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Toxicity of Ambient Particulate Matter
III. Acute toxicity study in (asthmatic) mice following 3-day exposure to ultrafine and fine ammonium nitrate, a model compound 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 are part of the Dutch "Wintertime Smog and Traffic Program ", performed by order and at 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_{10}, i.e. the "Pentagon" model for possibly main critical fractions with respect to particle size and chemical composition.

This research will be a meaningful help to prove causality and biological plausibility of effects of PM_{10} in healthy people and, in particular, asthmatics as a possible susceptible group, especially when used in combination with ambient PM10deposition models and exposure-dose relationships.

The 1st phase of these toxicity studies has been started in 1996 and the results of the third 3-day inhalation study (with ammonium nitrate) in healthy and asthmatic are presented in this report. The results of these and successive studies performed in this framework will be integrated later on in a review report on the toxicity of carbonaceous and secondary aerosols.

Samenvatting

In dit derde rapport over acute inhalatiestudies met modelcomponenten van fijnstof in de buitenlucht worden de resultaten gepresenteerd van een studie met ammoniumnitraat in gezonde en astma dieren. Ammoniumnitraat is de belangrijkste component van het secundair gevormde fijnstof in Nederland. Secundair stof is een verzamelnaam voor aerosolen die vanuit gasvormige componenten in de atmosfeer worden gevormd. In deze studie is gebruikgemaakt van een diermodel voor astma, omdat epidemiologisch onderzoek astmatici identificeren als een groep die gevoelig is voor acute expositie aan fijnstof.

We hebben gezonde en "astmatische" muizen blootgesteld aan fijn (CMD = 0.3 μm; 4 x 10^3 deeltjes per cm^3) en ultrafijn (CMD = 0.03 μm; 2 x 10^5 deeltjes per cm^3) ammoniumnitraat gedurende 4 uur per dag drie dagen lang. De gemiddelde massa concentratie bedroeg respectievelijk 140 en 250 $\mu g/m^3$. De muizen werden 1 dag na de laatste blootstelling opgeofferd om de effecten vast te stellen middels longspoelvloeistof (BALF) analyse, luchtweg-reactiviteitsmetingen en histopathologie.

De behandeling met ovalbumine (om astma te induceren) was succesvol, wat bleek uit hypertrofie van Goblet cellen in bronchusepitheel en mononucleair infiltraat en duidelijk aantoonbare verhogingen van specifiek IgE in serum. De belangrijkste parameter in deze studies was *in vitro* luchtwegreactiviteit, de mate van contractie van tracheaspieren na toediening van methacholine. Deze bleek significant verhoogd te zijn na expositie aan fijn ammoniumnitraat, waarbij er geen verschil optrad tussen gezonde en gecompromitteerde dieren. In gezonde of gecompromitteerde dieren blootgesteld aan ultrafijn ammoniumnitraat werd geen effect op de reactiviteit waargenomen.
Er zijn geen aanwijzingen gevonden voor het optreden van cytotoxiciteit (gemeten met de indicatoren lactaat dehydrogenase (LDH), eiwit- en albumine gehaltes in BALF) als gevolg van de blootstelling aan (ultra)fijne ammoniumnitraat. Wel waren macrofagen in alle blootgestelde groepen geactiveerd waarbij N-acetylglucosaminidase alleen statistisch significant verhoogd was in gecompromitteerde dieren na expositie aan ultrafijn ammonium nitraat. Er zijn geen significante verhogingen van immunologische markers als TNF-α, Interleukine 4 en 6 vastgesteld. Het celdifferentiatiebeeld laat zien dat er relatief minder macrofagen en meer neutrofielen in BALF werden aangetoond na blootstelling aan fijn ammoniumnitraat.
Pathologisch onderzoek kon geen effect van de blootstelling aan aerosolen vaststellen.
De hier gepresenteerde gegevens suggereren dat bij concentraties die vergelijkbaar zijn met eerdere studies met ammoniumbisulfaat en ammoniumferrosulfaat pulmonaire effecten in normaal gezonde en gecompromitteerde muizen (als model voor astma) optreden. Hierbij blijkt dat deze effecten met name bij fijn en niet bij ultrafijn nitraat optreden. Uit het feit dat de massa concentratie van fijn ammoniumnitraat lager was dan die van ultrafijn nitraat kan worden opgemaakt dat de invloed van de grootte van het deeltjes niet verwaarloosd mag worden. Depositiemodellering zal hier meer inzicht in geven. Er zijn geen indicaties voor een versterking van de allergische reactie.

Summary

In this third report on acute inhalation studies with model compounds for particulate matter (PM) in ambient air results are presented of a study with ammonium nitrate in healthy and asthmatic mice. Ammonium nitrate is the most prominent component of secondary PM10in the Netherlands. Secondary PM10is a collective term for aerosols formed out of gaseous air pollutants. In the present study we have used an animal model for asthma, since epidemiological research has identified asthmatics as a subpopulation sensitive for acute exposure to PM.

Healthy and asthmatic mice were exposed to fine (CMD = 0.3 μm; 4×10^3 particles per cm^3) and ultrafine (CMD = 0.03 μm; 2×10^5 particles per cm^3) ammonium nitrate during 4 hr a day for three consecutive days. The mean mass concentrations were 140 and 250 $\mu g/m^3$, respectively. The animals were sacrificed 1 day after the last exposure to establish effects on the airways using bronchoalveolar lavage fluid (BALF) analysis, airway reactivity measurements and histopathology.

The ovalbumin treatment (to induce asthma) was successful as seen by hypertrophy of Goblet cells in bronchus epithelium and mononuclear infiltrate, as well as marked IgE levels in serum. *In vitro* airway reactivity, indicative for the seriousness of asthma and the most important parameter in this study, appeared to be statistically significantly increased in mice exposed to fine ammonium nitrate. However, no differences were observed between healthy and asthma mice. Ultrafine ammonium nitrate did not induce an effect on this parameter. There were no signs of cytotoxicity (as measured with lactate dehydrogenase (LDH), protein- and albumin contents in BALF) in any of the exposed groups. Macrophages were activated in all groups exposed to the test atmospheres, though the indicator N-acetyl glucosaminidase (NAG) was only statistically significantly enhanced in asthma animals exposed to ultrafine ammonium nitrate. Immunological markers like TNF-α, Interleukin 4 and 6 were not changed. Cell differentiation profiles showed that there were relative less macrophages and more neutrophils in BALF of mice exposed to fine ammonium nitrate.
No effects due to ammonium nitrate were observed after histopathological examination.
The presented results suggest that at exposure levels comparable with previous studies with ammonium bisulfate and ammonium ferrosulfate pulmonary effects were observed in both healthy and asthma mice. The effects were mainly found after exposure to fine rather than to ultrafine nitrate. From the fact that the mass concentration of fine was even lower than of the ultrafine aerosols we conclude that not only mass concentrations but also the specific size of the particles are important for the development of adverse effects. Dosimetry models will be useful for the foundation of this conclusion.
There were again no signs for asthma mice being more sensitive for secondary aerosols than healthy mice.

1. Introduction

The PM10toxicity studies are conducted within the framework of a toxicity research program on health effects of relevant components of ambient particulate matter (PM). This program is part of the Dutch "Wintertime Smog and Traffic Program". This is the third report on acute inhalation studies with model components of ambient particulate matter.

Recent experimental animal studies show that intratracheal instillation (high dosage of 2.5-5 mg/rat) as well as *in vitro* incubation of fine or coarse particles (~ 0.4-10 μm; mineral dusts, PM10collected from ambient air, fly ash samples) cause (pulmonary) cytotoxicity, inflammation, and production of reactive oxygen species, also in an animal model for pulmonary hypertension (Hatch et al. 1985; Ghio et al. 1992; Ghio and Hatch 1993). Although nitrate particles are not as good correlated with fine PM (PM2.5) as sulphates, sufficient levels of nitrate aerosols can be found in the Netherlands (RIVM, 1997)

The studies described in this report were aimed to identify adverse effects of ammonium nitrate aerosol in healthy and asthmatic mice. We hypothesised that fine and ultrafine ammonium nitrate particles as a model compound for secondary aerosol fraction of PM_{10} enhances inflammation and hyperresponsiveness in a mouse model that mimics asthma. We have exposed mice 4 hr/day for 3 consecutive days and investigated the adverse effects of fine and ultrafine particles on the lung. In this study we have focused on biochemical, histopathological, cytological and immunological parameters indicative for cytotoxicity and asthmatic symptoms.

2. Experimental

2.1 Testing facilities

The studies described in this report were conducted at the department of Toxic Effects of the Laboratory of Health Effects Research, RIVM, the Netherlands.

2.2 Test material

Crystalline ammonium nitrate (NH_4NO_3) was supplied by Aldrich-Chemie, Steinheim, Germany and has the following characteristics:

CAS nr.	: 7783-85-9
Batch nr.	: 06774-065
Purity	: 98 %
Density	: 1.720 kg/l
Molecular weight	: 81
Melting point	: 210°C.
Solubility	: 1200 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 acclimatisation period was at least 7 days. The room temperature was maintained at 22 ± 2°C and the relative humidity at 40-70% with a 12 hr light/dark cycle. The mice were fed a cereal-based rodent diet (SMR-A; Hope Farms, Woerden, the Netherlands). The day before the start of each substudy (see 2.4), the mice were identified with an earmark to be able to discriminate between sensitised and non-sensitised mice. One day prior to the challenge procedure each mouse was marked according to the allocation list with a unique number, which was coded by placing stripes on the tail using felt-tips.

2.4 Location of the study

Mice arrived at the Institute's animal housing facilities at least 7 days prior to the start of the study and checked for body weight variation. In addition, animals were random allocated and then sensitised. 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 was used for airway reactivity measurements and histopathology and the rest the rest was used for bronchoalveolar lavage and serum collection.

2.5 Conduct of the study

The study was performed with 60 mice. Before exposure to the test material, 30 mice were compromised with ovalbumine (OVA). 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. All animals were sacrificed one day after the last exposure. Mice were either used for bronchoalveolar lavage and serum collection or airway reactivity measurements and histopathology.

Group code	Sensitisation with OVA	Challenge with OVA	Test atmosphere exposure	Number of animals
A	-	-	3x Sham	10
B	7x	8x	3x Sham	10
C	-	-	3x Fine	10
D	7x	8x	3x Fine	10
E	-	-	3x Ultrafine	10
F	7x	8x	3x Ultrafine	10

Group A/B: control, sham exposed.
Group C/D: fine ammonium nitrate aerosol.
Group E/F: ultrafine ammonium nitrate aerosol.

2.6 Sensitisation and antigen challenge

Animals were sensitised 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 33 these mice were exposed to ovalbumin (0.2 % w/v) aerosol for 5 min. at 8 consecutive days. On days 38, 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 (Hessel *et al.* 1995).

2.7 Exposure system

Mice were individually, nose-only exposed in a small exposure chamber of the Institute's design. During exposure the animals were restrained in animal holders (modified Battelle tubes) which closely fit to the exposure chamber. The animals were allowed to breathe freely from the stream of fresh test atmosphere. During exposure animals were deprived from food and water. Animals were trained for 2 hr a day during the five days prior to the first exposure (days 33 - 37).

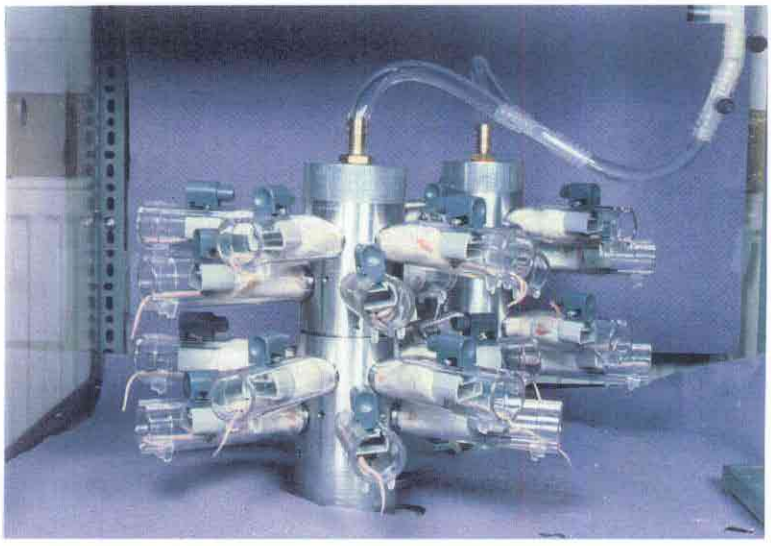

Fig. 1 The nose-only exposure system used in this study

2.8 Generation of the atmosphere[1]

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

2.9 Analysis of the test atmosphere[2]

The mass concentrations of the test atmospheres were determined wet chemically by analysing sodium carbonate coated filters on which particles were collected.
Particle size, total number of particles and size distribution were determined with a scanning mobility particle sizer (SMPS) 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).
Relative humidity and temperature of the test atmosphere were measured conventionally. The morphology of the fine particles was visualised by scanning electron microscopy (SEM). Freshly generated particles were sampled on a Teflon filter and processed for SEM.

2.10 Observations and measurements

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

[1] A detailed generation report is added to the file of this study.
[2] A detailed exposure report is added to the file of this study.

2.11 Body weights

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

2.12 Bronchoalveolar lavage and measurements

Mice were i.p. anaesthetised with sodium pentobarbital. Each lung was lavaged three times with one volume saline corresponding with 40 ml/kg body weight 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 (LDH), N-acetyl glucosaminidase (NAG) and albumin were determined using a commercially obtained reagent kit (Boehringer Mannheim (Mannheim, Germany). Total protein was determined using a reagent kit 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 albumin and total protein levels in BALF were measured as indicators for increased permeability of the alveolar-capillary barrier.

2.12.2 Immunology

Cytoscreen immunoassay kits (Biosource international, Camarillo, CA) were used to determine TNF-α, IL-3, Il-4 and Il-6. 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 and IL-6 is released by TH2, fibroblasts and other cells and augments inflammation. The primary cell sources of TNF-α are macrophages and will result in increased phagocytosis and enhancement of immune- 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 granulocytes, eosinophilic granulocytes and lymphocytes) was calculated based on total viable cells per BALF sample.

2.13 Serum IgE[3]

A sandwich micro-ELISA was used for the quantification of mouse IgE to ovalbumin. 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.

[3] A detailed description of the procedure is added to the files of this study.

2.14 Airway reactivity

Airway reactivity was measured according to a method published by Garssen *et al.* (1990). A cumulative contractile concentration-effect curve was determined for each trachea using methacholine. The maximum contraction (E_{max}) as well as an EC_{20}, EC_{50} and EC_{80} (the concentration that induces 20, 50 and 80 % of the maximum contraction, respectively) were determined from this curve.

2.15 Histopathology

At autopsy the animals were anaesthetised 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. Sextions were stained with haematoxylineosin 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 Netherlands). The (log-transformed) data were analysed using multiple one-way analysis of variance. 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 were analysed separately. Since homogeneity of variance was not obtained for the IgE data, these data were analysed using the Kruskal-Wallis non-parametric test.
Airway reactivity data were analysed with an ANOVA with all data points of the concentration-effect curves. EC_{20}, EC_{50} and EC_{80} as well as E_{max} were calculated from fitted curves and these values were analysed with a t-test.

2.17 Deviations from the protocol

OVA-challenges were performed with 10 animals at the same time in stead of 6 as mentioned in the protocol. Mass concentrations of the ammonium nitrate aerosols were initially determined by collection particles on coated filter as described in section 2.8. However, the deviations from the estimated mass concentrations for ultrafine and fine were considered too large. Consequently, the test atmospheres were generated again using approximately the same number concentrations and particle diameters. The filters were weighted and an additional chemical analysis was performed. The results were used for regression analysis and based on the original number concentrations and particle size, mass concentrations were calculated. Based on this additional analysis it was concluded that the chemical analysis was not reliable and only gravimetric determined mass concentrations were used for analysis of the effect parameters. 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 summarised in Table 1.

Table 1 Actual exposure concentrations (#/cm^3) of fine and ultrafine ammonium nitrate aerosol. Values are expressed as means, 5 percentile and 95 percentile.

Day	Fine (#/cm^3) . 10^3	Ultrafine (#/cm^3) . 10^5
1	4.0 (2.7- 4.8)	2.3 (1.9 - 2.9)
2	3.8 (2.5 - 5.1)	2.6 (2.4 - 3.0)
3	3.9 (2.8 - 5.1)	1.9 (1.8 - 2.0)
mean	3.9 ± 0.1	2.3 ± 0.3

3.1.2 Particle size and mass measurement

The results of the size and mass measurements are summarised in Table 2. The mass determinations during the exposure period were considered to be irreliable. Therefore, additional measurements were performed in which the test atmosphere was generated again. Based on the same stock solution, approximately the same number of particles with the corresponding CMD was generated. Both gravimetrical measurements (using particle collection on Teflon filters) and chemical detection of ammonia were used to determine the aerosol mass concentrations.

Table 2 Count Median Diameter (CMD) and Mass Median Diameter (MMD) as determined with SMPS of fine and ultrafine ammonium nitrate aerosol. Values are expressed as means ± geometric standard deviation.

	Mass concentration[4]	Particle Diameter (nm)	
Day	µg/m^3	CMD	MMD
Fine	140	313 ± 2	584 ± 2
Ultrafine	250	34 ± 2	219 ± 2

[4] Mass concentrations were determinded using the volume concentrations of the test atmospheres, and at a later stage a similar aerosol was generated with its accompanying gravimetically determined mass concentrations, since the original mass measurements failed. See also section 2.16.

3.2 Temperature and Relative Humidity

Table 3 The mean temperature and R.H. of the test atmospheres. Values are expressed as means ± SD of 3 consecutive exposure days.

	Temperature (°C)	RH (%)
Sham	24 ± 1	34 ± 13
Ultrafine	24 ± 1	12 ± 2
Fine	25 ± 1	20 ± 2

3.3 Clinical observations

No abnormalities were observed in mice that were either sensitised and challenged with ovalbumin, in mice that were exposed to the test material, or both.

3.4 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-day post exposure.

Table 4 Body weights of healthy and asthma mice exposed to fine or ultrafine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-7 for all groups).

Type	Healthy[a]	Asthma
Sham	29.0 ± 1.7	28.6 ± 2.2
Fine NH_4NO_3	30.2 ± 1.1	29.7 ± 1.8
Ultrafine NH_4NO_3	30.6 ± 1.8	29.1 ± 1.8

a. Values are expressed as gram body weight

3.5 Bronchoalveolar lavage

3.5.1 Biochemistry

The results of the BALF protein, LDH and NAG measurements are shown in Tables 5 and 6. No statistically significant differences between either control mice and mice exposed to ammonium nitrate or between healthy and asthma mice could be established with respect to LDH and protein levels. NAG levels were increased in mice exposed to ultrafine ammonium nitrate. A small and physiologically irrelevant decrease of albumin levels was observed due to fine ammonium nitrate exposures in asthma mice.

Table 5 BALF levels of NAG, LDH, albumin and protein in healthy and asthma mice exposed to fine or ultrafine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5).

Type	NAG[a]	LDH[a]	Albumin[b]	Protein[b]
Healthy , sham	0.54 ± 0.17	114 ± 98	101 ± 28	180 ± 41
Asthma, sham	0.56 ± 0.25	157 ± 74	127 ± 25	219 ± 49
Fine ammonium nitrate	0.58 ± 0.14	139 ± 130	114 ± 27	193 ± 40
Asthma, fine ammonium nitrate	0.78 ± 0.24	179 ± 162	134 ± 63	198 ± 107
Ultrafine ammonium nitrate	0.53 ± 0.16	61 ± 21	88 ± 10	158 ± 12
Asthma, ultrafine ammonium nitrate	0.84 ± 0.24	142 ± 112	114 ± 32	202 ± 53

a. Activities are expressed as U/l BALF
b. Levels are expressed as mg/l BALF

Table 6 BALF levels of NAG, LDH and albumin in healthy and asthma mice exposed to fine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all other groups). ** P<0.01

Type	NAG[a]	LDH[a]	Albumin[b]
Healthy, sham	2.96 ± 0.57	589 ± 397	0.56 ± 0.05
Asthma, sham	2.79 ± 0.31	698 ± 255	0.58 ± 0.02
Fine ammonium nitrate	3.02 ± 0.39	646 ± 486	0.59± 0.02
Asthma, fine ammonium nitrate	3.48 ± 0.81	638 ± 326	0.54 ± 0.04**
Ultrafine ammonium nitrate	3.31 ± 0.81	383 ± 109	0.56 ± 0.04
Asthma, ultrafine ammonium nitrate	4.13 ± 0.43**	643 ± 360	0.56 ± 0.02

a. Activities are expressed as U/g protein
b. Levels are expressed as g/g protein

3.5.2 Immunology

The results of BALF TNF-α measurements are shown in Table 7. Large inter-group variation was observed in all groups. Correction for protein contents did not result in lower inter-group variation (data not shown).

Table 7 BALF levels of interleukines in healthy and asthma mice 1 day post exposure to fine or ultrafine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n = 5-7 for all other groups).

Type	TNF-α (x10)[a]	IL-3	IL-4	IL-6 (x10)
Healthy, sham	54 ± 58	8.1 ± 0.4	82 ± 65	14 ± 9
Asthma, sham	69 ± 34	8.1 ± 0.2	92 ± 34	13 ± 7
Fine ammonium nitrate	71 ± 87	8.5 ± 1.0	111 ± 93	18 ± 18
Asthma, fine ammonium nitrate	35 ± 19	7.9 ± 0.1	66 ± 31	9± 4
Ultrafine ammonium nitrate	19 ± 10	7.9 ± 0.1	49 ± 10	7 ± 3
Asthma, ultrafine ammonium nitrate	67 ± 63	8.1 ± 0.6	88 ± 53	15 ± 10

a. Levels are expressed as pg/ml BALF

3.5.3 Cytology

The results of the cell measurements in BALF are summarised in Tables 8 - 10.
Asthma mice tend to have slightly lower cell viability compared to healthy animals. No treatment-related effects were observed in total cell counts or the percentage of the different cell types that were distinguished.

Table 8 Viability of lavaged cells from lungs of healthy and asthma mice exposed to fine and ultrafine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n = 5 for all other groups).

Type	Healthy[a]	Asthma
Sham	78 ± 13	85 ± 9
Fine NH_4NO_3	91 ± 3	86 ± 9
Ultrafine NH_4NO_3	92 ± 4	80 ± 13

a. Values are expressed as percentage of total lavaged cells.

Table 9 Total number of macrophages, neutrophils, eosinophils and lymphocytes in BALF of healthy and asthma mice exposed to fine or ultrafine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5).

Type	Macrophages		Neutrophils		Eosinophils		Lymphocytes	
	Healthy[a]	Asthma	Healthy	Asthma	Healthy	Asthma	Healthy	Asthma
Sham	92 ± 63	69 ±14	6 ± 5	5 ± 2	0	1 ± 0	1 ± 1	2 ± 2
Fine NH_4NO_3	80 ± 31	93 ± 49	17 ± 14	12 ± 16	0	0	1 ± 0	3 ± 2
Ultrafine NH_4NO_3	96 ± 22	71 ± 23	9 ± 5	5 ± 3	0	0	1 ± 1	2 ± 1

a = number x 10^3 cell/lung

Table 10 Relative number of macrophages, neutrophils, eosinophils and lymphocytes in BALF of healthy and asthma mice exposed to fine or ultrafine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as mean percentage ± SD (n= 5).

Type	Macrophages		Neutrophils		Eosinophils		Lymphocytes	
	Healthy[a]	Asthma	Healthy[a]	Asthma	Healthy[a]	Asthma	Healthy[a]	Asthma
Sham	93 ± 4	91 ± 2	7 ± 4	6 ± 3	0	1 ± 0	1 ± 1	3 ± 2
Fine NH_4NO_3	83 ± 7	87 ± 7	16 ± 7	10 ± 8	0	0	1 ± 1	4 ± 4
Ultrafine NH_4NO_3	91 ± 3	92 ± 4	8 ± 4	7 ± 4	0	0	1 ± 1	2 ± 1

a = expressed as percentage

3.6 Serum IgE

The results of the serum IgE determinations are summarised in Table 11. Significant increased serum IgE directed against ovalbumin could be detected in mice that were sensitised and challenged with ovalbumin compared to their controls. No significant differences were observed between sham and nitrate exposed mice. It should be noted that the variation in ovalbumin specific IgE levels ranged from 3 to 293 % of the standard within a group.

Table 11 Serum Ig-E levels (specific for ovalbumin) of asthma mice exposed to ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n = 5-7 for all other groups).

Type	Healthy [a]	Asthma
Sham[a]	n.d.	100 ± 62
Fine NH_4NO_3	n.d.	86 ± 66
Ultrafine NH_4NO_3	n.d.	67 ± 41

a. Mice that were not sensitised and challenged with ovalbumin had no detectable (n.d.) IgE directed against ovalbumin (<1%)
b. Values are expressed as percentage of a standard (see experimental section)

3.7 Airway reactivity

A slight increase in airway reactivity in trachea induced by methacholine was observed in compromise (asthmatic) animals compared to healthy animals (Fig 2A versus Fig 2B; Table 12). However, this increase was statistically not significant, neither for the whole

concentration-effect curve nor for E_{max}, EC_{20}, EC_{50}, or EC_{80}. It should be noted that the values of control animals were somewhat higher compared to historical data.
Mice exposed to fine ammonium nitrate appeared to respond with an increased contraction of the smooth airway muscles: E_{max}, EC_{50} and EC_{80} values were statistically significantly increased compared to sham exposed animals. This was not observed in animals exposed to ultrafine ammonium nitrate. However, based on a comparison of the EC values, no enhancement of the effect of OVA was found as a result of ammonium nitrate aerosol exposure: EC values are not higher in asthma animals compared to healthy animals. (Table 12). Thus, no statistically significant increase of the *in vitro* reactivity of the trachea in asthma mice compared to the healthy animals was observed, regardless the kind of exposure atmosphere.

Table 12 Values for maximum contraction (E_{max})and concentrations of methacholine needed to induce 20, 50 or 80% of the E_{max} (EC_{20}, EC_{50}, EC_{80}) in healthy and asthma mice exposed to fine or ultrafine ammonium nitrate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5).

Type	E_{max} [a]		EC_{20} [b]		EC_{50} [b]		EC_{80} [b]	
	Healthy	Asthma	Healthy I	Asthma	Healthy	Asthma	Healthy	Asthma
Sham	2691 ± 715	2566 ± 718	9.8 ± 2.4	14.6 ± 5.6	48 ± 9	60 ± 22	372 ± 94	452 ± 128
Fine NH_4NO_3	2916 ± 605	3289 ± 233	9.8 ± 1.6	9.3 ± 4.5	33 ± 5	32 ± 14	230 ± 27	241 ± 44
Ultrafine NH_4NO_3	2606 ± 502	2792 ± 268	10.6 ± 4.1	9.2 ± 1.0	48 ±15	41 ± 8	376 ± 139	317 ± 52

a = mg contraction force
b = x 10^2 µM metacholine

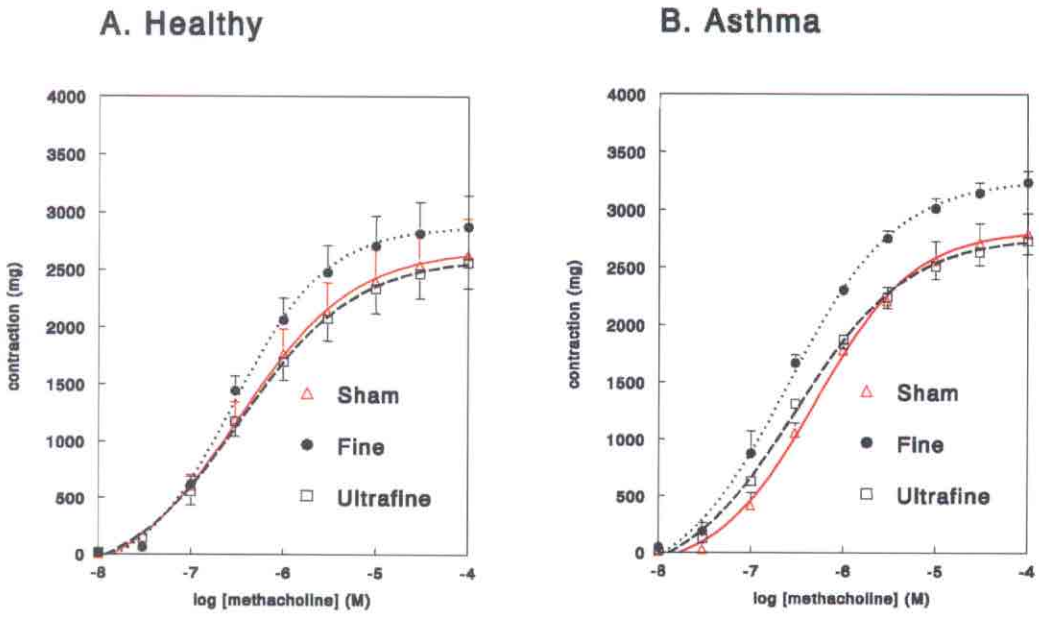

Fig. 2 Tracheal responsiveness to methacholine measured in healthy and asthmatic mice exposed to clean air or ammonium nitrate aerosol atmospheres.

3.8 Pathology

3.8.1 Autopsy

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

3.8.2 Histopathology

Half of the animals showed mild perivascular oedema, which could have been the result of the intratracheal fixation. Hypertrophy of the bronchiolar epithelium, mainly in the larger airways of the lung, and peribronchiolar inflammatory infiltrate of monocytes was spotted in all asthma mice (either sham exposed or mice exposed to nitrate). This was accompanied by mucus secretion in the bronchiolar lumen in some animals. In some animals no hypertrofic bronchiolar epithelium was spotted, since no larger airways were sliced of the lungs of these animals.

No abnormalities were observed in lungs of healthy or asthma asthmatic that could be related to ammonium nitrate aerosol atmospheres.

Table 13 Semiquantitative registration of hypertrophy of bronchiolar epithelium and mononuclear cell infiltrate in healthy and asthma mice exposed to fine or ultrafine ammonium nitrate. Data expressed as incidences.

Type	**sham exposed**	**fine ammonium nitrate**	**ultrafine ammonium nitrate**
Healthy mice			
Number of animals	*5*	*5*	*5*
Perivascular edema	2	0	4
Bronchus hypertrofy	0	0	0
Peribronchiolar infiltrate	0	0	0
minimal	0	0	0
slight	0	0	0
moderate	0	0	0
marked	0	0	0
Asthma mice			
Number of animals	*5*	*5*	*5*
Perivascular edema	1	2	4
Bronchus hypertrofy	5	4	4
Peribronchiolar infiltrate	5	4	5
minimal	2	1	1
slight	1	3	3
moderate	2	0	0
marked	0	0	1
Mucus in bronchiolar lumen	2	1	3

4. Discussion and Conclusions

We hypothesised that ammonium nitrate particles as a model component of the secondary fraction of PM_{10} will worsen asthmatic symptoms like inflammation and hyperresponsiveness in a asthma mice. From the results of our study we cannot confirm this hypothesis, although fine ammonium nitrate seems to induce hyperresponsiveness in both healthy and asthma mice. Interestingly, ultrafine ammonium nitrate aerosol dosed at a slightly higher mass concentration did not induce this effect.

Despite the observed association between exacerbation of asthma and particulate matter in recent epidemiological studies, no causal relationship has been established so far (Gamble and Lewis, 1996) and its existence is highly disputed. We have demonstrated in the present study that, using a mouse model that mimics asthma, exposure to fine and ultrafine ammonium nitrate did not enhance the symptoms of asthma at concentrations of this aerosol around the lowest-observed-effect level. Similar results were observed in two previously reported studies using the ammonium bisulphate and ammonium ferrosulfate in the same mouse model. However, in contrast to those studies, fine (but not ultrafine) ammonium nitrate caused clear adverse effects at comparable exposure concentrations in the present study.

Ambient nitrate is nearly all in the accumulation mode (0.1 - 2.5 μm) of the trimodal distribution. The 24 h ammonium nitrate concentration ranged from 4 to 6.5 $\mu g/m^3$ whereas concentration as high as 45 $\mu g/m^3$ can be reached during episodes (RIVM, 1997). The sizes of the solid spherical shaped nitrate particles in the present study were 34 and 313 nm CMD with the respective mass concentrations of 250 and 140 $\mu g/m^3$.

The effectiveness of the ovalbumin treatment is shown by significant ovalbumin specific IgE levels in asthma mice. IgE could not be detected in healthy mice. However, the variation in these levels was rather high which might implicate that the asthmatic status of the animals was variable as well. In addition, a significantly increased airway reactivity was observed in asthma mice compared to the healthy controls and histopathologic examination showed a slightly inflammation in asthma mice, that must have been caused by the ovalbumin treatment.

LDH, total protein, and TNF-α levels in BALF were unaffected by exposure to the test atmosphere, meaning that no lung injury and edema have been evoked by the nitrate exposures. In mice exposed to nitrate aerosol increased NAG levels indicated that macrophages were activated due to the test atmosphere. This effect was most prominent in asthma mice. This increase was only statistically significant for ultrafine nitrate. This induction was not observed in sham exposed animals. Therefore, we consider the effects to be caused by the test atmosphere alone, irrespective the size of the particles. This could implicate that the effect of nitrate particles can be independent of the site of deposition in the airways and will be more or less contingent with the mass concentration. Ammonium nitrate particles are highly hygroscopic and will increase in size during transport through the airways. If ultrafine grow faster than fine nitrate aerosols, the difference in size of the particles beyond the nasal passages may be smaller than the initial differences. The relative higher potency of nitrate aerosols compared to sulfate has also been shown by Kleinman *et al.* (1995).
The immunological indicators for inflammation TNF-α, IL-4 and IL-6 showed very high intra-group variation. Moreover, the levels of these cytokines were often close to the detection limit. A positive control could have revealed the possibility of observing an effect regardless the intra

group variation. However, histopathology showed that the inflammation was only observed in asthma mice and the response was not influenced by exposure to nitrate particles. Despite these observations, counting of inflammatory cells in BALF cytological examinations of BALF showed that the balance between macrophages and neutrophils were significantly altered in mice exposed to fine ammonium nitrate. Notwithstanding the fact that macrophages seems to be activated, the total number of these cells is not increased in either ultrafine of fine ammonium nitrate exposed mice. Ziegler *et al.* (1994) have shown that rats chronically exposed to 90 $\mu g/m^3$ ammonium nitrate did not affect the pulmonary macrophage function, as measured by the IgG binding capacity of macrophages. However, ammonium nitrate can have effects on other hematopoetic derived cells in the lung. Charles and Menzel (1975) have showed an increase of histamine release by mast cells.
The present study also shows that the inflammation as seen by the neutrophil influx in BALF was only induced by fine ammonium nitrate. Although the influx is very moderate, this clearly shows that the adverse effects of PM10are not a result of the number of particles but an effect dependent of the mass concentration within a specific size range.

In this study we sought to determine if acute exposure to ammonium nitrate aerosols resulted in a more prominent effect in asthma, asthmatic mice than in healthy mice. *In vitro* trachea reactivity was selected as endpoint that reflects the airway hyperresponsiveness. We have shown that parameter of this endpoint can be altered due to fine nitrate aerosols, but no differences were observed between healthy and asthma animals. The results of this study differ from our previous study in which we could not demonstrate any effect due to exposure to ultrafine and fine ammonium bisulfate and ammonium ferrosulfate in healthy and asthmatic mice at comparable mass concentrations and particle sizes (Cassee *et al.*, 1998a/b). Loscutoff *et al.* (1985) have shown that ammonium sulfate resulted in significant lung function changes whereas no exposure-related changes were observed after ammonium nitrate exposures in rats after subchronic exposure to 1 mg/m^3 (0.3-0.4 MMAD).). The different exposure protocol and animal model used in that particular study might be responsible for this outcome. Despite this difference in sensitivity, this MMAD is comparable with the ultrafine aerosol of our study. Using a 4 times higher mass concentration than in the here presented study, still no effects of ammonium nitrate was observed. Although the animals were exposed to only one mass concentration for a particular particle size, we feel confident to conclude that ammonium nitrate did not enhance the asthma-like symptoms in this study.

Collectively, these preliminary data of this study suggest that ammonium nitrate model particles yield marginal pulmonary responses in mice model, but there are no indications of enhanced allergic response following exposure to PM. Since effects of fine ammonium nitrate were observed at lower mass concentration as used for ultrafine aerosols, particle size is most probably also an important parameter in order to characterise and to understand the causality of secondary aerosol associated health effects.

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