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Toxicity of Ambient Particulate Matter (PM_{10})

I. Acute toxicity study in asthmatic mice following 3-day exposure to ultrafine and fine ammonium bisulfate, a model compounds for secondary aerosol fraction of PM_{10}.

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Preface

Toxicity studies on components of ambient particulate matter (PM_{10}) are conducted within the framework of a research program on adverse health effects of ambient particulate air pollution. These studies, conducted by RIVM, TNO Toxicology, and University of Nijmegen (KUN) Toxicology, are part of the Dutch "Wintertime Smog Program", performed by order and for the account of The Ministry of Housing, Spatial Planning and Environment. The first part of this program included epidemiological studies on health effects of ambient PM_{10} and the possible role of traffic emissions. The second part of this program includes toxicity studies on ambient PM_{10} and has been designed in accordance with the simplified strategy of the health risk assessment model for ambient PM, i.e. the "Pentagon" model of possibly main critical fractions with respect to particle size and chemical composition.

The PM_{10} toxicity program, directed to (oxidative) cardiorespiratory toxicity as an important pathogenic mechanism, is focussing on the following aspects.

1. Determination of cardiorespiratory toxicity in laboratory animals following short-term inhalation of concentrated "real-world" $PM_{2.5}$ at various urban and rural locations (RIVM-LEO/LLO) (a mobile Harvard ambient PM concentrator has been installed).
2. Determination of cardiorespiratory toxicity in laboratory animals after short-term inhalation of carbonaceous or ammonium sulfate/nitrate particles as representative components for primary and secondary fractions of ambient PM (RIVM-LEO/LPI, TNO Toxicology). (**This report**)
3. Determination of respiratory toxicity (in vitro/instillation) of sampled (ultra)fine PM collected on filters from ambient air at various urban, rural, industrial, and traffic locations and from motor vehicle (diesel) exhaust emissions (KUN Toxicology, RIVM-LEO/LLO/LOC/LAC/LPI).
4. Determination of the relative importance of particle mass versus particle size/number for PM_{10} health effects (RIVM, TNO, KUN).
5. Determination of differences in cardiorespiratory toxicity between healthy animals and animal models mimicking various cardiopulmonary diseases of specific human risk groups for PM_{10} (RIVM,TNO).
6. Development and use of ambient PM deposition models and exposure-dose relationships for quantitative risk assessment in healthy people and risk groups with compromised airways (RIVM-LBO/LEO).

The 1st phase of these toxicity studies started in 1996 and the results of one of the studies mentioned under 2 are presented in this report. The results of this and successive studies performed in this framework will be integrated later on in a review report on the health effects (and in particular the toxicity) of carbonaceous secondary aerosols.

Samenvatting

Dit rapport beschrijft de resultaten van één de eerste van een serie inhalatiestudies die tot doel hebben inzicht te krijgen in de potentiële acute toxiciteit van het secundair deel van deeltjesvormige luchtverontreiniging (PM) in de buitenlucht. In dit onderzoek is gebruik gemaakt van een dierexperimenteel model voor astma als representative aandoening voor één van de risicogroepen in de humane polulatie. Gezien de complexiteit van PM_{10} is deze eerste studie beperkt tot ammoniumbisulfaat, een belangrijke component van PM_{10} in Nederland. We hebben gezonde muizen (BALB/c) en muizen met milde astmatische symptomen blootgesteld aan ultrafijn (1.70×10^5 deeltjes per cm^3, 85 nm MMD) en fijn (2.74×10^3 en 3.58×10^4 deeltjes per cm^3, 531-543 nm MMD) ammonium bisulfaat gedurende 4 uur per dag drie dagen lang. De gemiddelde actuele massa concentraties bedroegen respectievelijk 255, 78 and 972 $\mu g/m^3$. De muizen werden 1 en 4 dagen na de laatste blootstelling opgeofferd om de pulmonaire reactie vast te stellen middels analyse van onstekings- en celschade markers in longspoelvloeistof (BALF), luchtwegreactiviteitsmetingen en histopathologie.
Er werden geen veranderingen van lactaat dehydrogenase (LDH), N-acetyl glucosaminidase (NAG) en eiwitgehaltes in BALF gevonden als gevolg van de blootstelling aan ultrafijne en fijne aerosolen. De veranderingen in interleukine IL-4, IL-6, TNF-α en celdifferentiatiebeeld waren slechts marginaal en worden niet relevant bevonden.
Pathologisch onderzoek kon geen effect van de blootstelling aan aerosolen vaststellen. Wel was er een duidelijk effect van de behandeling met ovalbumine, namelijk hypertrofie van Goblet cellen in bronchus-epitheel en mononucleair celinfiltraat in gecompromitteerde dieren. Er kon geen IgE specifiek gericht tegen ovalbumine worden aangetoond in gezonde dieren, maar wel in de gecompromitteerde muizen. Er kon geen statistisch significant effect van de ammoniumbisulfaat blootstellingen op IgE spiegels worden aangetoond.
Concluderend kan worden gesteld dat muizen waarin een milde vorm van astma werd geïnduceerd geen effecten van blootstelling aan (ultra)fijnstof konden worden aangetoond. Dit geldt ook voor de niet-gecompromitteerde dieren.
De hier gepresenteerde gegevens suggereren dat ammoniumbisulfaat, zijnde een modelstof voor het secundaire deel van PM_{10}, bij realistische concentraties en bloostelllingsduur niet leidt tot pulmonaire effecten in gezonde dan wel licht-astmatische muizen. Er zijn geen indicaties voor een versterking door ammonium bisulfaat van de allergische reaktie in de muizen. Dit impliceert dat deze component van PM_{10} niet verantwoordelijk zou zijn voor de verergering van astmatische symptomen in de humane populatie zoals dit uit epidemiologisch onderzoek naar voren komt.

Summary

This report describes the results of the first of series of 3-day inhalation studies aimed to generate data on the health effects of inhaled ultrafine and fine ammonium bisulfate aerosols as model compound for the secondary fraction of particulate matter (PM_{10}). Epidemiologic studies have identified asthmatics as a risk group for PM exposure. Therefore, we have exposed healthy and compromised mice (BALB/C; ovalbumin-IgE-induced respiratory allergy mimicking asthma) to ultrafine (1.70×10^5 particles per cm^3, 85 nm MMD) and fine (2.74×10^3 and 3.58×10^4 particles per cm^3, 531-543 nm MMD ammonium bisulfate for 4 hr a day on 3 consecutive days. The mean actual mass concentrations were 255, 78 and 972 $\mu g/m^3$ respectively. Mice were sacrificed 1 and 4 days post-exposure to determine their pulmonary response using inflammatory and cell damage markers in bronchoalveolar lavage fluid (BALF), airway reactivity measurements and histopathology.
No changes in N-acetyl glucosaminidase (NAG), lactate dehydrogenase (LDH) and protein levels in BALF were observed following exposure to fine or ultrafine NH_4HSO_4. Changes in interleukines (IL-4, IL-6, TNF-α) and cell differentials were marginal and considered not to be biologically relevant. Gross and histopathological examination showed no treatment-related abnormalities in lungs except for a hypertrophy of Goblet cells in bronchus epithelium and mononuclear infiltrate in compromised mice. This latter observation was a result of the ovalbumin treatment. Ovalbumin-specific serum IgE levels were undetectable in healthy mice but significantly increased in compromised mice. Compared to 1 day post-exposure, IgE levels increased at 4 days post-exposure. Exposure to (ultra)fine particles did not lead to alteration in tracheal reactivity in mice in which asthma-like symptoms were induced.

These preliminary data suggest that ammonium bisulfate in realistic concentrations and exposure durations as a model compound for the secondary fraction of ambient PM10 exert no pulmonary effects in normal healthy mice and in a mouse model of asthma, and there are no indications of ammonium bisulfate-induced enhancement of allergic response. This could mean that the excercerbation of asthma as identified in epidemiological studies is not caused by this component of PM_{10}.

1. Introduction

The database of adverse health effects associated with exposure to particulate air pollution PM10 (particles less than 10 μm in diameter) is rapidly growing. There are major concerns for human health including de effects on breathing and respiratory functions, aggravation of existing respiratory and cardiovascular disease, alterations in the body's defense systems against foreign materials, damage to lung tissue, carcinogenesis and premature death. Epidemiological studies have reported associations between relatively low levels of PM with excess cardiovascular and respiratory effects, morbidity and mortality (Dockery and Pope, 1994; RIVM, 1995). The major subgroups of the populations that appear likely to be most sensitive to the effects of particulate matter include individuals with chronic obstructive pulmonary and cardiovascular disease, individuals with influenza, asthmatics, the elderly, and children. Specifically the health effects in (young, aged) subpopulations with cardiopulmonary diseases like athma, is the subject of serious debate with respect to causality and biological plausibility. Although the relative risk is quite low (RR ≈ 1.03 - 1.15), the risk factors for the same effect between the various studies may sometimes differ by a factor 3-4, which might point to different exposures to PM, on differences in air quality.
The possible link is further complicated by the fact that the associations are found at various geographical urban and rural areas with a contrast in air quality, e.g. wood smoke, wind blown dust, industrial emissions, (heavy) traffic emissions, acid aerosols, all below the current standards for PM_{10} Remarkably, the observed health effects are associated with different indicators for PM air quality such as total suspended matter (TSP), PM_{10}, $PM_{2.5}$, SO_4^{2-}, and H^+, but also with NO_2, NO, SO_2, and O_3.

PM include dust, dirt, soot, smoke, and liquid droplets directly emitted into the air by sources such as factories, power plants, transportation sources, construction activity, fires, and windblown dust. Particulates are also formed in the atmosphere by condensation or transformation of emitted gases such as sulfur dioxide, nitrogen oxides, and volatile organic compounds into tiny droplets. Neither the responsible PM fractions, their size and chemical composition, nor their emission sources are presently established. The size fraction can roughly be described as coarse (2.5 - 10 μm), fine (0.1 - 2.5 μm) and ultrafine (< 0.1 μm) particles. A limited number of studies suggests that fine particles (measured as $PM_{2.5}$ or SO_4^{2-}) are of more importance to induce adverse health effects than the coarse particles and that responsible PM fractions have adsorbed acids, transition metals, and/or reactive PAHs (RIVM, 1995). There is, however, at present an insufficient toxicity data base that 1) could explain the observed exposure-response relationships, featured by low PM levels, apparent linearity, lack of a threshold value, and the specific susceptibility of risk groups, and 2) could give a biologically plausible view on the occurrence of adverse health effects.

The PM toxicity studies are conducted within the framework of a toxicity research program on health effects of relevant components of ambient PM_{10}. This program, conducted by RIVM, TNO Toxicology, KUN Toxicology and Leiden Universiteit Medical Center, is part of the Dutch "Wintertime Smog and Traffic Program". It has been designed in accordance with the simplified strategy of a PM health risk assessment model, i.e. the "Pentagon" model (fig. 1) to possible main critical fractions with respect to particle size and chemical composition.

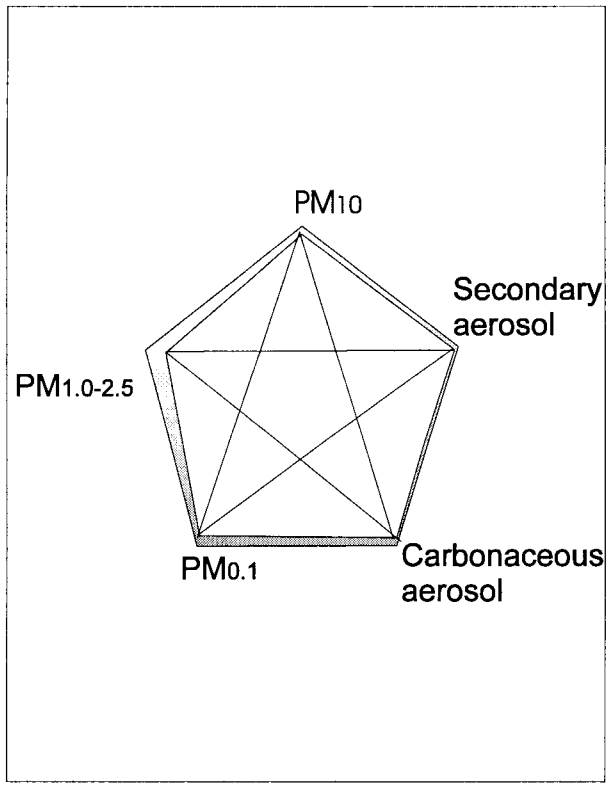


Fig. 1 The "Pentagon" model: a visualization of the strategy of a quantitative health risk assessment of ambient particulate matter as performed by the RIVM. In this assessment a simplified strategy is followed on characterization of emissions, exposures, doses and health of ambient particulate matter

The studies described in this report were aimed to identify adverse effects of ammonium bisulfate aerosols in healthy mice and mice with asthma, representative for one of the identified risk groups of PM_{10}. We hypothesized that ammonium bisulfate particles as a model compound for secondary aerosol fraction enhances inflammation and hyperresponsiveness in a mouse model that mimics asthma. Secondly, we hypothesis that fine PM will result in stronger effects (if any) than ultrafine effects. This hypothesis was inspired by dosimetric consideration (Freijer *et al.*, 1997): fine particles will deposit in mainly the lower airways, while ultrafine will behave more like a gass and (depending on the hydroscopic properties) will deposit to a greater extent in the upper airways. The exposure duration of 4 hr/day for 3 consecutive days was used to represent an acute exposure to secondary aerosol. We investigated the health effects of fine~500 nm and ultrafine ~ 50 nm particles on the lung. In this study we have focused on biochemical, histopathological, cytological and immunological parameters related to toxicity and asthma.

2. Experimental

2.1 Testing facilities

The studies described in this report were conducted between April and June, 1996 at the department of Toxic Effects of the Laboratory of Health Effect Research, RIVM, The Netherlands.

2.2 Test material

Crystalline ammonium bisulfate was supplied by Aldrich-Chemie, Steinheim, Germany and has the following characteristics:

CAS nr.	: 7803-63-6
Batch nr.	: 03210-094
Purity	: 98%
Density	: 1.790
Molecular weight	: 115.11
Melting point	: 146.9 °C.
Solubility	: 1000 g/l
Storage conditions	: room temperature
Toxicity	: irritating for skin, eyes and the respiratory tract

2.3 Test animals

Specified pathogen free male BALB/c mice (6-8 weeks old) were obtained from the breeding colony of the National Institute for Public Health and the Environment, Bilthoven, the Netherlands. The mice were housed in macrolon cages and food and water was provided *ad libitum* during non-exposure periods. The acclimatization period was at least 7 days. The room temperature was maintained at 22 ± 2°C and the relative humidity at 40-70%. Animals were subjected to a 12 hr with light on from 8 am - 8 pm. The mice were fed a cereal based rodent diet (SMR-A; Hope Farms, Woerden, the Netherlands). The day before the start of each substudy, the mice were identified with an earmark to be able to discriminate between sensitized and non-sensitized mice. One day prior to the challenge procedure each mouse was marked according to the allocation list with a unique number with was coded by placing stripes on the tail using felt-tips.

2.4 Location of the study

Mice arrived at the animal housing facilities at least 7 days prior to the start of the study. The check for body weight variation, the random allocation and the sensitization procedure were performed at the facilities of the Central Animal Laboratory. At day 33 groups of mice were transported to another building, where they were trained in the restraining tubes, challenged with ovalbumin and exposed to the test atmosphere. Part of the animals of each substudy was used for airway reactivity measurements, histopathology and serum collection and the rest was used for bronchoalveolar lavage.

2.5 Conduct of the study

The study was split into three substudies with different exposure levels. All substudies were performed with 96 mice. Before exposure to the test material, half of the mice were compromised as described under 2.6. The animals were exposed to the test material for 4 hr a day on 3 consecutive days. Immediately after exposure, the animals were returned to their living cages, 4-6 animals per cage.

Number of mice	Healthy	Compromised
Sham exposure	16	16
Exposure to NH_4HSO_4	32	32

Half of the mice were sacrificed 1 day post exposure and the other half 4 days post exposure. Mice were either used for bronchoalveolar lavage and serum collection or airway reactivity measurements and histopathology (Appendix C).

2.6 Sensitization and antigen challenge

Animals were sensitized by seven intraperitoneal 0.5 ml injections of 0.02 mg ovalbumin (Grade II; Sigma, St.Louis, MO) per ml saline vehicle on alternate days (days 0, 2, 4, 6, 8, 10 and 12). From day 34 these mice were exposed to ovalbumin (0.2 % w/v) aerosol for 5 min. at 8 consecutive days. On day 39 and 40 challenges were performed right after the exposure to the test material. The aerosol was generated with an ultrasonic nebulizer (Devilbiss 2000, particle size 5 μm) connected to a Plexiglas whole-body exposure chamber (27 l). The challenge was given in groups of maximal 6 animals.

2.7 Exposure system

A schematic representation of the exposure system is shown in Figure 2. Mice were individually, nose-only exposed in a small exposure chamber. During exposure the animals were restrained in animal holders (modified Battelle tubes) which closely fit to the exposure chamber. The animals were allowed to breath freely from the stream of fresh test atmosphere. During the 4 hr exposures animals were deprived from food and water.

2.8 Generation of the atmosphere

The aerosols were generated by ultrasonic nebulization (Omron NE-U12, Omron Corporation, Tokyo, Japan) of an aqueous solution of ammonium bisulfate. The molarity of this solution determined the size of the final aerosols. Large droplets were removed by impaction. The main aerosol stream was led through a heating tube to evaporate the water. Water vapor was removed in a diffusion drying chamber filled with silica beads. This resulted in solid sulfate particles. To improve its performance, the dryer was cooled with a cooling device. The final concentration was reached by diluting the aerosol stream with purified air. A detailed description of the generation is retained in the archives of the Institute.

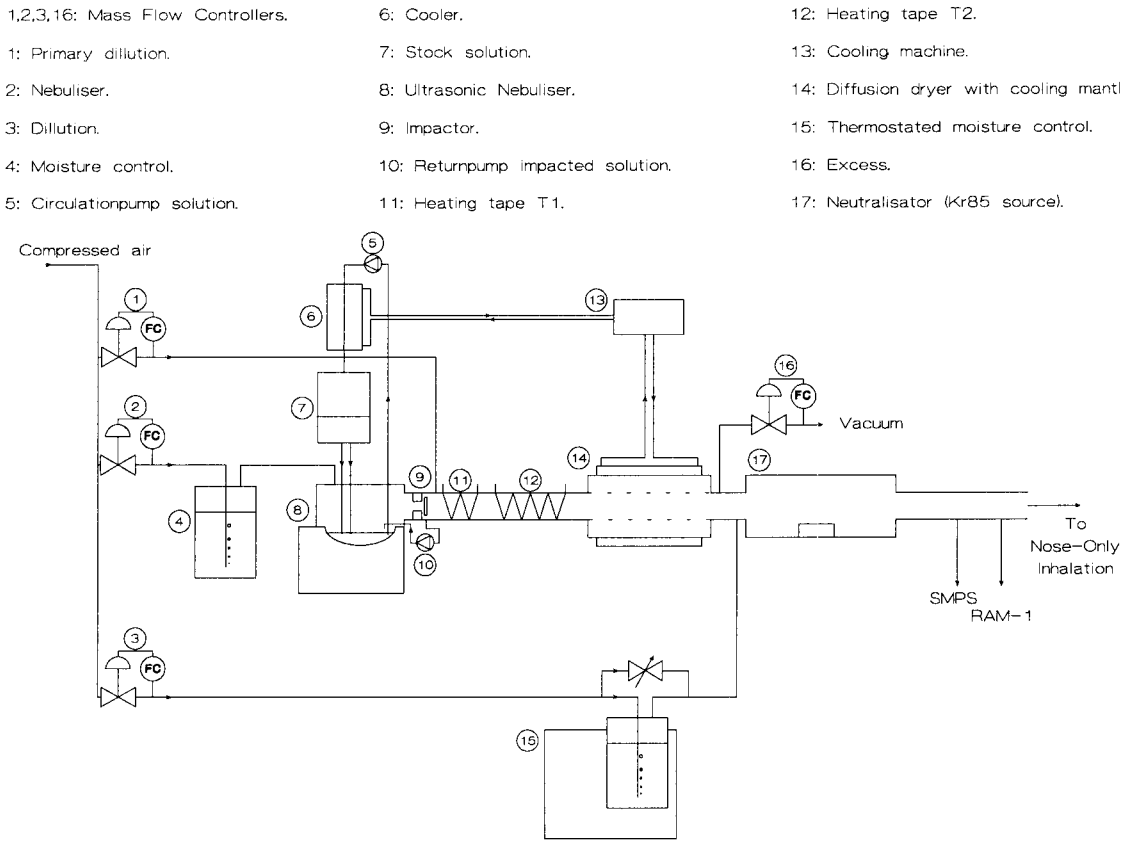


Fig. 2. Schematic representation of the generation of the test atmosphere

2.9 Analysis of the test atmosphere

The mass concentrations of the test atmospheres were determined using a Real-time Aerosol Monitor (RAM-1, MIE Inc., Bedford, Ma, USA) for fine particles or chemically by collecting ultrafine particles on a filter during a 4 h period. After dissolving the filter in water the ammonium concentration was determined with annular denuder sampling and on line analyses (AMANDA, ECN, Petten, NL). The concentration of ammonium bisulfate was subsequently calculated from this concentration and the total volume of air that passed the filter during the 4 hr of sampling.

Particle size, total number of particles and size distribution were determined with a scanning mobility particle sizer consisting of an electrostatic classifier (TSI 3071, TSI Inc., St. Paul, Min., USA) and a condensation nucleus counter (TSI 3022A, TSI Inc., St. Paul, Min., USA). In addition, an aerodynamic particle sizer (APS, TSI 33, TSI Inc., St. Paul, Min., USA) was used to check the number and size of particles above the 500 nm.

Relative humidity and temperature of the test atmosphere were measured conventionally. The morphology of the fine particles were visualized by scanning electron microscopy (SEM). Freshly generated particles were sampled on a Teflon filter and processed for SEM. In this study we have used 3 different exposure atmospheres based on the particle size and mass concentration. See for details Results 3.1

2.10 Observations and measurements

The mice were visually daily inspected for reactions to treatment (sensitization and challenge with ovalbumin and exposure to the test material).

2.11 Body weights

Body weights were recorded prior to the start of each substudy and just before sacrifice.

2.12 Bronchoalveolar lavage and measurements

Mice were anaesthetized with sodiumpentobarbital. Each lung was lavaged three times one volume saline corresponding to 40 ml/kg body weight with saline at 37 °C. This procedure was repeated and the collected fluid (BALF) was centrifuged for 10 min. at 200 g. The supernatant was used for biochemical and immunological measurements. The pellet was resuspended in saline and was used for total cell number and cell differentials.

2.12.1 Biochemistry

Lactate dehydrogenase was determined according to a procedure by the Scandinavian Committee on Enzymes (1974), N-acetyl glucosaminidase was using a reagent kit (cat. no. 875406 obtained from Boehringer Mannheim (Mannheim, Germany), total protein was determined using a reagent kit (Cat. no. 23225) obtained from Pierce (Oud-Beijerland, the Netherlands). LDH was measured as a marker for cytotoxicity, NAG was determined as an indicator for macrophage activation, and total protein levels in BALF were measured as an indicator for increased permeability of the alveolar-capillary barrier of the lung.

2.12.2 Immunology

The following cytokines were determined using Cytoscreen immunoassay kits (Biosource international, Camarillo, CA): IL-4, IL-6 and TNF-α. These cytokines were measured as being mediators involved in the communication network of the cells of the immune system. They are indicators for pro-inflammatory responses. IL-4 is released by TH2 cells and promotes IgE reactions and mest cell growth; IL-6 is released by TH2, fibroblasts and other cells and augments inflammation. The primary cell source of TNF-α are macrophages and will result in increased phagocytosis and enhancement of immuno- and inflammatory responses.

2.12.3 Cytology

Total number of cells and the viability were counted using a Bürker-Türk chamber. For differential cell counts cytospin preparations were made and stained with May-Grunwald Giemsa. Per cytospin preparation 400 cells were counted and the proportion of each cell type (macrophages, neutrophilic granolucytes, eosinophilic granulocytes and lymphocytes) was calculated based on total viable cells per BALF.

2.13 Serum IgE

A sandwich micro-ELISA was used for the quantification of mouse IgE to ovalbumin. A detailed description of the procedure is given in appendix B. Briefly, microtiterplates were coated with anti-immunoglobulin solution (Monoclonal Rat Anti-mouse IgE, 2 µg protein/ml, clone LO-ME-2, Zymed) and incubated with diluted sera samples, Ovalbumin-DIG (DIG Antibody Labeling Kit, Boehringer Mannheim, Germany) and Anti-Digoxigenin-POD Fab

fragments (Boehringer Mannheim, Germany), respectively. Finally, the peroxidase activity was visualized by an incubation in tetramethylbenzidine (TMB-H_2O_2 solution (6 mg per 100 ml sodiumacetate buffer + 20 µl of 30% H_2O_2)). The IgE levels are given as a percentage of a standard.

2.14 Airway reactivity

Airway reactivity was measured according to a method published by Garssen et al. (1990). Briefly, mice were killed with an intraperitoneal injection of 0.25 ml Nembutal (pentobarbitone sodium 60 mg/ml, Abbott Laboratories, North Chigago, Il, USA). The tracheas, resected in toto, were transferred to a petri dish containing a modified oxygenated Krebs'carbonate solution (118.1 mM NaCl; 4.7 mM KCl; 2.5 mM $CaCl_2.6H_2O$; 0.5 mM $NaHCO_3$; 1.0 mM NaH_2PO_4; 11.1 mM glucose). The trachea was prepared free of excess tissue under a binocular preparation microscope and a piece of approximately 6 mm length (8 trachea rings, just beneath the larynx) may taken. Tracheas were immediately slipped onto two supports in an organ bath, of which one was connected to an isometric transducer. The organ baths were filled with 20 ml of the Krebs' solution. The solution was continuously gassed with a mixture of 95% O_2 and 5% $CO_2$to mainatin a pH of 7.4. The temperature was maintained at 37°C with a constant temperature circulating unit.
Isometric tension was measured with a force displacement transducer (Servogor transducer, Harvard Bioscience, Boston, USA) and a two-channel recorder (Servogor type SE-120) and was expressed as changes in grams force. The optimal basic force is 1 g (Bartell et al., 1980; Hooker et al., 1977). The tracheas were allowed to equilibrate for at least 45 min. before methacholine was applied. During equilibration, the bath fluid was changed once every 15 min.. Metacholine was prepared in Krebs' solution and kept on ice for the duration of the test. A cumulative contractile concentration-effect curve was determined for each trachea.

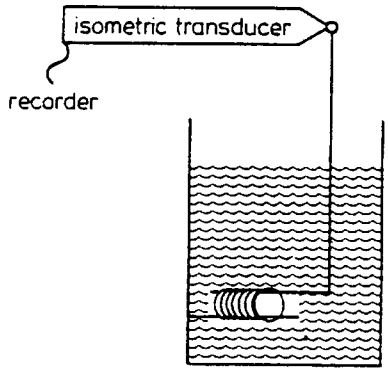


Fig.3 Drawing of the organ bath with the isometric transducer and the trachea (Garssen et al., 1990).

2.15 Histopathology

At autopsy the animals were anaesthetized and the trachea was removed for determination of airway reactivity. The lungs were removed and a canule, inserted in the rest of the trachea or in one of the bronchi, was fixed. The lungs were fixed intratracheally with 2% glutaraldehyde in a 0.1 M phosphate buffer at a pressure of 20 cm for one hour. After immersion fixation in formalin the lungs were embedded in Paraplast and sliced in 5 µm sections. Slides were stained with haematoxylin, eosin and periodic acid Schiff-Alcian blue.

2.16 Statistical analysis

All values are expressed as the mean ± SD. All statistical analysis were performed using STATA (version 2.05, CRC, Oasis, Nieuwegein). The (log-transformed) data were analyzed using multiple one-way analysis of varianc andva. The assumption of homogeneity of variance was tested with the Bartlett test. For some parameters, analysis of the control animals showed differences between the three sub-studies. Therefore responses of dose (ultrafine, fine high and fine low) were analyzed separately. Also the response of the asthmatic status was analyzed separately for each dose and for both autopsy days. Since homogeneity of variance was not obtained for the IgE data, these data were analyzed using the Kruskal-Wallis non-parametric test.
Airway reactivity data were analyzed with an ANOVA with all data points of the concentration-effect curves. EC_{20} (the concentration metacholine that induces 20 percent of the maximal effect), EC_{50} and EC_{80} as well as E_{max} were calculated from fitted curves and these values were analyzed with a t-test.

2.17 Deviations from the protocol

The deviations encountered in the present study are not considered to have influenced the validity of the study.

2.18 Retention of the records

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study, will be retained in the archives of the Laboratory of Health Effect Research of the RIVM for a period of at least 5 years after the reporting of the study.

3. Results

3.1 Analytical results of the test atmosphere

3.1.1 Particle number concentration

The actual concentration was determined by measuring the number of particles per volume and the results are summarized in table 1.

Table 1 Actual exposure concentrations (#/cm^3) of the 3 substudies with ultrafine and fine ammonium bisulfate aerosols. Values are expressed as means ± SD of 3 consecutive exposure days.

Type	Number concentration (#/cm^3)	Relative to ultrafines
ultrafine	$1.70 \cdot 10^5 \pm 0.20 \cdot 10^5$	100 %
fine (low mass)	$2.74 \cdot 10^3 \pm 0.87 \cdot 10^3$	1.6 %
fine (high mass)	$3.58 \cdot 10^4 \pm 0.28 \cdot 10^4$	21 %

3.1.2 Particle size measurement

The results of the size measurements are summarized in Tables 2 and 3. Differences in size between a scanning mobility particles sizer (SMPS) and an aerodynamic particles sizer (APS) are a result of the different techniques and are therefore not comparable. The lower detection limit for an APS is approximately 0.5 µm and consequently, no CMAD and MMAD can be determined for ultrafine particles.

Table 2 Count Median Diameter (CMD) and Mass Median Diameter (MMD) as determined with SMPS of the 3 substudies with ultrafine and fine ammonium bisulfate aerosols. Values are expressed as means ± geometric standard deviation (GSD) of 3 consecutive exposure days.

	Particle Diameter (nm)	
Type	CMD	MMD
ultrafine	55 ± 2	85 ± 2
fine (low mass)	267 ± 2	531 ± 2
fine (high mass)	222 ± 2	453 ± 2

Table 3 Count Median Aerodynamic Diameter (CMAD) and Mass Median Aerodynamic Diameter (MMAD) as determined with APS of the 3 substudies with ultrafine and fine ammonium bisulfate aerosols. Values are expressed as mean ± GSD of 3 consecutive exposure days.

	Particle Diameter (nm)	
Type	CMAD	MMAD
fine (low mass)	638 ± 1.	841 ± 2
fine (high mass)	634 ± 1	699 ± 2

3.1.3 Particle mass measurements

The results of the mass measurements for the fine particles is given in Appendix B. The measurements for fine particles were taken right before or straight after a 4 hr exposure period.

The mass concentration of the ultrafines was determined only once because the minimal sampling time was 4 h. Prior to this study, a gravimetric determination of ultrafine particles showed a mass of 386 $\mu g/m^3$ right after sampling and 117 $\mu g/m^3$ after a two hour equilibration period in a petri dish with silica gel. In order to check the mass measurements, we have calculated the mass from total particle counts (Table 1), the CMD (Table 2) and the density of ammonium bisulfate assuming spherical shaped single particles ($V= 4/3.\pi r^3$) as shown in Figure 4. A second calculation is based on the volume of the particles. These results in a higher calculated mass concentration than the one based on CMD. The results of these calculations are presented in Table 4.

Table 4 The mean calculated and measured mass concentration of exposures to particles of the 3 substudies with ultrafine and fine ammonium bisulfate aerosols. Values are expressed as time weighted average (ultrafine) or means (fine) ± SD of 3 consecutive exposure days.

	Calculated mass concentration ($\mu g/m^3$)		Measured mass concentration ($\mu g/m^3$)	90% confidence limits
	radius based	volume based[a]		
ultrafine	26.5	53.7	235	N.D., n=1
fine (low mass)	48.2	199	78	(58 - 114)
fine (high mass)	400	1915	972	(754 - 1054)

N.D. = not determined

[a] this calculation is based on the measurements of the first day of the exposure only

3.1.4 Temperature and Relative Humidity

The temperature and the relative humidity (RH) were recorded once per hour. During the exposures to ultrafine particles the RH varied between 70 and 100% with a mean of 93%. This was in part caused by the unexpected relative high room temperature during the exposure days. The performance of the diffusion dryer decreased with increasing room temperature and this problem was solved by cooling the dryer during the rest of the exposures. The mean RH for the fine particle exposures was 43% and varied between 26 and 57% (Tabel 5).

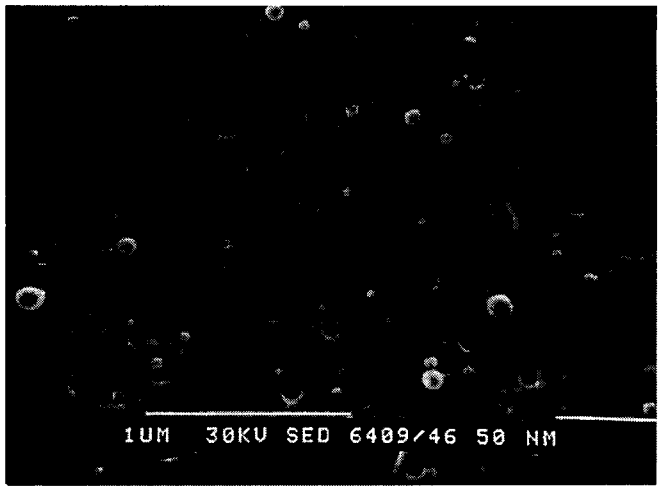
Table 5 The mean temperature and RH during exposure of the 3 substudies with ultrafine and fine ammonium bisulfate aerosols. Values are expressed as means ± SD of 3 consecutive exposure days.

	Temperature (°C)	RH (%)
ultrafine	27.0 ± 1.8	92.7 ± 22.7
fine (low mass)	23.9 ± 1.0	43.0 ± 10.1
fine (high mass)	23.9 ± 0.9	42.3 ± 13.2

3.1.5 Shape of the particles

The fine and ultrafine aerosol test atmospheres appeared to consist of spherical shaped solid particles (Fig.4)

A.



B.

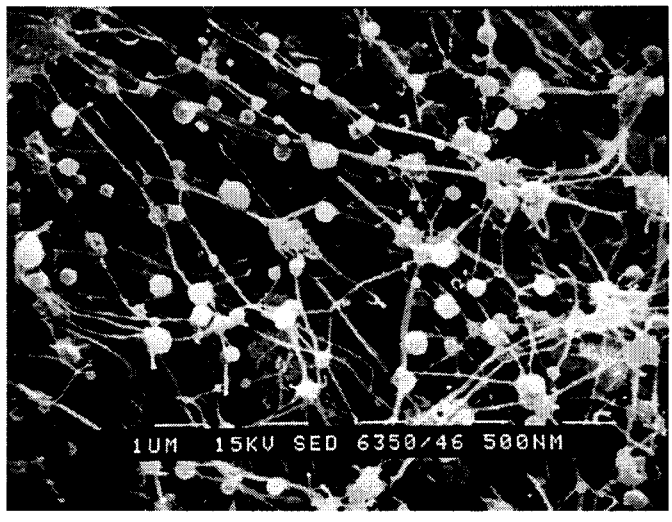


Fig.4. Scanning electron microscopic photographs of ultrafine (A) and fine (B) ammonium bisulfate particles. Ultrafine particles were collected on a VMTP filter (pore size= 50 nm) and fine particles on a VALP Teflon filter (pore size 1 μm). Both pictures show a white line that can be used to calculate the magnification and thus the diameter of the particles.

3.2 Clinical observations

No abnormalities were observed in mice that were either sensitized plus challenged with ovalbumin or in mice that were exposed to the test material.

3.3 Body weights

No differences in body weights were observed between controls and asthmatic mice. Neither were there treatment-related changes in the body weights 1 and 4 days post exposure. The overall mean body weight was 28.6 ± 2.1 g.

Table 6 Body weights of healthy and asthma mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and 5-6 for all other groups).

Type	Days post-exposure 1	
	control[a]	asthma
ultrafine	28.9 ± 2.2	29.4 ± 2.1
fine (low mass)	28.2 ± 1.9	27.6 ± 2.3
fine (high mass)	29.0 ± 2.0	28.7 ± 1.9

a. Values are expressed as gram body weight

3.4 Bronchoalveolar lavage

3.4.1 Biochemistry

The results of protein, LDH and NAG levels determined in the BALF are shown in Tables 7-9. No statistically significantly differences between either control mice and mice exposed to ammonium bisulfate or between healthy and compromised mice were observed.

Table 7 NAG levels in BALF in healthy and asthma mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and 5-6 for other groups).

Type	Days post-exposure			
	1		4	
	control[a]	asthma	control	asthma
control	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.3
ultrafine	1.0 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
control	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.3	1.6 ± 0.4
fine (low mass)	1.1 ± 0.2	1.2 ± 0.2	1.4 ± 0.5	1.3 ± 0.1
control	1.3 (n=2)	1.4 ± 0.1	1.4 ± 0.2	1.8 ± 0.1
fine (high mass)	1.3 ± 0.2	1.3 ± 0.3	1.2 ± 0.1	1.5 ± 0.2

a Activities are expressed as U/l BALF

Table 8 LDH levels in BALF in healthy and asthma mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and 5-6 for all other groups).

	Days post-exposure			
	1		4	
Type	control[a] (x10)	asthma (x10)	control (x10)	asthma (x10)
control	8 ± 2	12 ± 4	8 ± 5	13 ± 9
ultrafine	8 ± 5	7 ± 1	8 ± 5	9 ± 5
control	13 ± 12	12 ± 10	17 ± 7	15 ± 14
fine (low mass)	15 ± 8	25 ± 15	30 ± 22	23 ± 27
control	10 ± 4	12 ± 6	22 ± 22	22 ± 12
fine (high mass)	11 ± 7	13 ± 15	15 ± 10	13 ± 7

a Activities are expressed as U/l BALF

Table 9 Protein levels in BALF in healthy and asthma mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and 5-6 for all other groups).

	Days post-exposure			
	1		4	
Type	control[a] (x10)	asthma (x10)	control (x10)	asthma (x10)
control	16 ± 1	16 ± 1	17 ± 1	19 ± 6
ultrafine	17 ± 2	18 ± 2	21± 5	18 ± 2
control	25 ± 8	24 ± 6	27 ± 8	26 ± 6
fine (low mass)	27 ± 3	30 ± 11	33 ± 14	25 ± 5
control	21 ± 4	22 ± 4	23 ± 5	30 ± 8
fine (high mass)	22 ± 4	20 ±3	24 ± 7	25 ± 6

a Values are expressed as mg protein/l BALF

3.4.2 Immunology

The results of BALF cytokine measurements are shown in Table 10. Measurements of Il-4 and Il-6 indicated that these very close to the detection limit.

Table 10 TNF-α, IL-4 and IL-6 levels in BALF in healthy and asthma mice 1 day post exposure to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and 5-6 for all other groups).

Type	TNF-α[a]		IL-4[a]		IL-6[a]	
	control (x10)	asthma (x10)	control (x10)	asthma (x10)	control (x10)	asthma (x10)
1 day						
control	33 ± 17	41 ± 23	7 ± 5	12 ± 4	6 ± 5	11 ± 9
ultrafine	39 ± 19	33 ± 12	9 ± 8	5 ± 8	7 ± 8	3 ± 1
control	79 ± 34	61 ± 11	15 ± 14	11 ± 5	11 ± 8	5 ± 3
fine (low mass)	54 ± 14	73 ± 14	13 ± 9	15 ± 8	9 ± 8	16 ± 8
control	73 ± 36	64 ± 22	7 ± 5	14 ± 2	7 ± 6	8 ± 5
fine (high mass)	64 ± 14	44 ± 12	8 ± 8	7 ± 4	10 ± 10	3 ± 1

[a] All values are expressed as pg/ml BALF

3.4.3 Cytology

The results of the measurements in BALF are summarized in Tables 11 and 12.
No treatment-related effects were observed in total cell counts or the percentage of the different cell types that were distinguished.

Table 11 Viability of lavaged cells from lungs of healthy and asthma mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and n=5-6 for all other groups).

Type	Days post-exposure			
	1		4	
	control %	asthma %	control %	asthma %
control	92 ± 2	80 ± 9	92 ± 2.1	80 ± 9
ultrafine	87 ± 6	87 ± 14	89 ± 5	90 ± 8
control	69 ± 20	88 ± 16	69 ± 20	88 ± 16
fine (low mass)	85 ± 8	80 ± 18	69 ± 19	88 ± 8
control	71 ± 15	84 ± 7	71 ± 15	84 ± 7
fine (high mass)	84 ± 5	81 ± 12	86 ± 13	82 ± 14

Table 12 Total number of macrophages, neutrophilic granulocytes, and eosinophilic granulocytes plus lymphocytes in BALF of healthy and asthma mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and 5-6 for all other groups).

Type	Macrophages		Neutrophilic granulocytes		Eosinophilics granulocytes and Lymphocytes	
	control x 10^3 cell/lung	asthma x 10^3 cell/lung	control x 10^3 cell/lung	asthma x 10^3 cell/lung	control x 10^3 cell/lung	asthma x 10^3 cell/lung
1 day post exposure						
control	111 ± 12	102 ± 48	1.2 ± 0.5	0.5 ± 0.5	0.9 ± 0.4	1.4 ± 1.0
ultrafine	100 ± 52	96 ± 62	0.4 ± 0.3	0.4 ± 0.4	3.3 ± 2.7	0.9 ± 0.8
control	97 ± 54	69 ± 67	0.6 ± .01	0.5 ± 0.2	0.6 ± 0.6	0.5 ± 0.5
fine (low mass)	48 ± 11	51 ± 28	1.1 ± 0.7	0.8 ± 0.7	0.3 ± 0.3	1.1 ± 0.9
control	57 ± 29	96 ± 48	0.6 ± 0.7	1.0 ± 1.1	0.6 ± 0.7	2.9 ± 3.4
fine (high mass)	77 ± 51	77 ± 54	0.5 ± 0.4	0.7 ± 0.3	0.4 ± 0.4	1.5 ± 1.4

Table 12 (Continued) Total number of macrophages, neutrophilic granulocytes, and eosinophilic granulocytes plus lymphocytes in BALF of healthy and asthma mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and 5-6 for all other groups).

Type	Macrophages		Neutrophilic granulocytes		Eosinophilics granulocytes and Lymphocytes	
	control x 10^3 cell/lung	asthma x 10^3 cell/lung	control x 10^3 cell/lung	asthma x 10^3 cell/lung	control x 10^3 cell/lung	asthma x 10^3 cell/lung
4 day post exposure						
control	93 ± 32	39 ± 19	5.6 ± 1.1	1.3 ± 0.7	0.6 ± 0.1	0.2 ± 0.1
ultrafine	88 ± 26	105 ± 53	2.4 ± 0.8	0.8 ± 0.5	0.8 ± 0.8	2.3 ± 1.4
control	32 ± 18	92 ± 55	1.7 ± 1.3	0.7 ± 0.3	0.4 ± 0.2	1.3 ± 0.6
fine (low mass)	39 ± 31	67 ± 31	0.8 ± 0.6	1.8 ± 2.1	0.5 ± 0.5	3.7 ± 7.7
control	68 ± 76	83 ± 30	6.9 ± 7.2	1.4 ± 0.9	1.4 ± 1.0	0.8 ± 1.2
fine (high mass)	86 ± 33	59 ± 38	4.5 ± 1.8	0.9 ± 0.7	0.5 ± 0.2	3.4 ± 2.4

3.5 Serum IgE

The results of the serum IgE determinations have been summarized in Table 12. Significant increased serum IgE directed against ovalbumin could be detected in mice that were sensitized and challenged with ovalbumin compared to their controls. Although the IgE levels in mice that were exposed to aerosol test atmospheres tended to be lower compared to non-exposed mice, the differences were not statistically significant. IgE levels 4 days post-exposure are higher, but not statistically significant, in comparison to 1 day post exposure.

Table 12 Serum IgE levels (specific for ovalbumin) of asthma[a] mice exposed to fine and ultrafine ammonium bisulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n=3 for controls and n=5-6 for all other groups).

Type	Days post-exposure	
	1	4
control	23.2 ± 34.6[b]	20.2 ± 14.3
ultrafine	6.8 ± 7.4	18.1 ± 16.8
fine (low mass)	12.8 ± 7.2	29.5 ± 26.6
fine (high mass)	14.5 ± 11.4	29.8 ± 19.5

a Mice that were not sensitized and challenged with ovalbumin had no detectable IgE directed against ovalbumin

b Values are expressed as percentage of a standard (see Experimental section)

3.6 Airway reactivity

Concentration-effect curves of *in vitro* reactivity of the trachea to methacholine in mice, in which asthma-like symptoms were induced, were not influenced by exposure to any of the exposures, irrespective the type of analysis. The concentration-effect curves of methacholine in non-compromised mice were unexpectedly high compared to previous results of our laboratory in which mice were not strained in exposure tubes or exposed to an test atmosphere by inhalation. The effects were so high that they were not distinguishable from "asthma-mice".

However, the effects in these mice were not caused by the exposure to particles, because these phenomenon was also observed in mice exposed to clean air.

3.7 Pathology

3.7.1 Autopsy

Macroscopic examination of the mice at autopsy did not reveal abnormalities.

3.7.2 Histopathology

No abnormalities were observed in lungs of healthy, sham-exposed animals.
Hypertrophy of Goblet cells in bronchus and bronchiolar epithelium was observed in all asthma mice. The hypertrophy was only observed in bronchioles with a relatively large diameter (Table 13). In addition, mononuclear cell infiltrate was observed near the bifurcation's of these large airways. In 40% of the compromised mice mucus (neutral mucopoly-saccharides) was spotted in the lumen of the alveoli.

In healthy mice exposed to ultrafine particles minimal peribronchiolar and perivascular infiltrate was observed in only one animal. No treatment-related abnormalities were detected in healthy mice exposed to fine ammonium bisulfate.
No treatment particle-exposure-related effects were found in compromised mice.

Table 13 Semiquantitative registration of hypertrophy of bronchiolar epithelium and mononucleair cell infiltrate in asthma mice exposed to ultrafine and fine ammonium bisulfate. Data expressed as mean ± SD.
(minimal, slight, moderate, marked 1,2,3 and 4 respectively)

Type	Days post-exposure	Hypertrophy	Infiltrate	Number of animals examined
control	1	2.5 ± 1.0	2.1 ± 0.9	15
	4	2.5 ± 0.9	2.1 ± 0.8	15
ultrafine	1	2.4 ± 0.8	2.1 ± 0.9	10
	4	2.0 ± 1.1	1.5 ± 0.7	10
fine (low mass)	1	2.1 ± 0.6	2.5 ± 0.7	10
	4	1.4 ± 0.5	1.4 ± 0.5	10
fine (high mass)	1	2.1 ± 0.7	2.6 ± 0.8	10
	4	2.0 ± 0.0	1.6 ± 0.8	10

4. Discussion and Conclusions

Since especially people with compromised airways experience adverse effects of exposure to PM, we used a mouse model that mimics asthma. This report describes the results of a series of short-term inhalation studies with mice. Healthy and asthma mice were exposed to ammonium bisulfate aerosol atmospheres. We have used three different exposure atmospheres to discriminate between various particle size, and mass concentrations.

Although the MMD's of fine and ultrafine were not exactly 50 and 500 nm as described in the original proposal, the particle sizes used in the present study can be classified as ultrafine and fine aerosols. Table 4 shows results of the mass calculations based on the CMDs and MMDs. Significantly lower masses were calculated for ultrafine particles. The relative largest particles in a test atmosphere account for a significant part to the total mass. CMD and MMD were determined with a SMPS that was equipped with an impactor that excluded all particles with a diameter larger than 1 μm. Particles mass measurements can therefore result in a larger mass compared to the mass calculations based on CMD or MMD. In case of calculation with CMD, all particles were considered to be spherical shaped and strictly monodisperse. This will result in an underestimation of the calculated mass, because the aerosol test atmosphere has a certain distribution around the CMD. The difference between mass calculations between CMD and MMD is a result of the way the MMD is calculated from classes of diameters from the CMD measurements and thus accounting for the distribution around the CMD. The results clearly indicate that a mass concentration cannot be derived accurately from a CMD.

NAG, LDH and total protein levels in BALF were unaffected in all exposure groups, suggestive for the absence of macrophage activation, lung injury and edema. BALF changes in the pro-inflammatory markers IL-4, IL-6, and TNFα were not observable. There were no differences observed between healthy and asthma mice. However, in asthma mice exposed to the highest mass concentration of fine NH_4HSO_4 these parameters tended to be slightly (but not statistically significantly) decreased compared to healthy mice exposed to the same test atmosphere. Both histopathologic examination and counting of inflammatory cells in BALF fluid showed a moderate to marked inflammation in asthma mice. However, this inflammation was not changed by exposure to NH_4HSO_4 particles. Moreover the inflammatory response was not confirmed by changes in cytokine levels one-day post exposure.

Serum ovalbumin-specific IgE levels are indicative for the asthmatic status of the compromised mice. This was confirmed by the fact that in control (non-compromised) mice the IgE levels were undetectable, whereas in asthma mice these levels were significantly elevated. It is also hypothesized that asthma mice are hyperresponsive to metacholine. Despite this hypothesis, no simple correlation could be established between serum IgE levels and the maximally observed contraction of a trachea *in vitro*. Exposure to the test atmosphere did not affect the IgE levels 1 day post-exposure. Four days post-exposure we have observed that IgE levels in asthma non-exposed mice had not changed, but the levels in asthma mice exposed to (ultra) fine particles had increased in comparison with 1 day post-exposure. This result suggests that exposure to ammonium bisulfate aerosols may have a stimulating effect on the IgE response. However none of the other parameters measured in this study justifies such an effect and we consider this effect as non-treatment related.

Histopathologic examination showed no particle-related effects in either healthy or asthma mice. Control groups showed clean, healthy lungs. The method to induce symptoms that refer to asthma proved to be effective: both hypertrophy of bronchiolar epithelium and mononuclear cell infiltrate were observed.

The third criteria to investigate the effectiveness of the method to induce an animal model that mimics the symptoms of asthma was the cytological examination of cell lavaged from the lungs. There is a distinct trend that asthma mice have more eosinophils in their lungs compared to mice that were not treated with ovalbumin. Again, no particle-exposure related effect was observed in either healthy or compromised mice, suggesting that there are no adverse effects on lungs at the concentration levels of ammonium bisulfate used in this study.

An important parameter in these studies was airway reactivity, related to (hyper) responsiveness in patients with (enhancement) of allergic asthma. However, in the present study it seems to have been influenced by a non-treatment-related phenomenon. The asthma model was originally developed to study the development of asthma rather than to use it in an inhalation study. In the present studies, mice were restrained in nose-only exposure tubes. Although the mice were trained in the tubes prior to the exposure to the test atmosphere, the possibility that restraining did influence the airway reactivity measurements can not be excluded. Moreover, the differences between the controls of the three studies forced us to evaluate the results of the studies separately. Since there is always a significant intra-group variation for this parameter, the statistical analysis did not result in any significant differences between any of the experimental groups
Besides comparing the complete concentration-contraction curves for each experimental group, additional analysis has been carried out to discriminate between airway sensitivity and airway reactivity, by determination of an EC_{20}, EC_{50}, EC_{80} and an E_{max} for each curve. This additional analysis did not reveal exposure-related effects.

Collectively, these preliminary data suggest that ammonium bisulfate model particles exert only marginal, if any, pulmonary responses in a mouse model of allergic asthma and there seem to be no indications of an enhanced allergic response following exposure. No differences were observed between fine and ultrafine particles in the exposure range tested (up to ~ 1000 $\mu g/m^3$). Neither can we make a statement on the effect of number of particles in relation to adverse health effects. Large variations in most parameters that are inherent to the use of mice in this experimental setting prevent us from any statistically well-founded conclusions.

5. References

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Appendix

"Sandwich" micro-ELISA for Quantification of Mouse IgE to Ovalbumin

Wells of polystyrene microtiterplates (Greiner, high binding) were coated by adding 100 µl of anti-immunoglobulin solution (Monoclonal Rat Anti-mouse IgE, 2 µg protein/ml, clone LO-ME-2, Zymed) in sodium carbonate buffer (0.1 M; pH 9.6) with 0.02 % NaN_3 as preservative. Plates were shaken on a plate shaker and covered with a plate sealer.
After incubation at 4°C for 18 hr, plates were washed by using PBS containing 0.1% Tween 20 (Microplate washer 120, Titertek; 5 repeated washes). After washing of the plates, 100 µl of blocking reagent (Boehringer Mannheim, Cat. No. 1096176; 1 % w/v in PBS + 0.1% tween 20) was added to each well. Plates were covered with a plate sealer and incubated at 37°C for 1 hr, in a thermostatically controlled water-bath (plates floating on the water). Incubated plates were washed with PBS+ 0.1% Tween 20 (5 repeated wash cycles). Sera samples were pre-diluted (1:4) with phosphate buffered saline (PBS; 0.01M pH 7.2) containing 0.02% NaN_3 and stored at -20° until analysis.

For analysis, pre-diluted samples were diluted (in an optimal dilution) with PBS. Quantities of 100 µl were added to each well, and plates were incubated in a water-bath (as above) at 37°C for 1 hr. After incubation, plates were washed as described above and 100 µl of an Ovalbumin-DIG* solution (0.01 µg protein/ml) in blocking reagent were added to each well and incubated at 37°C in a water-bath for 1 hr.
After incubation, plates were washed, 100 µl of the conjugate (Anti-Digoxigenin-POD Fab fragments, Boehringer Mannheim Cat. No. 1633716; 40 mU/ml) in blocking reagent solution was added. Plates were incubated in a water-bath at 37°C for 2 hr. After incubation, excess of conjugate was removed by washing. Finally, the amount of enzyme bound to the wells was determined by adding 100 µl of substrate.
The substrate for the peroxidase was prepared by:

A) Dissolve 6 mg tetramethylbenzidine (TMB) per ml dimethylsulfoxide (DMSO)
B) Buffer solution of 1.1 M sodiumacetate and citric acid to a pH of 5.5
Stored with prevention of light at 4°C

Immediately before use, solution B was diluted 10 times with distilled water.
1.67 ml of solution A was added to 100 ml of the diluted buffer and was 20 µl of 30% H_2O_2 was added to this mixture.
After incubation at room temperature for a period of 20 min. exact, the reaction was stopped by adding 100 µl of 2 M H_2SO_4 to each well. Extinctions were measured at 450 nm on a multichannel photometer (Multiskan MCC /340 MK II, Titertek)
For quantification of IgE levels a standard curve consisting of seven twofold serial dilution's of a pooled serum of "positive" mice, was prepared using logistic curvefit (Titersoft II, Flow Laboratories). Test sera were analyzed in one dilution (triplicate) of an optimal concentration.
In addition, a conjugate control and a substrate control were included. In order to eliminate variations between plates, a standard curve was included in each plate. To eliminate systematic variations within plates, samples were randomly distributed over the wells. This means that per microtiter plate 21 wells were used for standard curve, 3 for the conjugate controls, 3 for the substrate controls and 69 for the test sera (triplicate analysis of 21 sera).

* Ovalbumin-DIG (the ovalbumin-DIG was prepared by labeling of ovalbumin (albumin, chicken egg, grade VII, Sigma) with digoxigenin using the reagent kit (DIG Antibody Labelings Kit, Cat.No.1367200) obtained from Boehringer Mannheim, Germany.
Ovalbumin (Mw: approx. 40.000)
Molar reaction mix; 1 : 10
Protein concentration was measured conform Lowry.