, and increased the relative B cell numbers significantly more than the OVA+Lodz coarse fraction.

The IL-4 production from the PLN cells was significantly increased in all OVA+particle groups compared to the HBSS group, and in all groups given OVA+coarse fractions compared to the OVA group (figure 30A). The IL-10 production was significantly increased in all OVA+particle groups compared to the HBSS group and OVA groups (figure 30B). No statistically significant differences in IFN-γ production were seen between any groups (figure 30C). However, a weak trend of higher levels of IFN-γ in the fine fractions than in the coarse fractions was seen.

Taken together, the production of the Th2 associated cytokines IL-4 and IL-10 were increased in all OVA+particle groups compared to the HBSS group, and for IL-10 also compared to the OVA group. The IL-4 levels seemed to be higher in the groups given OVA+coarse fractions, which also showed statistically significant higher IL-4 levels than the OVA group. No significant differences in the IFN-γ levels were seen, however a trend of higher IFN-γ levels for the fine fractions were observed.
Fig. 30. *In vitro* cytokine production from lymph node cells on day 5 after a single injection into both hind footpads of HBSS, 50 µg OVA or 50 µg OVA + 100 µg particle fractions (Amsterdam (A’dam) coarse and fine, Lodz coarse and fine and Oslo2 coarse and fine). Single cell suspensions were prepared, and cells from 3 animals were pooled and cultured with ConA (5 µg/ml) for 24 and 48 hours. IL-4 (A), IL-10 (B) and IFN-γ (C) in supernatants were measured by ELISA, and data from optimal time points are shown (24, 24 and 48 hours for IL-4, IL-10 and IFN-γ, respectively). Each point represents a pool of three animals and columns represent the group median value. # and * indicate a statistically significant difference (p<0.05) compared to HBSS and OVA, respectively. Brackets indicate a statistically significant difference (p<0.05) between OVA-preparations with different particles. Data are representative for two independent experiments.

3.9 Mechanisms underlying modulation of signalling pathways by particles in rat and human epithelial lung cell cultures

Importance of particle-associated bacterial endotoxins

Endotoxins from different types of bacteria, or bacterial components, may be bound to ambient air particles and contribute to the induction of inflammatory responses in the lung. With respect to the particle suspensions used in these studies, analysis of the content of endotoxin did not reveal any marked site-specific differences. However, a slightly higher level was found in the coarse fractions when compared to the fine (Cassee et al., 2003).
Type 2 cells. Particles were treated with polymyxin B, which binds endotoxins from Gram negative bacteria, for 1 h before addition to the cell cultures. Figure 31A shows the IL-6 release after exposure of type 2 cells to coarse particles with or without treatment with polymyxin. LPS at a concentration above the highest concentration of endotoxins analysed in any of the collected particle sample was included as a control. The concentration of polymyxin used for particle-treatment (10 µg/ml) was chosen at a level above what is needed for binding the amount of LPS added. Addition of polymyxin abolished the LPS-induced cytokine release, demonstrating the LPS-binding effect of polymyxin. Treatment of the particles from Rome, as well as the reference particle Ottawa dust, resulted in a small, but insignificant decrease in the particle-induced cytokine release, whereas polymyxin-treatment had no effect on the particles from Lodz.

A549 cells. The results after exposure of A549 cells to particles with or without polymyxin-treatment, as well as LPS, are presented in Figure 31B. In contrast to what was seen with the type 2 cells, LPS was not able to induce any marked increase in cytokine release in A549 cells. This is in agreement with several other studies demonstrating this cell type to be insensitive to LPS. This may explain the observation that treatment with polymyxin had no effect on the IL-8 induced by coarse particles from Rome. Coarse particles from Lodz, however, demonstrated a reduced potency to induce IL-8 after treatment with polymyxin. It has been demonstrated that the response of lung cells to LPSs may be differently regulated depending on the E. coli serotype and species involved. Regarding the reduced effect of the polymyxin treated coarse fraction from Lodz, this may be explained by a possible content of toxin from a certain type of gram-negative bacteria to which A549 cells are more responsive. With respect to the fine particles, polymyxin-treatment did not seem to have any effect on their potency.
Fig. 31. (A) Release of IL-6 from type 2 cells and (B) release of IL-8 from A549 cells. The cells were exposed to PM or PM treated with Polymyxin B (10 µg Polymyxin per ml particle suspension of 5 mg/ml). Coarse fractions (C) (type 2 and A549 cells) and fine fractions (F) (A549 cells) collected in Rome and Lodz during spring, as well as Ottawa dust, were studied. LPS (10 ng/ml) was used as a positive control to verify the effect of Polymyxin B. PM was treated with Polymyxin B for 1 h before addition to the cells. Data are mean ± SEM (n = 3-4).

These results indicate that endotoxins of the type that bind polymyxin B make no, or only a small, contribution to the inflammatory potency of the particles tested. The observed differences in effect of polymyxin-treated particles between type 2 cells and A549 cells may be explained by intercellular variation in responses to specific toxins.

Importance of metals

Deferoxamine is a chelator of trivalent metals, and may interact with transition metal ions such as Fe$^{3+}$ and Al$^{3+}$. It was therefore of interest to study the involvement of such metal ions for the effect of the selected particle samples.

Type 2 cells. Figure 32A shows the release of IL-6 after exposure of type 2 cells to particles with or without treatment with deferoxamine. Treatment of the selected coarse fractions from Rome and Lodz with the metal chelator did not result in a reduced release of IL-6. In contrast, treatment of particles with deferoxamine seemed to induce higher levels of IL-6 compared to the levels induced by untreated particles in type 2 cells.

A549 cells. A549 cells exhibited a roughly similar pattern as type 2 cells after exposure to coarse particles from Lodz or Ottawa dust with or without deferoxamine-treatment (Figure 32B). However, a slight reduction in potency of the metal rich particles from Rome was observed after treatment with deferoxamine, indicating a possible effect of soluble metals for the release of IL-8 in A549 cells.
Taken together, treatment of the metal-rich particle sample from Rome and the less metal rich sample from Lodz with deferoxamine, did not influence the potency of the particles to induce cytokines to any certain extent. These observations are supported by the results presented in Deliverable 9 (Hetland et al., manuscript in prep.), in which aqueous extracts made from the summer samples did not induce marked increase in cytokine release. Accordingly, soluble trivalent metals do not seem to explain the observed toxic effects of the particle samples tested in these studies. However, deferoxamine has been suggested to interfere with the intracellular balance of free and protein-bound metal ions. The observed increase in cytokine
release with deferoxamine-treated particles, as well as in controls, may therefore be explained by such an effect.

Involvement of MAP kinases

Specific inhibitors of the MAP kinases ERK or p38 were added to the cell cultures 1h before addition of the selected particles. Cytokine release was measured after 20h of particle exposure. Experiments were also performed to study particle-induced phosphorylation of the MAP kinases in type 2 cells and A549 cells.

Type 2 cells. Pretreatment of type 2 cells with the PD98059, an inhibitor of the upstream kinase that activates ERK, did not have any effect on the level of particle-induced IL-6 when compared to untreated cells, whereas inhibition of p38 with SB202190 or PD169316 resulted in a reduced level of IL-6 after 20 h of exposure to the types of particles tested (Figure 33A). These results indicated a more prominent role for p38 compared to ERK with respect to particle-induced cytokine release in type 2 cells. However, an increase in particle-induced phosphorylation of both p38 and JNK, as well as ERK, was observed after 4 h of exposure (Figure 34A). Of the two particle samples tested, particles from Rome seemed equally, or slightly more potent than particles from Lodz. No significant difference in the potency to induce cytokines in type 2 cells was demonstrated (Deliverable 9).

A549 cells. In this cell type, pre-treatment with the ERK-inhibitor resulted in a marked reduction in the level of IL-8 release, whereas a smaller reduction was observed after treatment of the cells with the specific inhibitors of p38 (Figure 33B). With respect to the ERK-inhibitor, a marked reduction in the basal level of cytokine release was also observed. Exposure to the particle sample from Lodz stimulated phosphorylation of ERK and p38, as well as JNK, to a higher extent than the Rome sample (Figure 34B). This difference is in accordance with the higher potency to induce cytokines observed by the spring particles collected in Lodz compared to particles collected in Rome described in Deliverable 9. At the exposure dose used in experiments presented in this report, a difference between the two particle samples is not so obvious. This may be explained by some difficulties with the A549 cell line during the last weeks of experimental studies (reported in annual report 2003). Cell toxicity was observed at exposure to lower doses of both particles and other substances compared to results from previous experiments. It turned out to be difficult to find the exposure dose that was high enough to induce cytokine responses without being toxic. Therefore, the apparent small difference between cytokine releases induced by the Lodz sample compared to the Rome sample presented may be due to a certain toxicity of the Lodz sample after 20 h of exposure. However, no toxicity was present at 4 h, the time point for analysis of the particle-induced phosphorylation of the MAP kinases.
A

![Graph showing IL-6 release from type 2 cells after 20 h of exposure to coarse (C) particles.](image)

B

![Graph showing IL-8 release from A549 cells after 20 h of exposure to coarse (C) particles.](image)

Fig. 33. (A) Effect of the ERK-inhibitor PD 98059 (25 μM), and the p38 inhibitors SB202190 (2.5 μM) and PD169316 (1.0 μM) on IL-6 release from type 2 cells after 20 h of exposure to coarse (C) particles and (B) effect of the ERK-inhibitor PD 98059 (50 μM), and the p38 inhibitors SB202190 (5 μM) and PD169316 (1.5 μM) on IL-8 release from A549 cells after 20 h of exposure to coarse (C) particles. Data are mean ± SEM (n = 3 – 5).
Fig. 34. Stimulation of the MAP kinases p38, ERK, JNK and total ERK-2 analysed by immunoblotting in type 2 cells (A) and A549 cell (B) after 4 h of exposure to coarse (C) particles. Densitometric analysis of the blots is presented as fold increase compared to control. Data are mean ± SEM (n = 3) with p38 and ERK and mean (n = 2) with JNK.
Taken together, the MAP kinase pathways seem to be involved in particle-induced cytokine release in the two cell types used in this study. Furthermore, the potency of the selected particles to induce cytokine release may be, at least partly, a result of their potential to stimulate the MAP kinase pathways.

Involvement of EGF receptor/Src

Activation of tyrosine kinases and the involvement of epidermal growth factor (EGF) receptor may be involved in the induction of inflammatory responses. Therefore, effects of an inhibitor of the Src family of protein tyrosine kinases and an EGF-receptor kinase inhibitor were studied in both cell types (Figures 35A and 35B). Reduced responses when the cell cultures were treated with the inhibitors before exposure indicated activation of the Src family of protein tyrosine kinases, as well as involvement of the EGF-receptor, in the observed cytokine responses induced by coarse particles in both cell types. A problem in these studies was, however, the observed ability of both inhibitors to interfere with the basal activity. Additional studies are therefore required to study particle-induced activation of Src and EGF.

A
Involvement of ROS

Intracellular production of ROS was measured by analysing the generation of DCF-fluorescence after exposure of A549 cells to the coarse or fine fractions collected during spring in Rome and Lodz (Figure 36). All particle samples except the fine fraction from Rome generated an increased production of ROS compared to control, with the coarse fraction from Rome the least potent. Treatment of the cell cultures with the antioxidant N-acetylcysteine (NAC) before addition of particles did not seem to influence the levels of IL-8 release after exposure to the selected particle samples (Figure 37). NAC is a stable precursor of cysteine for intracellular synthesis of the effective ROS scavenger glutathione. The lack of effect of NAC may be due to the relatively high content of glutathione in A549 cells.
Fig. 36. Formation of ROS as measured by DCF-fluorescence in A549 cells after exposure to coarse (C) particles (50 µg/ml), fine (F) particles (50 µg/ml) or Ottawa dust (250 µg/ml) for 45 min. The fluorescent probe DCFH-DA (100 µM) was added to the cells 30 min before addition of the particles. Results are mean ± SEM of three parallels in one representative experiment.

Fig. 37. Effect of the antioxidant NAC on particle-induced IL-8. A549 cells were treated with NAC (40 mM) for 1 h before addition of coarse (C) or fine (F) particles (50 µg/ml). IL-8 release was analysed after 20 h of exposure. Data are mean ± SEM (n = 3).

Activation of NF-κB
Preliminary results indicated an increase in nuclear translocation of the transcription factor NF-κB in A549 cells after 4 h of exposure to the coarse fraction from Lodz compared to control (Figure 38). A minor increase was also observed after exposure to the coarse sample from Rome. This observed difference in levels of nuclear NF-κB is supported by the higher
potency to induce cytokine release (Deliverable 9) and higher production of ROS observed with the Lodz particles compared to the Rome particles.

![Graph showing translocation of NF-κB](image)

Fig. 38. The subunit p50 of the transcription factor NF-κB measured in nuclear extracts from A549 cells after exposure to coarse (C) particles (100 µg/ml) or positive control (provided by the manufacturer of the ELISA-kit) for 4 h. The result from one experiment is shown.

3.10 Mechanisms underlying modulating effects of particles on in vivo respiratory immune responses

In our study it was hypothesized that the adjuvant activity is due to an activation of macrophages. Our results showed that the wild type Balb/c mice compared to the Nramp1<sup>r</sup> and Nramp1<sup>s</sup> mice showed a similar adjuvant activity of antibodies to ovalbumin in the subclasses of IgE, IgG1 and IgG2a. In contrast, the histopathological lesions showed for the wild type group a significant increase due to the co-exposure to ovalbumin and PM, while in the Nramp1<sup>r</sup> and Nramp1<sup>s</sup> mice the adjuvant activity was not statistically significant increased. Similar results were found for the number of eosinophils in the BAL. These results provide information that the adjuvant activity seems not to be due to the macrophage activating stage as no differences in adjuvant activity appear in these Nramp1<sup>r</sup> and Nramp1<sup>s</sup> strains of mice. In the study of Whitekus et al. (2002), it was shown that thiol oxidants (N-acetylcysteine, NAC), prevented the adjuvant activity of co-exposure to ovalbumin and DEP. Administration of NAC suppressed directly complexes to electrophilic DEP chemicals and exerted additional antioxidant effects at the cellular level (Whitekus et al., 2002; Xiao et al., 2003). The results the study of Whitekus et al. (2002) is in contrast with our study in which co-exposure of ovalbumin and EHC-93 after pretreatment with NAC showed adjuvant activity in antibody response to ovalbumin in all subclasses. Moreover, the IgG2a response was significantly increased compared to non-pretreated group. In addition, histopathological lesions and the influx of eosinophils were still statistically significantly increased compared the non-pretreated group. The discrepancy may be the different schedule of the immunisation and challenge. From our results, it is concluded that the adjuvant activity observed after co-exposure to ovalbumin and EHC-93 cannot be diminished when mice are pretreated with
NAC. This suggests that the adjuvant activity of ambient particles is not due to an antioxidant activity.

iNOS-deficient mice were used to study the role of NO-synthase in the process of adjuvant activity of co-exposure to ovalbumin and PM. Our results show that the adjuvant activity expressed in IgE production was comparable in both strains and the production of IgG1 and Ig2a antibodies to ovalbumin were even higher in the iNOS-deficient mice (Figure 39). Moreover, the histopathological lesions and the influx of inflammatory cells in the BAL reflected in both strains an equal adjuvant activity of co-exposure to ovalbumin and EHC-93.

Our data strongly reject the hypothesis that iNOS and inducible NO play a role in the skewing of Th2 response resulting in an adjuvant activity. So, we must conclude that not only the induction and excitation phase is independent from iNOS, but also the adjuvant activity induced after co-exposure to ovalbumin and EHC-93.

![Fig. 39. IgE in serum to ovalbumin in C57BL/6 wild type and iNOS deficient mice. Mice were intranasally exposed to NaCl, ovalbumin (2 mg/ml) or co-exposed to ovalbumin and EHC-93 (3 mg/ml) during the sensitization phase (day 0 and 14) and challenged with ovalbumin only (day 35, 38 and 41). Significant different from OVA exposed group: * P<0.05.](image)

We used an IL-4 deficient mouse, which lack IL-4, while recruitment of airway eosinophils in response to aeroallergens is diminished, but not abolished. No antibody response can be elicited after inhaled ovalbumin However with epicutaneous application the immunological response was intact (Herrick et al., 2000). Moreover, the characteristic airway damage and hyperreactivity normally resulting from allergen inhalation are not attenuated (Hogan et al., 1997). Indeed, in our study, no antibodies (IgE and IgG1) to ovalbumin were measured in the IL-4 deficient mice even after co-exposure to ovalbumin and EHC-93. IgG2a was produced, although no adjuvant activity was determined in this Th1 compartment of the immune system as shown also by others (Hogan et al., 1997). Our data show that co-exposure to EHC-93 could not circumvent the reduced IgE response as shown for epicutaneous application (Herrick et al., 2000). As IgE in this respiratory model is dependent on IL-4, we cannot conclude that the adjuvant activity is IL-4 dependent. However, histopathological lesions were observed after respiratory application with ovalbumin only in wildtype and IL-4 deficient mice, while no increased lesions (except for hypertrophy of mucous cells) were found after co-exposure to ovalbumin and EHC-93 in IL-4 deficient mice. We may therefore conclude that IL-4 is a key player in the adjuvant activity based on histopathological lesions, but other processes involved such as hypertrophy of the mucous cells seem to be not under the control of IL-4. IL-5 was still present, although reduced compared the wild type group co-exposed to ovalbumin and EHC-93. This reduction of IL-5 may reflect the reduced eosinophilia in the lung, as seen in our study.
4. Discussion

4.1 Discussion on sampling campaign and chemical characterization

A successful PM sampling campaign was performed in the period of March 2001 – April 2002 in which 4 locations (Amsterdam, Lodz, Oslo, Rome) were visited three times (Spring, Summer and Winter). An additional background location (De Zilk) was sampled once. Most of the PM quantities were sufficient to use them for both chemical analysis and in vitro and in vivo studies in experimental animals. It has to be noted here that we have applied total elemental composition. This is in particular of importance to know since often the water soluble part of transition metals (i.e. Fe, Ni, Cu etc) are thought to be more biologically effective than the none-water soluble part (i.e. Fe$_2$O$_3$). The results of the chemical characterisation have shown distinct differences in composition among the locations and between the fine and coarse fractions that can be used to estimate the source contribution to the total PM and to explain the biological effects observed in the in vitro and in vivo studies. In addition, seasonal differences were observed in all of the locations, but were most striking for Lodz, Poland. This is explained by the low temperatures during the cold (winter) season. Obviously, the emissions of combustion for heating purposes will increase with decreasing temperatures. In contrast to the other sampling sites, the local energy sources in Lodz are brown and black coal burning with probably a lack of effective emission reduction technologies.

Also striking was the lack of contrast between the four traffic-dominated locations and the background station in De Zilk. Even though great care was taken to avoid sampling under conditions that the wind was not coming from the directions of the North Sea, we cannot exclude that substantial influences were present of inland sources.

4.2 Discussion on immunological characterization

Morphology and elemental composition

Analytical electron microscopy carried out on specimens from the four European cities (Lodz, Amsterdam, Rome and Oslo) during the three seasons (Spring, Summer and Winter) showed that for both PM$_{10}$ and PM$_{2.5}$ specimens, the majority of the ambient particles consisted of combustion particles (soot aggregates).

Combustion particles are small particles (<50 nm in diameter) that aggregate into grape-like clusters that can be transported over long distances. It was, therefore, expected to find such particles also in specimens from the rural seaside location of de Zilk. Apart from gypsum fibres, probably from plasterboards found in some of the specimens, there were not observed any other inorganic fibres such as asbestos or man-made-mineral fibres. In addition to combustion particles, sulphur particles probably formed from acidic aerosols, were present in all specimens except from the spring sample from Amsterdam. Most of the sulphur dioxide is released from coal-burning power plants and other industrial sources, such as smelters, industrial boilers, and oil refineries. Similar to soot these particles are transported over long distances. During wintertime, soot particles from heating may also contribute to the total ambient particle mass. In Oslo, wood burning is common during wintertime, whereas burning of coal still takes place in Lodz. Still, XRMA-analysis did not reveal any significant differences in the spectra of soot aggregates from the different sampling sites. However, the relative intensities of carbon, sulphur and oxygen (C:S:O), was higher in the spring for all sampling sites, except for Oslo.
Immunogold labelling

Antibodies to all allergens tested, except the monoclonal antibodies to birch, labelled the combustion aggregates and particles of organic origin from all four cities and seasons. As seen from Table 4, soot aggregates from all filters, except the spring filter from Amsterdam, were weakly labelled with anti-latex (Hev b 3). This labelling was independent of season, and may therefore, be due to elution of latex allergens from car tires. Since latex is known to share epitopes with allergens of vegetal origin, the possibilities of cross-reactions must be considered. The fact that the antibody to timothy (Phl p I) labelled carbon aggregates from all seasons on filters from Oslo, also points to a cross-reactive epitope. However, the presence of the actual allergen even in wintertime cannot be ruled out. The particles judged to be of organic material, were probably of vegetal origin since they were labelled by the antibodies to pollens and plants. Plant materials on filters from all cities and seasons were especially strongly labelled with anti-β-glucans. β-Glucan is a polysaccharide in the cell wall of fungi, but β(1-3) glucans are also found in many plants which may explain the heavy labelling. The combustion particles and aggregates are highly hydrophobic and the adsorbance of allergens may therefore be charge dependent. The absence of labelling using the present antibodies to birch may therefore be due to a charge effect. Similarly, the absence of adsorbance of the purchased birch allergen used as positive control, support an unfavourable charge of the allergen to the combustion particles. All the other allergens used as positive controls labelled the combustion particles only and not the filter.

Summary

Analytical electron microscopy of PM₁₀ and PM₂.₅ LV specimens collected from the four cities in Europe showed no marked differences, with regard to morphology and elemental composition. Carbon extraction replicas showed that the majority (>90%) of the ambient particles consisted of combustion (soot) particles that aggregate into grape-like clusters. Immunolabelling showed that airborne antigens corresponding to several allergens were adsorbed to, and carried by, combustion (soot) particles and micronic particles of vegetal origin in the ambient air. The results indicate that the allergens adhere to the particles to different extent depending on charge. Air pollution may, thus in predisposed subjects, increase airway reactivity and bronchial responsiveness to inhaled allergens.

4.3 Discussion of allergy results

Allergy screening

The allergy screening for adjuvant activity show that ambient particles, with exception of a few of the coarse fractions, collected in the four cities in Europe; Oslo, Rome, Lodz, and Amsterdam, in three seasons (spring, summer, and winter), have an adjuvant activity in the doses examined (100-200 µg per mouse). It seems, however, as the fine fractions have a stronger adjuvant activity than the coarse ones. This stronger adjuvant effect of fine particles in the assay employed was statistically highly significant when all particle preparations were compared. No marked differences were observed neither between locations, nor between seasons. It should be noticed that the statistical analysis could have been more conservative with regard to the comparison of individual test groups. We cannot exclude, however, that the popliteal lymph node assay and the allergen specific ELISA-based IgE assay, with the immunisation protocol used, are not sensitive enough to differentiate between smaller differences in the adjuvant capacity of ambient particulate matter. Alternatively, ambient particles from different locations may not differ substantially in qualitative characteristics. The adjuvant effect of several particle types on IgE production has earlier been shown both
after intraperitoneal, intranasal and intratracheal exposure in mice. However, the subcutaneous (s.c.) PLN model is particularly well suited for studies of the cellular response in a draining lymph node, as the popliteal lymph node is the only node draining the footpad. Moreover, the retained dose varies less after s.c. injection than after exposure in the airways. The footpad injection model is an established screening model for the immunostimulating ability of chemical allergens and immunotoxic substances.

Our findings support the notion that the physical particle by itself has an adjuvant activity on antibody production, an activity that may be further modulated by chemical factors. Further, our findings are in line with earlier observations that small model particles give a stronger adjuvant effect than larger particles, and that adjuvant activity is determined not by particle mass but by particle surface or number. Small particles have, per mass unit, much larger numbers and larger surface area than larger particles. Our finding of a stronger adjuvant effect from small than large particles is also compatible with epidemiological observations that fine particles have stronger effects with regard to acute respiratory disease than coarse particles.

Interestingly, along with the finding that apparently all particles have some adjuvant effect, our findings indicate that different particles appear to stimulate distinct immune responses to an allergen. DEP appears to skew the immune response in a Th2-direction, according to our findings and earlier published data. Silica particles have been reported to skew the immune response in a Th1-direction. The ambient particulate matter samples in our experiments, on the other hand, which are composed of different types of particles including both combustion and silica particles, elicit a Th2- as well as a Th1-immune response.

The antibody response to Ottawa dust was highly variable. By transmission electron microscopical analysis the Ottawa dust was found to contain many large particles, or aggregates of particles. When given as weight dose, these large particles will contribute more than the small ones. The resulting variable amount of small particles, which in our test system seem to have a stronger adjuvant activity than large ones, might explain the variable antibody response to Ottawa dust.

The results obtained using the relatively simple six days popliteal lymph node assay seem to correlate fairly well with the results obtained using the more sophisticated 26 days ELISA-based allergen specific IgE assay. Both assays, in which mice are sensitised by footpad inoculation, seem to be suitable as allergen screening assays. The popliteal lymph node assay may be further simplified by leaving out measurements of proliferative responses, which are time-consuming and costly considering the information obtained. Importantly, it should be noted that there were some clear discrepancies between the popliteal lymph node assay and the specific IgE response. In the present experiments, the relatively strong IgE adjuvant activity of fine particulate samples from Rome (spring) and Lodz (summer) was not reflected by the popliteal lymph node response. The popliteal lymph node assay does not distinguish between an allergic and a non-allergic immune response. Further, the popliteal lymph node response has a non-immune contribution from inflammation caused by the particles. Finally, IgE represents a biologically relevant endpoint for allergy. The IgE assay may therefore be both more specific for IgE adjuvant activity and be more sensitive.

Finally, it should be noted that the footpad immunisation results at first glance may appear to be contradictory to the result obtained by intranasal instillation. The former found that fine particles had the strongest IgE adjuvant effect, whereas by intranasal instillation the coarse fraction had the strongest effect. The footpad injection technique shows what happens when allergen and particles are introduced directly into the tissues without having to pass a mucosal barrier. To pass the mucosal barrier, inflammation is needed. Inflammation will in the intranasal model be elicited by the coarse particles that have stronger inflammatory capacity.
than the fine particles, and this may possibly explain why coarse particle had stronger effect than fine particles by intranasal instillation. However, also in the intranasal model fine particles had a strong adjuvant effect when the doses given were high enough to provoke inflammation. In real life, fine particles will always be accompanied by irritant gases such as ozone and nitrogen oxides. It is therefore likely that in real life, there will always be enough inflammatory substances present to allow to become manifest the strong adjuvant capacity of the fine particles that we have demonstrated.

**Allergy adjuvant mechanisms**

Overall, the data obtained indicate that ambient air particulate matter increase the allergy-related immune response by modulating the expression of cell membrane molecules and the pattern of Th2 associated cytokine production. With few exceptions, the measured parameters were changed in the OVA+particle groups compared to the OVA group. DEP seemed to induce a stronger effect than the RAIAP particles, as was also observed for the Th2 antibody responses. DEP has been shown to quite exclusively exert a Th2 antibody adjuvant effect in BALB/cA mice, whereas the collected RAIAP particles induced both a Th2 and a Th1 adjuvant effect according to the results obtained in the allergy screening experiments. The Th1/Th2 antibody “bias” observed between some of the RAIAP particle fractions in allergy screening was not associated with significant differences in the primary cellular response to OVA preparations with the different city particles. It should be noticed that the statistical test used was conservative. Therefore non-significant trends are also included in the discussion.

In general, particles alone did not significantly alter the measured cellular parameters. However, trends were seen that Oslo1, more than Rome, and more than DEP fractions did increase the total cell numbers compared to the HBSS levels. Rome and Oslo1 also seemed to weakly increase the expression of MHC class II molecules. The particles alone thus seemed to exert an unspecific, irritative effect measured as increased lymph node cells numbers and increased MHC class II expression, that may be caused by different particle characteristics. On the other hand, DEP (together with OVA) did stand out from the RAIAP particles, inducing the strongest “adjuvant” effect on the expression of surface markers on B cells. This increased expression of the surface markers MHC class II, CD86 and CD23 is likely to be associated with a specific OVA-response. Also, a trend of increased IL-4 and IL-10 production induced by the RAIAP particles alone was observed. In human *in vivo* systems, DEP alone has been shown to increase a broad array of cytokines including IL-4 and IL-10 (16). The RAIAP particles’ different capability of increasing the production of these cytokines indicates that different chemicals on the collected particles from different sites and seasons or other particle characteristics (i.e. size distribution, particle numbers, total surface area, surface charge, etc.) may influence these parameters.

The selected RAIAP particle fractions together with OVA did increase most of the cellular parameters, however few significant differences between the RAIAP particles were observed. All RAIAP particles (with few exceptions among the coarse fractions) had an adjuvant effect on the antibody responses in allergy screening. We cannot exclude the possibility that the assays we use are insufficiently sensitive to differentiate between smaller differences in the adjuvant activity of the particles. However, as the particle fractions have been shown to differ in chemical composition, our data suggests that the particle core strongly contributes to the adjuvant effect on the allergic response. This is supported by recent studies showing that polystyrene particles or carbon black particles, models of the particle core, exert an adjuvant effect on the IgE response, as well as the primary cellular response, in different mouse models.
In the antibody screening experiments, all selected particles exerted an adjuvant effect on production of OVA-specific IgE and IgG1 (allergy (Th2) associated antibodies) and IgG2a (non-allergic (Th1) associated antibodies). However, the fine Rome spring particles seemed to have more of a Th2 profile than the other particles, whereas the fine Oslo summer particles seemed to have more of a Th1 profile than the other particles. In BALB/cA mice, subcutaneous injection of DEP and allergen have been shown to increase the levels of Th2-associated antibodies and not the Th1-associated antibody. This pattern of a Th2, Th2>Th1, and Th1>Th2 adjuvant effect of the particles on antibody response was in the present WP found not to be associated with a particular pattern in surface marker expression or cytokine production. The “pure” Th2 adjuvant DEP was overall inducing the largest changes in the measured cellular parameters, followed by the Th1-biased Oslo1 and the Th2-biased Rome. Similarly, for the second set of particles tested in our studies, the Th1/Th2 biased antibody responses observed in WP 3 could not be associated with a particular pattern in the cellular parameters. Thus, the cellular parameters of the primary response studied here did not predict the particle’s potency to affect the balance between a Th1 versus a Th2-dependent antibody response.

We have found that DEP or RAIAP particles with OVA increased the levels of the Th2 associated cytokines IL-4 and IL-10, but not the Th1 associated cytokine IFN-γ. This is in accordance with earlier reports, where nasal exposure to DEP together with a neoallergen in humans was shown to induce sensitisation to this new allergen, including increased levels of allergen-specific IgE as well as increased levels of IL-4, but not IFN-γ.

We further found that the RAIAP particles with OVA increased the expression of both MHC class II molecules, CD86 molecules and CD23 molecules on the B lymphocytes compared to the control groups. MHC class II expression is crucial for antigen presentation to the T lymphocytes, and increased expression will presumably result in a more effective antigen presentation. Our data also suggests, although the effect was not statistically significant, that particles alone may induce a small increase in MHC class II expression, which could possibly be enough to start a self-reinforcing circle of increased immune response. CD86 is a co-stimulatory molecule expressed on antigen-presenting cells (APC), and several investigators have proposed that CD86, as compared to CD80, preferentially provide costimulation in Th2 responses. All particles with allergen increased the expression of the low-affinity IgE receptor CD23 on the B lymphocytes. In contrast, injection of OVA with the known Th1 adjuvant CpG-DNA (25) in our system significantly decreased the level of CD23 expression (to be published). This supports the notion that up-regulated CD23 expression on B cells may be an important regulatory mechanism for increasing the allergic IgE response. The observed up-regulation of the measured surface molecules on B cells from the draining lymph node may all be contributing to the adjuvant effect of particles on the allergic (Th2) antibody response.

Thus, in WP 7 we have shown that when particles are given with allergen, particles cause upregulation of several parameters in part associated with an immune response in general (cell surface MHC class II), in part associated with an allergy-related (Th2) immune response (the cytokines IL-4 and IL-10), as well as parameters possibly involved in the regulation of the Th2 response (CD86 and CD23 expression). However, we did not find differences between particle preparations that had shown some Th2 or Th1-bias in the allergy screening, nor did we find differences between coarse and fine particles that could explain the stronger antibody adjuvant effect of fine particles. This could be due to the parameters selected, the sensitivity of the detection systems, or because the crucial effects are exerted very early in the immune response.
4.4 Discussion of inflammation results

The outcome of these studies supported our proposed hypothesis that difference in short-term respiratory health effects is related to differences in particle composition. The combined in vivo and in vitro studies show that exposure to dusts of different origin and constituents may cause similar effects but of different magnitude. It was shown that the Lodz particles contained more inflammation-generating components than did those from other Europeans cities. These compounds, beside their direct cytotoxicity, may also induce inflammatory changes by modulating the numbers of viable inflammatory cells and the levels of cytokines. However, the identification of one single constituent responsible for the observed effects in a mixture of components is difficult. The data suggest that metals may be of importance, and they are also found in the Lodz PM. However the in vitro data do not indicate that soluble factors (endotoxins or metals) explain the observed differences in effects of different PM samples, though a contributing role of soluble factors cannot be excluded. The importance of endotoxins and soluble metals has been emphasised in a variety of different studies (e.g. the groups of S. Becker and D. Costa), but others have indicated that also insoluble factors may be of importance (groups P. Borm, A. Aust and P. Schwarze). Though the data with antioxidants did not show large significant effects of antioxidants on cytokine release the demonstrated activation of NFkB might indicate that the ROS-sensitive pathway of signal transduction had been activated. In addition MAP kinases were activated and upstream src family protein kinases seemed to be involved. It has been shown previously that IL-8/MIP-2 production depends on NFkB activation and that MAPK can enhance that response via AP-1/jun phosphorylation. The studies also indicated the involvement of several cell types in the response as indicated by the use of different cells types in vitro and changes in CC16 levels in BAL from the rats. The importance of CC16 in the lung defence system and its anti-inflammatory effects have been indicated by several groups. The increased levels of cytokines found in the in vivo studies have previously been related to the regulation of this protein.

4.5 Discussion of integrated results

In the preliminary analyses of the intergrated data, it appears that a high number of constituents of PM can be clustered, based on their effects, as follows: the industry combustion/incinerators, traffic, crusted material, wood smoke, long range transport elements and sea salt. It was observed that individual and group-wise health effect parameters were associated with these clusters. These data indicated that the respiratory allergy model is associated with sources which produce particle components related to combustion processes, e.g. industrial combustion, traffic, and wood fire. In contrast, there is a distinctive difference between the irritating and toxicity health effect parameters being sensitive for the crustal material. In the clusters traffic and wood fire, Lodz appeared to be associated very strongly, while Rome was associated with crustal. The coarse fraction was confined mostly to the clusters crustal and sea salt, and the fine fraction to the other clusters. An association with season appear not to be very strong, except for winter related to the cluster traffic. Further analysis will reveal the relevance and implications of this source-oriented health effect assessment.
5. Conclusions

Sampling and chemical characterization

PM$_{10}$ and PM$_{2.5}$ samples were successfully collected using high-volume cascade impactors and a low-volume sampler in the cities of Amsterdam (The Netherlands), Lodz (Poland), Rome (Italy) and Oslo (Norway) during Winter, Spring and Summer Seasons. In addition, the background location in De Zilk (The Netherlands) was sampled once. The samples were characterized by physical, chemical and immunological parameters. The results of the chemical characterization show distinct differences in composition among the locations and between the fine and coarse fractions. These findings will be used in further analyses to estimate the source contribution to the total PM and to explain the biological effects observed in the *in vitro* and *in vivo* studies.

Allergy screening

The allergy-enhancing (adjuvant) activity was measured in mice both as a cellular response in the popliteal lymph node and as an allergen-specific IgE antibody response in serum. All ambient particulate matter fractions, with the exception of a few coarse ones, had an adjuvant effect in the examined doses (100-200 $\mu$g per mouse). The IgE assay is more specific for allergy, and appeared to be more sensitive. The fine fractions had a stronger adjuvant activity than the coarse ones measured as the allergen specific IgE response. Few marked differences were detected between locations, except for the coarse particles from Lodz, which appeared to have a stronger adjuvant effect than the rest. Particulate matter from the background location de Zilk did not appear to be different from that of the other locations. In addition to the increased allergen specific IgE and IgG1 antibody responses to ovalbumin (an allergy-related Th2-immune response), a significant increase in allergen specific IgG2a antibody response was observed (a non-allergic Th1 immune response). Thus, ambient particles, at least the fine fractions, stimulated both an allergic and a non-allergic immune response.

Inflammation screening

The respiratory inflammatory potential of the collected samples was studied in different types of freshly isolated rat lung cells, isolated human lung cells and a human lung cell line. Both site-specific and seasonal variations in the potential to induce release of inflammatory signalling substances (cytokines) were demonstrated in all cell types. Even though responses to the fine fractions were more obvious in the human lung cells compared to the rat lung cells, the order of potency among the PM samples was relatively similar in all cell types. Particle-bound bacterial components were shown not to be a major cause of the observed effects. With respect to chemical characteristics, content of metals found in both soil surface and combustion products was positively correlated with increased levels of cytokines. The observed cytokine-release was not caused by water-soluble components of the particles, which implies that the effects are predominantly due to material tightly bound to the particles or the particles themselves.

Allergy *in vivo*

Co-exposure to ovalbumin with 4 out of 12 collected PM samples (3 mg/ml) resulted in an increase of mainly IgE and IgG1 indicating an increased allergy. The pathological changes consisted of a small to severe inflammatory responses which was confirmed by increased numbers of inflammatory cell in lung washes. Statistical evaluation of the above-
mentioned parameters showed associations between coarse and fine PM, geographical location and season. In addition, adjuvant activity of the PM, irrespective the size fraction (i.e. coarse of fine) can be ranked as Lodz > Rome = Amsterdam > Oslo. When the different seasons were compared for IgE, PM sampled during the winter, they were found more active compared to PM sampled during spring and summer. Only for the pathological lesions, statistically significant difference in effects was found between coarse and fine PM (coarse>fine). No associations were found between the endotoxin content and the biological effect parameters, although endotoxin was much more confined to the coarse fraction.

**Inflammation in vivo**

In covariance statistical analysis the highest response was noted for the coarse fraction at the higher concentration level. No marked inflammatory effects were demonstrated since only significantly higher responses were shown for two of the nine inflammation markers investigated (MIP-2 and TNF-α). No marked seasonal differences in inflammatory responses were noted. From covariance analysis by city, the most potent PM with respect to inflammation were found for particulate matter collected in the city Lodz. This indicates that the Lodz particles contained more inflammation-generating components than those from the other European cities. Statistical analyses investigating the toxicity of PM when taking into account the impact of season, city and PM size, showed a dose-response relation. However, for CC16 and TNF-α a high relationship was found with respect to size form (coarse, fine) and location. With respect to seasonal variations, a high relationship was found for MIP-2 only.

**Correlation of chemical composition and effect parameters**

By using the extensive chemical composition database of both the coarse and fine fractions of PM, the observed effects in *in vitro* and *in vivo* assays were linked with sources of emission. In general, less secondary inorganic aerosol (sulphate, nitrate) was measured in the coarse fraction than in the fine traction. The contrast in chemical composition observed was considerable. The components were clustered based on similar response patterns of the health effect parameters to be able to link the health effects to groups of chemicals. By cluster analysis, the correlation between health effects and individual chemical components were grouped using statistical criteria. These clusters of components were subsequently associated to the source of emission that dominates the cluster: [industry/combustion/incinerators (IAI)], [traffic], [crusted material(CM)], [combustion of brown and back coal and wood (BBW)], [secondary inorganic aerosol (SIA) and long range transport aerosol (LTA)] and [sea salt]. Significant differences were found for the contributing amounts of cluster components between the coarse fraction and the fine fraction. A preliminary analysis indicated that individual and group-wise health effect parameters could be associated with these clusters. Further analysis will reveal the relevance and implications of this source-oriented health effect assessment.
6. Exploitation and dissemination of results

An important output of the RAIAP project has been to disseminate the results from the project, not only to the scientific community, but also to regulatory authorities, industry and other stakeholders within and/or interested in air pollution and health. To this end a workshop was arranged in Bilthoven, The Netherlands, 11-12 March 2004 together with the EU-funded projects HEPMEAP (Health Effects of Particles from Motor Engine Exhaust and Ambient Air Pollution) and AIRNET (A Thematic Network on Air Pollution and Health). The workshop was well attended with the maximum of 80 participants. The workshop consisted of overview lectures and specific research results on the following topics: 1) The mechanisms by which particulate matter can cause health effects, 2) reaction of specific lung cells and animal models on particulate matter exposure, 3) reactions of specific animal models on particulate matter exposure, 4) health effects of particulate matter exposure, 5) integration of research outcomes, and 6) implications of toxicology research findings. A report is being prepared with extended abstracts from the various presentations. This report will be widely distributed to the research community, regulatory agencies, industry and other stakeholders.

Members of the RAIAP team have also been heavily involved in the AIRNET project, a network of scientists and stakeholders which has the overall objective to help create a foundation for public health policy on improving European air quality. An important outcome of the AIRNET project has been to produce a non-specialist assessment of toxicology research findings in the field of air pollution and health, including those related to particulate matter. The RAIAP co-ordinator has led the AIRNET Toxicology Workgroup and is thus responsible for the publication and dissemination of the report.
7. Policy related benefits

The outcome of the RAIAP project has given new and important understanding on how exposure to ambient particulate matter may result in respiratory inflammation and allergy. This is reflected by the scientific papers evolving from the project, of which at least half are still in preparation. Clearly, it was demonstrated that PM collected from various high-traffic density locations expressed different toxic responses, and that the composition as well as the size fraction with PM10 are more closely related with the effects than the total PM mass. It was also demonstrated that exposure to PM in combination with natural allergens results in more than additive effects. Thus, valuable additional insights into the complex interactions between particles and biological systems have been gained. In addition, the increased competence related to particulate exposure and health effects developed through the RAIAP project, has been of importance for risk assessment and advisory functions. RAIAP partners were strongly involved in the WHO EURO project ‘Systematic Review of Health Aspects of Air Quality in Europe’. This project aimed to provide the Clean Air for Europe (CAFE) programme of the European Commission (DG Environment) with a systematic, periodic, scientifically independent review of the health aspects of air quality in Europe. Significant contributions were given to the reports ‘Health Aspects of Air Pollution with Particulate Matter, Ozone and Nitrogen Dioxide’ (2003), ‘Health Aspects of Air Pollution – answers to follow up questions from CAFÉ’ (2004) and ‘The Effects of Air Pollution on Children’s Health and Development: A Review of the Evidence’ (in press).
8. Literature cited


9. List of scientific publications from the project


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