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Indoor dust and hot surface contact: Biological effects in vitro of heated dust and heat-generated emissions

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Indoor dust and hot surface contact:

Biological effects *in vitro* of heated dust and heat-generated emissions.

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Thesis submitted in partial fulfilment of the requirements for the academic title

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Acknowledgement

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Abbreviations

A549   -   Cell line from alveolar epithelial tissue

ATD   -   Automatic thermal desorption unit

DMSO   -   Dimethyl sulfoxide

DNA   -   Deoxyribonucleic acid

ELISA   -   Enzyme-linked immuno sorbent assay

GC   -   Gas chromatography

LDH   -   Lactate dehydrogenase

LPS   -   Lipopolysaccharide

MTT   -   3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-salt

PBMC   -   Peripheral blood mononuclear cells

PBS   -   Phosphate buffered saline

TNFα -   Tumour necrosis factor alpha

VOCs   -   Volatile organic compounds. WHO's definition for volatile organic compounds (VOCs) is based on boiling point range from 50 - 100 °C to 240 - 260 °C (WHO, 1989).

Word list[1]

Aerodynamic diameter	The diameter of a sphere of density $1 \ g \cdot cm^{-3}$ with the same terminal velocity due to gravitational force in calm air, as the particle, under the prevailing conditions of temperature, pressure and relative humidity.
Fine particles	Particles smaller than 2,5 μm (aerodynamic diameter).
Inhalable dust	Airborne particles that is inhaled through the nose and mouth.
Respirable dust	Inhaled particles penetrating to the unciliated airways, less than 5 μm (aerodynamic diameter) if reaching the alveoli and less than 10 μm (aerodynamic diameter) if reaching the bronchioli.
Sub micron particles	Particles less than 1 μm (aerodynamic diameter).
Ultra fine particles	Particles less than 0,1 μm in particle diffusion diameter (the diameter of a sphere with the same diffusion coefficient as the particle under the prevailing conditions of temperature, pressure and relative humidity).

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[1] The definitions are cited from European Committee for Standardization (CEN) (1993) and Morawska (2000).

Table of contents

List of papers

This thesis includes the following publications, which are referred to in the text by the respective Roman numerals:

I   Mathiesen M, Pedersen EK, Syversen T, Bjørseth O (1999). Finding a suitable *in vitro* system for testing differences in biologic effects of settled household dust. In: *Proceedings of Indoor Air '99*, Vol 4, pp. 1122-1127.

II   Mathiesen M, Pedersen EK, Bjørseth O, Egeberg KW, Syversen T. Heating of indoor dust causes reduction in its ability to stimulate release of IL-8 and TNFα *in vitro* compared to non-heated dust. *Indoor Air, accepted.*

III   Mathiesen M, Pedersen EK, Urfjell B, Bjørseth O, Syversen T. Emissions from heated indoor dust: An approach for sample preparation and *in vitro* toxicity testing. *Atmospheric Environment, in press*.

IV   Mathiesen M, Pedersen EK, Bjørseth O, Syversen T. Emissions from indoor dust inhibit proliferation of A549 cells and TNFα release from stimulated PBMCs. *Environment International, in press.*

Background

Electrical heaters are common in homes, day-care centres and offices in Norway and other cold-climate-countries. Indoor dust settling on surfaces of heaters is likely to become heated once the device is switched on. This may lead to a well-known characteristic smell of "burned dust". Such heating is also likely to occur on other surfaces in the indoor environment, e.g., light fixtures and electrical appliances.

This thesis has been a part of the multidisciplinary project "Indoor environment- health effects of aerosols and settled dust". The project was established in order to characterise the physical and chemical changes of the dust caused by heating (subproject 1) and to investigate the biological effects of indoor dust subjected to heat including the emissions (subproject 2). Two doctoral students and two supervisors (Olav Bjørseth and Tore Syversen) were part of the project group representing the fields of technology and toxicology. Professionals within occupational medicine, chemistry and ventilation were available for advice and discussions.

The two subprojects have resulted in two independent, but closely related doctoral theses. Dr.Ing. Ellen Katrine Pedersen completed her thesis '*Dust in the indoor environment. Physical and chemical changes due to hot surface contact*' in 2001. The present thesis is concerned with the biological effects of indoor dust subjected to heat.

Aim of the thesis

Knowledge of various factors' influence on indoor air environment is important in order to reduce sources and processes that can elicit negative health effects. Due to the smell of "burned dust" when heaters are switched on, it is hypothesised that indoor dust subjected to heat will contribute to negative health effects due to production of reactive species. The aim of this thesis was to investigate:

- o The *in vitro* effect of heated indoor dust compared to non-heated dust.

- o The *in vitro* effect of the emissions generated during heating of indoor dust.

Indoor dust also consists of microorganisms and allergens. These are significant constituents of indoor dust when concerned with health effects. However, investigating the heating of such contaminants was beyond the scope of this study.

The following working goals were set:

- o Finding adequate methods for sampling indoor dust

- o Adaptation of the dust-heating systems for collection of residuals as well as emissions

- o Choosing representative cell cultures for testing of indoor dust

- o Establishing suitable protocols for exposure and effect-measurement of dust and emissions

- o Testing the *in vitro* effect of non-heated dust, residuals and emissions

The content of the study can be systemised in the following way:

Protocol and method development

- o Sampling methods, pre-treatment of dust, dust suspensions, cell culture exposure, endpoints (paper I and II)

- o Collection of emissions and cell culture exposure, endpoints (paper III)

Effect of heating

- o Residuals vs. non-heated dust (paper I and II)

- o Emissions from heated dust (paper III and IV)

Authorship

I am primarily responsible for the scientific quality of this dissertation. I hold the first authorship for all papers and I am responsible for the quality of the research, originality of the ideas, study design, accuracy of the data and quality of reporting. In paper II, III and IV Irene Bragstad has done the dry-sieving of dust. Tom Myran has provided 'Dust I' applied in paper IV. Bente Urfjell has done part of the cell culture experiments in paper II, III and IV and Liv Ryan has done part of the TNFα-cell culture experiments in paper II. Ragna Bogen Hetland performed the IL-8-analysis and Kjartan Egeberg is responsible for the choice of the analytical flow cytometry in paper II.

Introduction

Contaminants in indoor air

During the last decades, there has been an increasing concern among the public and within the scientific community over the health effects of indoor air quality. The relevance of indoor air quality to human health is emphasised by the fact that people in the western world spend 90 % of their time indoors. Small children, sick and elderly people, who are more susceptible to pollutants, spend even more of their time indoors (Jenkins et al, 1992; Simoni et al, 2003). The replacement of older naturally ventilated buildings with more energy efficient and airtight buildings is partly causing the increased concern for indoor air quality. In modern buildings, there is increased complexity of indoor air chemistry through new activities, equipment and products (Brooks, 1994; Jones, 1999; Pedersen et al, 1999). Short construction periods may not allow sufficient time for materials to dry, and buildings may still have high levels of humidity and high rates of emissions when residents move in (Bakke et al, 2000). These changes in building characteristics have lead to environments in which contaminants are more readily produced and may build up to much higher concentrations than are typically found outdoors (Jones, 1999).

This thesis is concerned with indoor dust and the consequences of its contact with hot surfaces. The use of electric heating in residents is assumed to influence the health of occupants (Krafthefer and MacPhaul, 1990; Sammaljärvi and Raunemaa, 1990; Raunemaa and Sammaljärvi, 1993). A recent epidemiological study has demonstrated an increase in ocular and nasal symptoms in dwellings with electric radiators (Engvall et al, 2003). It is suspected that heated indoor dust is responsible for such effects. The extent of dust-accumulation on surfaces of the interior is dependent on factors such as cleaning, ventilation and thermal conditions, and indoor dust is likely to be subjected to temperatures up to 300 °C or more when settling on the surfaces of heaters, light fixtures and electrical appliances (Pedersen et al, 2003: Amundsen, 1992; Frode Frydenlund, pers.comm.).

The dust particles, ranging from less than 1 μm to over 100 μm, are any small bits of material or droplets, inorganic or organic, viable or non-viable that can become airborne (Salvaggio, 1994). In addition to the core material of the particle, the dust consists of several organic compounds and allergens adsorbed onto the particle-surface (Hanson et al, 1983; Wilkins et al, 1993; Hirvonen et al, 1994; Ormstad et al, 1998; Mølhave et al, 2000; Wilkins et al, 2002).

A temperature-induced rise in the concentration of sub-micron particles is found has been detected in a laboratory dust-heating model and from electric heaters with layers of indoor dust (Sammaljärvi and Raunemaa, 1990; Pedersen et al, 2001). Furthermore, volatile organic compounds (VOCs)/thermal degradation products have been identified in the emissions from indoor dust heated

up to 300 °C (Hirvonen et al, 1994; Pedersen et al, 2003). Joshi and Wanner (1975) examined the physio-chemical changes caused by heating of indoor dust in a model apparatus at 200 – 600 °C. They found that among the emissions were CO, CO_2, ammonia and oxides of nitrogen.

Indoor air contamination may affect the respiratory system, the nervous system (sensory effects) and the immune system, or irritate and cause inflammation of the skin or the mucous membranes of the eyes, nose or throat (Berglund et al, 1992; SHD, 1998). Particles, gases and allergens are indoor air contaminants of special significance for asthma, allergy and other sensitivity reactions (SHD, 1998). An overload of particles in a healthy subject or an ordinary exposure in a sensitised subject can cause irritation of the lung cells and lead to release of cytokines, onset of inflammation in the tissue and tissue damage (McClellan, 1998). According to Hottinger (1924) the contact of dust with hot surfaces above 70 °C leads to irritation of mucous membranes of the eyes and airways (quoted in Joshi and Wanner, (1975)). VOCs and other volatile compounds which are not covered by the VOC definition, sampling and analysis technique, i.e., reaction products of VOC and oxidative agents, unsaturated VOCs, inorganic gases and aldehydes, are likely to cause irritation to mucous membranes in eyes and airways as well as odour irritancy (Berglund et al, 1992; Wolkoff and Nielsen, 2001).

Well-known indoor combustion products like nitrogen dioxide from gas cooking stoves, wood smoke and environmental tobacco smoke are associated with eye and airway irritation and respiratory illnesses (Jones, 1999). It is therefore reasonable to assume that heating of indoor dust may yield similar complex mixtures of pollutants with toxicological properties.

Biological testing

Pedersen (2001) described in her thesis physical and chemical characteristics of heated dust and emissions generated at temperatures up to 250 °C. Concerned with the possible biological effect of such contaminants in indoor air, it was important to find proper test methods. The influence of airborne contaminants can be investigated in different ways, and at present, there are no standard protocols.

Research into indoor air pollution health effects has involved human, animal and *in vitro* studies (Berglund et al, 1992; Jones, 1999). Human studies entail observations and reports of symptoms and other effects in individuals exposed to pollutants. The epidemiological studies observe effects under realistic exposure conditions. However, the critical issues of these studies are inhomogeneous populations, exposure misclassifications, insufficient power to signify the likely causality of associations and the control for confounding factors (e.g., psychosocial dissatisfaction, smoking,

stress). The advantages of experimental human studies are that the exposure conditions and the subject selection are controlled, but only slight, reversible, short term effects in healthy individuals can be investigated.

Animal studies are the assessment of health effects of exposures in laboratory animals. In this type of research, the investigators have reasonable control with exposure conditions and the health effects studied. Limitations in the applicability of results from animal studies are the need to extrapolate from animal to human and from high to low exposure levels.

When using *in vitro* studies, the effects of pollutants on cells or organ cultures are examined. These experiments are low costs compared to the use of laboratory animals and they are relatively quick to undertake. Cell cultures are in particular suitable for studying mechanisms of effects and relative toxicity, especially for modification of toxic agents. Because testing *in vitro* can be done efficiently and economically, some methods can be used to screen the potential toxicity of chemicals to be tested *in vivo* (Ehrich and Sharova, 2000). It can be difficult to predict the effects of the *in vitro* findings on the whole organism in a quantitative way. Still, *in vitro* data can be used as a basis for e.g., the parallelogram approach, which improves data interpretation and extrapolation to man (Environmental health criteria 180, 1996).

In vitro models

In the present thesis, short-term cell culture experiments were selected as assessment-method for health effects of heated indoor dust. The argument for this choice is that the biological effects of heated dust have not previously been tested. The cell cultures, a cell line and a primary culture, were easily obtained and are low cost tools for testing of various methods and screening of unknown mixtures.

Dust particles and chemicals in general can activate both the unspecific and the specific part of the immune defence system. As airborne contaminants like dust and its emissions are likely to be inhaled, cells relevant to the respiratory tract and the immune defence were used. *In vitro* assays have some limitations, particularly because the human pulmonary response of inhaled pollutants is the result of complex interactions involving many different cell types within the lungs (Wallaert et al, 1996). However, the use of cell culture in biological testing allows for the use of human cells.

The A549 cell line originates from alveolar epithelial tissue (Lieber et al, 1976). This cell type retains the main morphological features of human lung type II alveolar epithelial cells that may be exposed to aerosols *in vivo*. Epithelial cells are important in maintaining the integrity and fluid balance of the lung tissue and for the control of inflammation (Standiford et al, 1990; Nicod, 1999).

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Peripheral blood mononuclear cells (PMBC) consist of monocytes and lymphocytes after decontamination of blood platelets and were used as a model for primary immune cells.

Sampling of dusts

Sampling, by definition, means that only a portion of a population will be selected for study (Burge, 2003). Several methods for collecting settled and airborne dust exist (Jacobs, 1994; Macher, 2001; Burge, 2003). There is no sampling standard, although attempts to achieve this have been made, e.g., for the assessment of allergens in indoor dust (Dreborg et al, 1995). Common principles for dust sampling include collection of particles on filters or electrostatic precipitators and collection of settled dust. The dust samples in this study were collected with the intentions of having:

- o Sufficient amounts of dust for both subprojects, physical and chemical characterisation as well as biological testing

- o Dust from miscellaneous sources

- o Dust sampled with several different methods

- o Good collection efficiency of fine particles

The requirements to the indoor sampling locations were that they should be in buildings without:

- o Cigarette smoking

- o Moisture problems

- o Complaints about the indoor air quality

- o Pets

A total of seven different indoor dust samples denoted dust A through G, were applied. Two samples from outdoor sources (dust H and I) were included for comparison. All the sample locations where in Trondheim, Norway.

Dust A: University office building
Settled dust was sampled from textile surfaces of chairs in conference rooms, auditoriums and a cafeteria in a university office building with mechanical ventilation system with the air inlet on the roof (preheated air from heat exchangers, filter F7, mixed ventilation). The dust was collected onto filters (Versapor-1200, 1.2 μm, 293 mm dia, P/N X17928, Gelman Sciences, Michigan, U.S.) connected to a vacuum cleaner (Electrolux Excillio Z5045). The collection efficiency of these filters were determined to be 98 % for the 0.3 μm particles by counting particles of the filter inlet and outlet

(Laser particle counter, Model 200L, flow rate 472 cm³/s, MetOne, Pacific Scientific Instruments)(Pedersen et al, 2002). Sampling was performed in October 1997.

Dust B and C: School buildings

Indoor dust settled on hard surfaces like shelves and doorframes was collected by vacuuming with a special mouth piece and filter holder (ALK, Denmark: described in Dreborg et al (1995)) mounted on a vacuum cleaner (Electrolux Excillion Z5225 Super Silence or AEG Vampyr 751). Samples were collected on filters (M-65 Luklet 6GB) with a collecting efficiency of 96 % for 0.3 µm particles (Laser particle counter as described for dust A). Sampling was done in two schools in October 1999.

Dust D and E: Private home and day-care centre

Indoor, airborne dust was collected with an electrostatic air cleaner (EAC 10; Inlet: 240V, 50 Hz, 0.3 Ampere 55 W, Outlet: 5500 Volt DC, Series number EC 06106, Trion, Norway). The air cleaner was placed on a shelf 1.5 m above floor level in the living room of a private home (dust D) or in the play-room in a day care centre (Dust E) for 3 weeks in August/September and June 1999, respectively. There was forced ventilation in the private home and natural ventilation in the day care centre. The collection efficiency of the electrostatic air cleaner is 80- 90 % for 0.3 µm - 10 µm particles at a face velocity of 0.6 m/s (Olav Bjørseth, pers.comm.).

Dust F and G: Office buildings

Indoor, airborne dust was collected from the F7-bag filter (Camfil, Sweden) located in the outlet of the ventilation systems of two office buildings. The F7 filters have a collection efficiency of approximately 50 % for 0.3 µm particles (Camfil, Sweden). The filter with dust F was used for less than 6 months and the filter with dust G had been used for 3 months prior to collection in November 2000. The dusts were transferred onto Versapor filters by vacuuming as described for dust A.

Dust H: Air inlet ventilation filter

Outdoor, airborne dust retained in an inlet F7-bag filter (Camfil, Sweden) of an office building was collected by cutting the filter into pieces and transferring the dust to a glass container. The filter had been used for 2 months prior to collection in April 1999.

Dust I: Outdoor city dust

Airborne dust was collected outdoors 3 m above ground level in the city of Trondheim using an electrostatic air cleaner (Clean Tunnel Air) for 6 weeks in May and June 1998. The collection efficiency was 85 - 95% of the particle sizes 0.3 – 10 µm at face velocity 4.0 - 4.5 m/s (manufacturers description). The dust was transferred to a container by using a clean spatula.

Pre-treatment of dust samples

The dust samples collected from surfaces or from bag filters were fractionated in order to obtain a more homogenous sample from each source and to increase the reproducibility of experiments. This fractionation was initiated due to the poor reproducibility of dust suspensions as seen by particle counts and biological effect in paper I. In paper II, the dust was at first fractionated in a fluidised-bed method (Pedersen, 2001) and by wet sieving. In further work, the dust was fractionated by the dry sieving-method (37 μm cut off) (paper II, III and IV).

All the samples were radiated (10 kGy) in order to kill microorganisms, and stored in darkness at room temperature (approximately 22 °C).

Heating of dust

In order to simulate heating of dust on indoor hot surfaces, various laboratory devices have been used. Joshi and Wanner (1975) heated samples of indoor dust (200- 600 °C) in a porcelain boat situated in a quartz tube inside a furnace. Hirvonen et al (1994) placed indoor dust in small glass tubes and heated these in a thermal desorption apparatus up to 300 °C, while Rothenberg et al (1989) used a vacuum microbalance and a thermal analysis system at elevated temperatures (40 – 700 °C) for heating indoor dust.

The requirements for the heating systems applied in this project were the ability to:

- o Heat dust at temperatures relevant to surfaces of indoor equipment

- o Heat dust samples of variable size and sources

- o Ensure an even and thoroughly heating of the sample

- o Reproduce heating profiles

- o Collect heated dust for biological testing

The simulation of hot surface contact of dust was first performed in the controlled environment of an automatic thermal desorption unit (ATD). Pedersen et al (2002) applied this unit with the primary intention of collecting the emissions for chemical characterisation. The heating was carried out by placing the dust samples in Teflon tubes in the ATD-unit at 50 – 250 °C, with a constant purge of helium gas. Testing with a more realistic atmosphere, i.e., pressured air was performed in a rebuilt gas chromatography oven (HP 5790, GC-1).

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When heated, the dust was converted into three fractions; 1) the remains of the heated dust sample (the residuals), 2) the emissions, volatile at room temperature and 3) the condensate, condensed vapours of emissions. The residuals were collected from the Teflon tubes from both the ATD and GC-1 and used for biological testing *in vitro*. The heating system should also give the opportunity to collect the emissions from the heated dust, and due to practical purposes, a second GC-oven was rebuilt (Varian 3300, GC-2). In GC-2, the dust was placed in a special designed glass tube. The dust samples were heated to 50 – 250 °C with a constant purge of synthetic air through the dust tube. The emissions were brought out of the oven through a replaceable Pasteur pipette.

The labelling, source and equipment used for sampling and heating of each dust sample is summarised in table 1. The last column refers to the papers in which the analytical methods are described.

Table 1. Summary of dust sources, sampling, heating and application in papers (with corresponding labelling). ATD = automatic thermal desorption unit, GC-1 and GC-2 = gas chromatography oven 1 and 2.

Label	Dust source	Sampling	Heating	Paper
A	Settled particles, textile surfaces, university	Filter + vacuum cleaner	ATD, GC-1	I, II, IV
B	Settled particles, hard surfaces, school	Filter + vacuum cleaner	GC-1	II
C	Settled particles, hard surfaces, school	Filter + vacuum cleaner	"	II
D	Airborne particles, private home	Electrostatic air cleaner	"	II
E	Airborne particles, day-care centre	Electrostatic air cleaner	"	II
F	Filtered, indoor, airborne particles, office	Bag filter, vacuum transfer	GC-2	II, III, IV
G	Filtered, indoor, airborne particles, office	Bag filter, vacuum transfer	"	II, IV
H	Filtered, incoming airborne particles	Bag filter	"	IV
I	Outdoor airborne particles	Electrostatic air cleaner	"	IV

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Preparation and testing of dusts and emissions

The three fractions of heated dust were collected and tested separately in cell culture experiments.

Residuals and non-heated dust

There are several protocols in the literature concerning suspension and mixing of samples in order to investigate the biological effect *in vitro* of dust (Kondo et al, 1995; Becker et al, 1996; Jenssen et al, 1997; Allermann Hansen et al, 1997; Saraf et al, 1999). Aliquots of residuals or non-heated dusts were suspended in cell culture medium, followed by sonication and rotation of the test tubes in order to mix and disperse the particles before addition to the cell cultures. In the first study, the unfractionated dust was dosed as weight per volume when incubated with cells (paper I). In further experiments with dust A, fractionation was done by the fluidised bed-method and in addition, the resulting dust suspensions were sieved through a 40 μm cell strainer before addition to cell cultures according to a method by Jenssen et al (1997) (paper II). The dose of the sieved suspension was given as particles per volume based on particles counts in the size > 4.0 μm using a Coulter counter. In consecutive experiments, the dust samples were fractionated by dry sieving and the dose of the fine fraction was given as weight per volume (paper II).

Most of the physical and chemical characterisation of the dusts were performed and presented in the thesis by Pedersen (2001). Determination of endotoxin-content in extracts of dust samples A, B, C, D and E (both residuals and non-heated dust) and characterisation of particle size distribution and granularity of dust A-suspensions was performed to better understand the *in vitro* results (paper II).

Gram-negative bacteria, which are constituents of indoor dust, contain LPS in their cell wall and this molecule, also called endotoxin, can be measured in the *Limulus* assay. In this test, the endotoxin-molecule activates a pro-enzyme. The activated enzyme cleaves a colourless substrate (Ac-Ile-Blu-Ala-Arg-pNA) to the yellow coloured molecule *p*-nitroanilin (pNA) and the optical density of pNA at 405 nm determines the quantity of sample-endotoxin and the included endotoxin standard.

The flow cytometer is an analytical instrument equipped with a laser beam. When analysing the dust suspensions, the side scatter of the laser beam was used to quantify the granularity of the particles, while the forward scatter was applied to examine the particle size relative to 1 μm - 10 μm reference standards.

Emissions

In order to expose cells to the emissions from heated dust *in vitro*, both direct emission exposure and extraction procedures were applied.

Direct emission exposure

In direct exposure, there is no intervening layer of medium between the cells and the airborne contaminants to be tested as opposed to conventional, submersed culture conditions. This have been described and tested in several studies and the requirements that must be met in such an exposure system have been pointed out (Rasmussen, 1984; Dubar et al, 1993; Tu et al, 1995; Knebel et al, 1998; Aufderheide and Mohr, 2001).

The exposure chamber for direct testing of emissions from heated dust was constructed in our laboratory based on the models for aerosol or gas exposure and the demands set for such exposure (Massey et al, 1998; Knebel et al, 1998). The chamber was connected to the dust heating system. Cells growing on a microporous membrane of inserts in the culture wells were exposed directly to the emissions, and feeding of the cells was done basolaterally through the inserts supporting the cell-layers. After the exposure, the cells were recovered from the system, re-soaked in fresh cell culture medium and post-incubated before endpoint measurements (paper III).

Extracts of emissions and condensate

The extraction procedure used for collecting the emissions was based on protocols for extracting cigarette smoke into water-based solutions (Dubar et al, 1993; Masubuchi et al, 1998). The emissions generated during dust heating were bubbled through cell culture medium. The resulting extracts were added to the cell cultures (paper III and IV). The condensate was extracted in acetone by sonication of the replaceable Pasteur pipette of GC-2 (the collector). After volume reduction, the condensate extract was diluted in medium and added to cell cultures (paper III).

A drawing of the apparatus used for direct exposure and collection of emissions and condensate is given in figure 1.

1. Synthetic air
2. Heating oven
3. Dust tube
4. Condensate collector

5. Smoke extraction tube
6. Exposure chamber
7. Pump
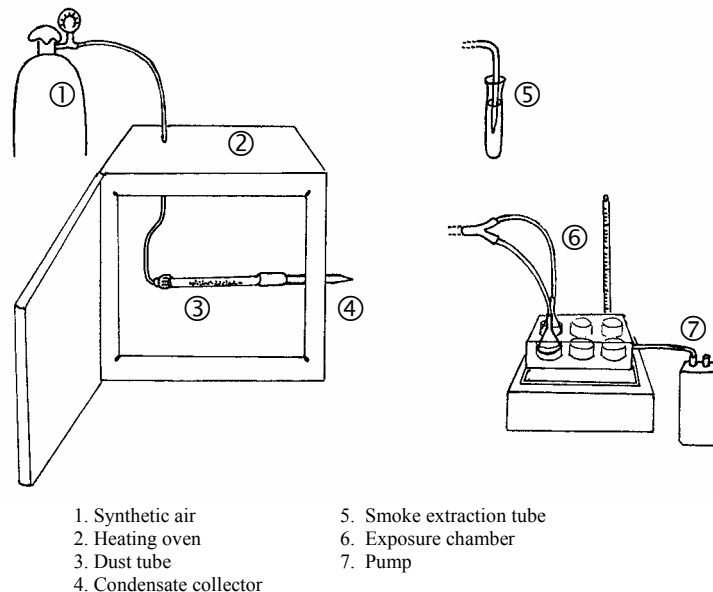
Figure 1. The set of apparatus for heating dust, collecting emissions and exposing cells directly to dust smoke.

In vitro endpoints

When choosing an endpoint to assess the effect of airborne contaminants, it should preferably be of biological or pathophysiological relevance to the effect detected *in vivo*. Knowledge of the *in vivo* effect of heated indoor dust and its emissions was not available; instead, endpoints relevant for similar airborne contaminants were applied.

Cytotoxicity tests

Cytotoxicity is a term that covers the effects test agents can have on cell growth, metabolic function, or viability. These tests are not specific indicators of the mechanisms associated with toxicant-induced effects. This means that they can be used to assess damage to various cell types under a wide variety of situations, including exposure to chemicals and particles (Ehrich and Sharova, 2000).

Cell viability was assessed by trypan blue dye uptake and lactate dehydrogenase (LDH) leakage. Live cells exclude the trypan blue dye and therefore this method is used to estimate the percentage of viable cells directly in a microscope. LDH is a membrane impermeable cytoplasmic enzyme, which is present in all mammalian cells. The LDH-leakage test is an indicator of increased cell membrane permeability and subsequent cell death. The assay used, is based on the cleavage of a yellow

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tetrazolium dye (2-p-iodophenyl-3-nitrophenyl tetrazolium chloride) by cell-free medium containing LDH, to a red formazan salt, which can be detected by absorbance measurements at 490 nm.

Tests for cell growth and cell proliferation are generally more sensitive to lower concentrations of toxicants than tests affecting cell viability. These tests can also be used at concentrations of toxicant that do not necessarily cause irreversible cell damage. Cell growth inhibition can be assessed by harvesting cultures treated with the same dose of test agent at different time points. The number of cells is counted microscopically. Cell proliferation is correlated to the amount of [3H]-labelled thymidine that can be incorporated during DNA synthesis. The radioactivity in DNA is measured by scintillation counting after cell harvesting. If the test agent inhibits cell proliferation, with or without affecting cell viability, the incorporated radioactivity will be decreased.

Cellular metabolism can be assessed by using MTT-salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The functional cell mitochondria are able to reduce this tetrazolium salt to an insoluble formazan precipitate, which causes a change in colour that can be determined optically at 570 nm. This bulk test indicates the fitness of the exposed cells.

Protein content is an indirect method for measuring cell numbers. The Bradford colorimetric method is based upon binding of the dye Coomassie brilliant blue to unknown cell proteins and comparing this binding to that of different amounts of bovine serum albumin. The protein concentration is quantified in a spectrophotometer at 280 nm based on the absorbance of UV light by aromatic amino acids in protein solutions (Simonian and Smith, 2001).

Cytokine release

Tumour necrosis factor α (TNFα), is a signal protein released from alveolar macrophages and monocytes and play a role as a mediator of the respiratory tract's response to particles (Driscoll et al, 1997). The TNFα-release from PBMCs was used to characterise the proinflammatory potential of the residuals and non-heated dusts. The TNFα-bioassay is a well-established method based on the cytotoxicity of TNFα upon the highly sensitive cell line WEHI 164 clone 13 (Espevik and Nissen-Meyer, 1986). The concentration of TNFα was determined by including a recombinant TNFα-standard.

Particles can also activate epithelial cells and cause the release of chemokines such as the polypeptide interleukin-8 (IL-8). Il-8 can elicit inflammation reactions in the airways (Driscoll et al, 1997). The levels of IL-8 from A549 treated with residuals or non-heated dust, were determined in the enzyme-linked immuno sorbent assay (ELISA) by using a recombinant IL-8 standard.

Cytokine-suppression

Some toxic agents are called immunosuppressants due to their ability to inhibit a normal immune response to foreign insult. Toxic agents can modulate the production of cytokines that normally elicit and regulate the immune response (Cohen et al, 1999). The effect of extracts of emission from heated dust was assessed by their suppression of LPS-stimulated TNFα-release from PBMCs. Quantification of TNFα was performed as described in the previous section.

Summary of individual papers

Paper I

"Finding a suitable *in vitro* system for testing differences in biologic effects of settled household dust"

The aim of this paper was to develop a protocol for exposing A549 cell cultures to suspensions of residuals and non-heated dust and compare their effects by means of LDH-leakage. Non-heated dust A and the corresponding 200 °C-residual from the ATD-heating were tested. Silica particles (0.5 – 10 μm) were included as a positive control.

The concentration of two parallel dust solution preparations, determined by particle counts between 4 and 10 μm particle diameter, varied by 25 % for the non-heated dust, by 5 % for the residuals, and 3 % by the silica particles. For the doses tested in the lactate dehydrogenase (LDH)-release assay, 250 μg and 1500 μg, no clear differences were seen between the non-heated and the residuals (results range: 19 – 40 % of total leakage). As a comparison, the silica particles caused 90 % LDH-leakage at the 250 μg/ml dose. We also demonstrated that non-heated dust suspensions caused an increase in the A549 doubling time (1.6 times) at 2000 μg/ml.

The results caused two major concerns for the test protocol, the reproducibility of the dust suspensions and the sensitivity of the LDH-test. The poor reproducibility of particles counts for parallel dust suspensions could be caused by sample heterogeneity and this might have concealed the possible difference in the membrane-damage effect of residual and non-heated dust. Moreover, the potential of the indoor dust to cause membrane damage to A549 cells is obviously low.

Paper I indicates the need for improvements, in particular by using the fine fraction of the dust samples. In addition, more sensitive endpoints than LDH-leakage should be tested.

Paper II

"Heating of indoor dust causes reduction in its ability to stimulate release of IL-8 and TNFα *in vitro* compared to non-heated dust"

In this paper, we studied the differences between residuals and non-heated dust by measuring cytokine release. The samples containing large particles were sieved before testing. Seven indoor dust samples (A through G) were heated at 50 – 250 °C and the residuals and the non-heated dusts were incubated with PBMCs. TNFα was determined in the cell culture medium at the end of incubation. For dust A the IL-8 release after dust-exposure to A549 cells was determined. Endotoxin, which is a potent stimulator of TNFα-release from PBMCs, was measured in residuals and non-heated dust of samples A, B, C, D and E. Particle sizes and granularity were assessed in the non-heated sample and residuals of dust A.

The residuals samples had less ability to induce the release of TNFα from PBMCs (dusts A through G) and IL-8 from A549 cells (dust A), with effects starting at 150 °C. All the dust samples tested had similar cytokine decline-pattern due to heating up to the maximum of 250 °C. The viability of the cells was not affected as assessed by trypan blue exclusion, MTT-test and LDH-release, and consequently, the decline in cytokine release could not be explained by cell impairment. The TNFα-decline could, however, partly be explained by the reduction of endotoxin content in the residuals compared to the non-heated samples and possibly by inhibitory decomposition products, but not by differences in measured particle size or granularity.

Paper II implies that residuals would promote a smaller inflammatory reaction in the airways if suspended and inhaled compared to the non-heated dust.

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Paper III

"Emissions from heated indoor dust: An approach for sample preparation and *in vitro* toxicity testing"

This paper describe methods for generating emissions from heated indoor dust, collecting the emissions and testing their effects in relevant *in vitro* models. Hot surface contact at 150 and 250 °C was simulated using dust F. The emissions were purged by synthetic air and tested directly in an exposure chamber or as extracts, using A549 cells or PBMCs.

The direct exposure of emissions at both temperatures reduced the viability and increased detachment of both cell cultures grown on inserts, the 250 °C-emissions being the more toxic. For the 250 °C-emission extracts, proliferation and mitochondrial activity were reduced for A549 cells exposed for 24 hours in cell culture wells. In a shorter incubation protocol (9 hours), developed for later cytokine-response measurements, the viability of PBMCs incubated in plastic wells were not affected by emission-extracts as assessed by trypan blue exclusion. However, an extract of high-boiling components condensing at room temperature reduced PBMC-viability in a 24 hour-exposure.

This paper demonstrates methods to generate and collect emissions from the heating of indoor dust, and that emissions are toxicologically active *in vitro*.

Paper IV

"Emissions from indoor dust inhibit proliferation of A549 cells and TNF-α-release from stimulated PBMCs"

In this paper, the effect of emissions-extracts from heated dust using PBMCs and A549 cells was further investigated. The method described in paper III was used and extracts of the volatile emissions from dusts A, F, G, H and I, heated at 50 − 250 °C. The effects on A549 cells were assessed by inhibition of proliferation or decrease in the total mitochondrial activity. The suppression of the LPS-stimulated TNFα-release was measured in PBMC culture incubated with extracts. The viability of the PBMCs was determined by trypan blue exclusion or mitochondrial activity.

The proliferation of the lung epithelial cell line A549 was inhibited by all the extracts. The indoor dust F yielded the most suppressive emissions and the outdoor dust I-emissions were least potent in this assay. The mitochondrial activity of A549 cultures was reduced after incubation with extracts from emissions from both dust F and G. Significant effects were seen in both assays for emissions released at temperatures below 200 °C.

The emission extracts also showed a clear suppressive effect upon the LPS-induced cytokine-response from PBMCs starting at 100 °C. The suppressive effect was most distinct for the emissions of the two indoor dusts F and G and weakest for the outdoor dust I. The mitochondrial activity was significantly reduced for the highest concentrations of the extracts generated at 250 °C. The viability as assessed by trypan blue exclusion was not affected. This indicates that the emissions suppress the TNFα-release stimulated by LPS in a manner that cannot be explained solely by decreased cell viability. Suppression of this proinflammatory cytokine by components of emissions from heated dust may increase the risk of infection.

The actual indoor air concentrations of emission from the heating of indoor dust have not been measured. Nevertheless, this paper suggests that temperatures relevant for hot surfaces in the indoor environment can cause toxic emissions from dust. The findings of the *in vitro* tests show that these emissions can affect cells and processes relevant for respiratory health.

Discussion

Methodological considerations

Sampling and pre-treatment of dust

In this project, several common dust-sampling methods were applied to collect airborne and settled dust. The main reason for sampling settled dust was the need for large samples for physical, chemical and biological characterisation. Vacuuming of chairs and shelves reflects settled indoor dust and potential airborne dust. Filtered, airborne dust sampled from ventilation bag filters also provided sufficient sample quantity for extensive method development. All particles that stay airborne long enough to settle on surfaces were relevant for this study. However, large particles were removed by fractionation in order to obtain a more homogeneous sample from each source and to increase the reproducibility of experiments. Three different fractionation methods were used (fluidised bed fractionation, wet-sieving and dry-sieving), where the dry sieving was the most suitable and time efficient method.

The electrostatic air cleaners provided airborne dust, but small quantities were collected despite long-term sampling. The dust samples obtained by this method were not fractionated due to small amounts, no visible clusters and a homogeneous appearance.

The collection efficiency of the samplers and methods used was controlled and showed high efficiency, which included fine particles.

Radiation of the dust samples prevented microbiological growth and consequent changes in the sample. This procedure does not change the chemical composition of the dust as verified by gas chromatography-mass spectrophotometry of dichloromethane-extracts of indoor dust before and after radiation at 10 kGy (Jenssen et al, 1997).

The storage of the samples in darkness at room temperature was chosen in order do avoid condensation of water that may occur if the samples are stored at a lower temperature. It was also important not to distort the adsorbed fraction due to light or due to great variation in temperature.

Sampling of indoor dust from offices, schools, a day-care centre and a private home ensured a variety of sources. The variation in dust source and collection methods may strengthen the overall conclusions of this study, since the components of indoor dust will always vary, depending on environment, activity, materials and time of year. Sampling locations contaminated by e.g., cigarette smoke, allergens from pets or fungi due to high humidity were avoided so that such special components would not dominate the dust samples.

The inclusion of two outdoor dusts was chosen for comparison, since these are representative for dust entering a building through windows and doors. Outdoor dust is also a major source for the indoor contamination in buildings without filtered air supply.

Heating of dust and collection of residuals and emissions

The three different sets of apparatus for heating of dust all provided adequate control of gas flow and heating profiles. These methods worked well for all the dust sources and for sample sizes up to 200 mg dust (GC-2). The ATD-method was suitable for modelling the heating, only, due to the use of inert gas. The GC-1 and the GC-2 methods allowed for heating in a more realistic air atmosphere. All three sets were adequate for collecting the air- or helium-residuals. For the collection of emissions, the GC-2 apparatus proved the most useful method.

Dust suspensions

The residuals resulting from heating at 200 °C or higher were dispersed in cell culture medium by sonication and rotation of the suspensions. Hence, this treatment was applied to all the samples. Health effects caused by particles correlate better to particle numbers than to weight (Morawska, 2000). It is therefore appropriate to give the doses as numbers of particles per volume of cell culture medium (paper I and II). However, the limitations in determining the particle size in suspensions ($\geq$ 1 μm in flow cytometry) and the similarity between the resulting TNFα-declining curves for particle-dose-response curves and weight-dose-response curves was the reason for choosing a weight-based dosage for the remaining experiments (paper II). The advantage of the weight-based dose was the independence of the particle size distribution of the sample, which could not be described entirely by the methods available.

Direct emission exposure

Due to the high cost of a commercial exposure chamber (Aufderheide et al, 2002), an apparatus suitable for emissions from heated dust was constructed in our laboratory (paper III). The dust heating system (GC-2) was coupled directly to the exposure chamber and this allowed exposure of cells to freshly generated emissions. The system could be controlled for dust sample size, airflow, exposure time and temperature.

The microenvironment for cells in culture is crucial for their survival and growth. Cells are optimally kept in an incubator at 37 °C, supplying at 95% relative air humidity and 5 % carbon dioxide (CO_2) for buffering the bicarbonate of the medium and keeping a constant pH of approximately 7.2. It was important to limit the effects on the cells due to deviations from the optimal levels of these parameters in the exposure chamber. The cells were kept humid by basolaterally feeding through the membrane support. No dehydration of the cells was observed. The temperature of a microplate-

heater surrounding the lower part of the chamber was kept at 37 °C. The synthetic air purging the heating system did not contain any CO_2, and pH of the culture was not monitored apart from visual observation of the colour indicator in cell culture medium. However, the viability determined for the control cells from both A549 and PBMC cultures after air exposure was only slightly lowered. This verified that the handling of cells on inserts, the microenvironment and the gas flow in the chamber agreed with high survival rates. Thus, the reduction in viability in exposed cells was caused by the emission exposure. Detachment of cells was seen during the post-incubation, and dilution of emissions and consequently more sensitive endpoints in future research would improve the existing exposure protocol.

Emission extracts

Extracts of emission is a common method to test cigarette smoke *in vitro*. This method was also suitable for exposure of cells to dust-heating emissions and it provided means for diluting the emission before cell exposure. The extracts were not analysed chemically, but the biological effects proved the ability of the cell culture medium to extract compounds from the emissions. The extraction was not complete, as visible smoke escaped the extraction tube during the procedure. In the first experiments, acetone and dimethyl sulfoxide (DMSO) were added in order to increase the range of extracted compounds (paper III). The extracts made with and without these solubilizers were later compared and the solubilizers did not seem to cause any additional extraction as seen by the biological effects of the extracts (unpublished results). Thus, medium only was applied in further extractions (paper IV).

Condensate extract

The condensate was extracted from the Pasteur pipette by the use of sonication and a solubilizer stronger than cell culture medium was necessary. Acetone was chosen as the best of the three solvents tested (acetone, dichloromethane and carbon disulfide). DMSO was added to increase the solubility of the high-boiling components when the condensate-extract was diluted in cell culture medium.

In vitro methods and endpoints

Both the PBMCs and the A549 cells were suitable models for studying the effects of heated dust and emissions. The A549 cells were easily cultured and were suitable for growth in conventional submersed monolayer as well as on inserts for direct exposure. PBMCs are normal immune cells, and they had a quick and high response to the compounds tested. The whole PBMC culture was used in the testing of dust suspensions and emission extracts, while the suspended cells were drawn off

along with the medium leaving only the adherent monocytes on the inserts for the direct emission exposure.

Cell lines like A549 are often preferred to keep the variance of results low as compared to the greater variance expected in primary cultures. Since the PBMCs are primary cells, the isolation procedure for obtaining these cells performed prior to every experiment also made this model more time-consuming than the A549 model. However, the PBMCs appeared more sensitive to the dust-suspensions as seen by the extent of the cytokine decline in both models.

Each cell is observed when using the trypan blue exclusion method for determining viability. However, by using this method cells that are impaired but have not yet lost their membrane integrity will be assessed as viable. Furthermore, the PBMCs were not removed from the wells after exposure in order not to rupture the adherent sub-population, and assessing the viability of the heterogeneous PBMCs microscopically was time consuming since the cells were both adherent and suspended. As a result, the bulk methods (proliferation, MTT-test, protein, LDH-leakage) was used to replace some of the viability testing in further experiments and these also allowed more tests to be run simultaneously. Knowledge of PBMCs fitness was important since only viable cells can synthesise cytokines.

In contrast to many other airborne particles, the membrane-damaging potential of residuals and non-heated dusts in A549 culture was low. Consequently, the LDH-assay was not relevant for distinguishing between effects of dusts before and after heating (paper I). The proliferation assay in A549 culture was especially sensitive to emission extracts and was easy to perform (paper III and IV). In the MTT-test, the condensate extract led to instantaneous dark blue formazan formation in the absence of cells. The assay was therefore replaced by the proliferation assay (paper III). This type of interference with the MTT-salt has also been detected for other test compounds, e.g., antioxidants (Bruggisser et al, 2002). A549 cells and PBMCs treated with the emission extracts were washed before addition of MTT-salt, and interference with the dye was not observed (paper III and IV).

The TNFα- and IL-8-release was suitable for distinguishing between the changes in proinflammatory potential due to heating of dust (paper II). Degraded or denatured forms of endotoxin will not react with the pro-enzyme in the *Limulus* assay (Walters et al, 1994) and thus, this assay was convenient for determining a possible heat destruction of endotoxin in the residuals.

The assessment of TNFα-suppression of LPS-stimulated PBMCs treated with emission extracts was possible since the extracts did not induce any TNFα per se. This assay proved useful in ranging the effects of the extracts of emissions from various temperatures and dust-sources.

Statistics

Statistical analyses using the Minitab software (version 13.31) were performed to test for differences between the median or the mean value of a set of cell cultures treated with LPS and emissions or LPS and medium. Statistical significance was accepted when $p \leq 0.05$. Bonferroni's correction method was used to adjust the alpha level when several outcomes were tested using the same data collection.

When the values from a set of cytotoxicity testing or cytokine-measurements were normally distributed, the parametric ANOVA was used. This test is based on estimates of the mean and standard deviation parameters of each group of cell cultures and it tests for a difference between several groups that is greater than that attributed to random sampling variation. For values that were not normally distributed, a non-parametric test was used; the Mann-Whitney Rank Sum Test. The principle of this test is to rank all observations from the smallest to the largest without regard to which group each observation comes from. Then the ranks for each test group are summed and the rank sums compared. If there is no difference between the two groups, the mean ranks should be approximately the same. If they differ by a large amount, one can assume that the low ranks tend to be in one group and the high ranks in the other, and conclude that there is a statistically significant difference between the two test groups.

Main findings

Residuals and non-heated dust

The major biological effect of heating indoor dust (samples A through G) was the reduced ability of the residuals to stimulate TNFα-release from PBMCs compared to the non-heated dust. Significant TNFα-reduction was seen for residuals generated at 150 °C and higher. The IL-8 release from A549 cells treated with residuals from dust A showed a similar decline compared to the response to the non-heated dust (paper II).

The dust suspensions were not cytotoxic to the PBMC cells at the doses tested (paper II). The membrane-damaging potential to A549 cells of unfractionated residuals and non-heated dust A was low, and no differences caused by heat-treatment of the dust were seen (paper I). Due to growth inhibition caused by the unfractionated, non-heated dust A, the protein content of exposed cultures was measured and used for adjusting the LDH-release to cell number (unpublished data). This did not change the original conclusion for the LDH-leakage.

Other investigators studying the effects of indoor dust have measured release of both IL-8 and TNFα from A549 cells and PBMCs, respectively (Jenssen et al, 1997; Allermann Hansen et al, 1997; Saraf

et al, 1999; Jenssen and Espevik, 2001). Brown and co-workers treating cells *in vitro* with wool and grain dust, saw similar effects to those seen in this study. The wool and grain dusts did not cause lytic effects in A549 cells or macrophages, but the treated macrophages released TNFα (Brown and Donaldson, 1991; Brown and Donaldson, 1996).

The non-cytotoxic characteristics of the dust imply that the decrease in cytokine release must be explained by other factors rather than decreased viability (paper II). The suspensions of dust or residuals were tested with or without serum in PBMC culture (preliminary experiments). Similar TNFα release patterns were found in these cases, but the levels of cytokine were much higher when serum was present. This suggests that some organic compounds in the dust might be of biological origin as such serum-effects have been noted for other TNF-release activators like bacterial peptidoglycan (Mattsson et al, 1994).

Endotoxin is a potent stimulator of TNFα and IL-8 release (Hansen et al, 1999; Collins, 2000). Heat-treatment is a common technique for endotoxin-inactivation, e.g., heating at 255 °C for 20 seconds caused 90%-reduction of the endotoxin in cotton (Nakata, 1994). The measured content of endotoxin had clearly decreased in the residuals compared to the non-heated dust, and destruction of this stimulator can partly explain the declining cytokine release-pattern as the dust-heating temperature rises. Similar to the indoor dust-study, experiments with urban dusts incubated with alveolar macrophages conclude that the endotoxin content of such dust samples are pivotal for the cytokine response, as seen by a major decrease by endotoxin-depletors (polymyxin B or recombinant endotoxin neutralising protein) or heat (180 °C) (Becker et al, 1996; Imrich et al, 2000).

The particle size and granularity analysis performed by flow cytometry for dust sample A did not show differences between residuals and non-heated dust suspensions that could be related to the lower TNFα-stimulatory effect by the residuals (paper II). In addition, earlier findings in this project showed that on one hand surface areas of non-heated and residuals from dust sample A had no heat-related differences (Pedersen et al, 2001). On the other hand, emissions of sub micron particles were detected during heating of dust A, starting at 70 °C with the major release at 250 °C (Pedersen et al, 2003). *In vitro* studies with alveolar macrophages have shown that ultra fine particles have a greater potential to induce cytokines than larger-sized particles, as such effects are believed to relate to surface area (Oberdorster et al, 1992; Brown et al, 2001). The heat-treatment of dust may cause loss of particles due to agglomeration and/or emission, and thus any particle size/surface area relation to cytokine-release might have been overlooked.

The temperature at which the TNFα decrease is starting for the heated dust samples varies from 150 °C to 250 °C. This variation might be due to the differences in sources, sampling methods and

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treatment of the dusts, pointed out in paper II. However, the overall decline-pattern for the TNFα-release was similar for all the samples, indicating that the heat-treatment is an important factor for the PMBCs' response to the residuals.

The reduced cytokine release caused by residuals may imply that the inflammatory response *in vivo* will be much lower compared to that of non-heated dust. This might be due to the heat-destruction endotoxin (paper II). However, it is also possible that decomposition products remaining in the residuals could partly cause the cytokine suppression. Such effects are seen *in vitro* and *in vivo* for combustion products like acrolein in co-exposure with respirable particles, extracts of post-oil-fire airborne dust, diesel exhaust particles and cigarette smoke (Jakab and Hemenway, 1993; Ezeamuzie et al, 1998; Yang et al, 1999; Ouyang et al, 2001).

Emissions

The emissions from indoor dust heated at 150 °C and 250 °C were cytotoxic to both A549 cells and PBMCs in the direct exposure system (paper III). The reduction in viability was greatest for the 250 °C-emissions. This is most probably due to the higher concentration of ultra fine particles and VOCs and the higher number of different chemical components released at 250 °C compared to 150 °C, which has been shown for many of the dust samples (Pedersen et al, 2003).

Significant cytotoxic effects were seen in A549 culture for emissions generated at temperatures starting below 200 °C (paper III and IV). The selective retention of water-soluble compounds and the dilution of components in the medium may explain the lower toxicity of the extract of the 150 °C-emissions than the direct exposure from this temperature in the A549 culture (paper III). Similarly, the 250 °C-condensate extract affected the A549-proliferation, but there was little effect of the condensate extract generated at 150 °C (paper III), probably due to a very limited condensation of high boiling point compounds at this temperature. This is in accordance with findings in a study where high-boiling C_{16}-C_{25} aliphatic hydrocarbons extracted from indoor dust were not emitted when the dust was subjected to vacuum desorption at 150 °C (Hanson et al, 1983).

Epithelial cells are important in maintaining the integrity and fluid balance of tissue and for the control of inflammation (Standiford et al, 1990; Lannan et al, 1994). The results from the A549-assays imply that injury to the epithelium *in vivo* may be an effect following exposure to emissions from indoor dust subjected to heat. Other airborne combustion products, e.g., gasoline engine exhaust, cigarette smoke and nitrogen dioxide, also affect these epithelial cells as assessed by reduced plating efficiency or [3H] thymidine incorporation (Seemayer et al, 1987; Lannan et al, 1994; Tu et al, 1995).

The emission extracts generated at 100 °C or higher yielded significantly lower TNFα-release from PBMCs than the control (paper IV). The cytotoxicity-results implied that this suppression could not be explained by impairment of cells. The explanation for the non-toxic effect of the extracts in the PBMCs compared to the effect in A549 culture might be the shorter incubation protocol that was chosen for PBMCs (6 hours vs. 24 hours). However, the most concentrated extracts of the 250 °C-emissions of dust F and G caused a significant reduction in the mitochondrial activity of PBMCs (paper IV). The condensate was not toxic to the PBMCs in the short incubation protocol (paper III) and these high-boiling emissions did not have a suppressive effect on the TNFα-release at the concentrations tested (unpublished results).

The PBMCs might be interpreted as a model for macrophages in the airways as only the monocyte-fraction of the PBMCs release TNFα upon LPS-stimuli (Collins, 2000) and since blood monocytes and tissue-macrophages produce similar levels of TNFα upon LPS-stimulation *in vitro* (Terje Espevik, pers.comm.). Suppression of TNFα-release due to LPS-stimuli caused by the emissions from heated indoor dust may increase the risk of infection *in vivo* since this proinflammatory cytokine plays an important role in the host defence (Luster et al, 1999). The underlying mechanism(s) by which extracts of such emissions affect PBMC response to LPS challenge has not been explored. Nevertheless, as mentioned for the residuals, the suppressive effect of heated dust-emissions is consistent with several other studies concerned with the contaminants from combustion processes, e.g., nitrogen dioxide, cigarette smoke, the organic fraction of diesel exhaust particles in macrophage or PBMC culture (Dubar et al, 1993; Polzer et al, 1994; Yang et al, 1999; Ouyang et al, 2001; Ma and Kinneer, 2002).

The experiments included extracts of emissions from heated dust samples from both indoor and outdoor sources (paper IV). The outdoor sources were included for comparison since they contribute to the final composition of indoor dust in buildings without extensive filtration of the outdoor air. The extracts of the emissions from the outdoor sources behaved similarly to the extracts from indoor dusts, but on average the emissions from indoor dusts were more toxic as assessed both by inhibition of A549-proliferation and suppression of TNFα-release from PBMCs.

The biological effects compared to the physical and chemical changes due to heat

The temperatures, at which effects on the cells caused by emissions and residuals were seen, are in agreement with the temperatures for the major increase in emissions from dust detected in subproject 1 (Pedersen et al, 2001). The major decomposition and emission of VOCs and decomposition products from the dust were assumed to start below 150 °C for the indoor dust samples (Pedersen et al, 2003). Particle counts showed that the emissions of sub micron particles from heated dust

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increased considerably starting at approximately 70 °C (Pedersen et al, 2001). These temperatures are relevant for indoor equipment like heaters and light fixtures liable to collect indoor dust.

The emissions purged through the heating system, but not the high-boiling components condensing in the apparatus, had suppressive effect on the release of the proinflammatory mediator TNFα. This difference might be explained by the chemical composition of the two sets of emissions. For example, low-boiling-aldehydes like hexanal and the gas nitrogen dioxide, which have been identified among the purged emissions from heated indoor dust (Joshi and Wanner, 1975; Pedersen et al, 2003), is among several compounds that show inhibitory effect on TNFα-release from macrophages (Polzer et al, 1994; Girona et al, 1997).

Implications for health

The findings in subprojects 1 and 2 indicate that compounds are emitted from indoor dust subjected to elevated temperatures and that these may contribute to the total indoor pollution and thereby cause airway irritation. The chemical and physical characterisation of the emissions are fairly in agreement with previous studies, however, very few investigators have been concerned with the biological effects of these indoor air contaminants. Results from a chamber study performed by Raunemaa and Sammaljärvi (1993) indicated a rise in sub micron particles from an electrical flow-through heater that seemed to impair lung function for some of the healthy volunteers, and in a recent study by Engvall et al (2003), associations between increased eye and airway symptoms and electric radiators in dwellings are demonstrated. Other studies on health effects of the heating of indoor dust were not found.

In vitro effects seen for other combustion products in indoor air were also found for emissions from heated dust. Accordingly, such parallel findings may imply that heated indoor dust can cause signs and symptoms as seen in persons exposed to e.g., wood smoke, cigarette smoke and nitrogen oxide. However, the indoor air concentrations of emissions from heated indoor dust are not known, but might be far less than cigarette smoke presented to the passive smoker.

Conclusions

The aim of this study was to simulate hot surface contact for indoor dust and develop methods to heat and collect fractions of heated indoor dust for investigating their biological effects *in vitro*.

Methods

The collection of dust by vacuum cleaner, electrical air cleaner and on filters in the air ventilation duct provided samples that were suitable for this project. The variation of dust sources enabled generalisations of the effect of heating on indoor dust. Dry sieving is recommended to remove large particles that are not compatible with cell culture exposure. Radiation and the storage conditions were found necessary for minimizing microbial growth and changes in composition of the samples throughout the project.

The systems for heating and collection of the fractions of heated dust were useful for the investigations. The cytokine release assays in PBMCs and A549 cells were suitable tools for detecting differences between residuals and non-heated dust samples. The endotoxin measurements were important in order to explain the declining effects in the cytokine assays.

The direct exposure to fresh emissions from heated dust was the most relevant exposure-method and the viability endpoint made it possible to range the temperatures at which the emissions were generated. The self-constructed chamber for emission exposure represents a platform on which standardisation of direct exposure of emissions can be further developed. Some improvements to this direct exposure method are justified, i.e., dilution of emissions and more sensitive endpoints.

The extracts of emissions made it possible to test this fraction in conventional cell cultures. The proliferation assay and the MTT-test used in A549 cultures and the TNFα-suppression assay used in PBMCs were the best measures for effects of the emission extracts. The endpoints used made it possible to distinguish between heating temperatures and in part, distinguish between dust sources of the emissions.

Main findings

The contact of indoor dust with hot surfaces causes changes in the remaining dust that yields reduced proinflammatory potential as assessed by cytokine release *in vitro*. The residuals heated at 150 °C or higher cause decline in cytokine release compared to non-heated dust. The compounds emitted during the heating of indoor dust have toxic effects that increase parallel to the increase in temperature. Effects of the emissions are seen at 100 °C and higher in the PBMC-model and are

starting below 200 °C in the A549-model. The cytotoxicity of the direct exposure of emissions was much more pronounced than the effect of the corresponding emission extracts. This is most probably due to concentrations and composition differences between the direct emissions and the extractable portion of the emissions.

Although different methods were used to assess the effect of the three fractions resulting from the heating of dust, a careful comparison of the temperatures yielding effects of the different fractions indicates that the emissions are most toxic. The residuals may be resuspended, but emitted chemicals and sub micron particles will probably affect the indoor air quality the most.

Practical implications

The effects of heating of indoor dust are seen as low as 100 °C, and this temperature is frequently found on surfaces of heaters and light fixtures. The main findings in this study imply that fractions of heated dust could have biological effects. This entails that heating of indoor dust should be restricted. Several approaches are possible to obtain this:

- o Reduce the amount of dust indoors

- o Restrict the occurrence of hot surfaces

- o Decrease the temperature on available hot surfaces

Keeping the dust load on surfaces to a minimum will improve air quality and reduce the amount of dust subjected to heat. This can be achieved by frequent cleaning and by ventilation with delivery of filtered air. Boerstra et al (2000) have emphasized the advantage of low temperature-heat supply systems above their high temperature counterparts as they produce fewer airborne particles. Electrical heaters with low surface temperatures, constructed for the safety of children are among the heaters commonly sold. When obtaining new indoor equipment one should critically consider the maximum surface temperature available for dust accumulation.

Implications for further studies

Studies regarding heated indoor dust and its emissions are only at their beginning. This work has showed that these contaminants have effects *in vitro*. It would therefore be of interest to perform epidemiological or clinical investigations of possible health effects under similar exposure scenarios. Measurements and calculations of realistic indoor air concentrations of emission and residuals should then be included.

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Paper I

FINDING A SUITABLE *IN VITRO* SYSTEM FOR TESTING DIFFERENCES IN BIOLOGICAL EFFECTS OF SETTLED HOUSEHOLD DUST

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ABSTRACT

Particles in indoor air may affect human health. We have hypothesised that dust in the ambient indoor air may change properties, including biological effects, after contact with hot surfaces e.g. convection ovens. This change in properties may be related to desorption of substances from the particles and even pyrolysis of the dust. To study possible changes in biological effects we have exposed cultures of the human bronchoalveolar carcinoma-derived cell line A549 to doses of heated and non-heated dust. Cell membrane damage was assessed by lactate dehydrogenase release. This method has been useful for testing various dusts but our hypothesis also needs to be tested with other more sensitive methods.

INTRODUCTION

Indoor air pollutants may have an adverse human health effect depending on the ambient concentration [1]. Complexity and composition of household dust may vary and include organic, non-organic, viable and non-viable particles [1, 2, 3, 4]. The dust vary in size from respirable particles to particles that are deposited in the upper respiratory tract, the largest being visible, non-inhalable particles [4]. Sources of dust are diverse and include building occupants themselves and their activities, combustion, building materials and furnishings, biological agents, ventilation systems, cleaning habits and entry of contaminated outdoor air [1, 2]. Substances of either toxic or allergenic origin e.g. volatile or semivolatile organic compounds (VOCs and SVOCs) can be adsorbed onto the dust surface and organic emission from household dust is detected at 50 °C [2, 3, 4]. Combustion of compounds may produce a wide range of lower molecular weight species of different toxicity and irritancy [5] and thermal breakdown and chemical conversion (pyrolysis) of organic dust has been shown to start at 70-80°C. [2]. The contact of dust with a hot surface e.g. electric convection ovens or condensers of refrigerators, may therefore lead to decomposition of particles and desorption of substances from the particles. We have hypothesised that dust will have a more adverse effect on human health after contact with hot surfaces. To test this hypothesis we have used an *in vitro* system for exposing a human cell line to dust particles and assess the effect of heated and non-heated particles to the cells by means of cell membrane damage. The lactate dehydrogenase (LDH)-assay is a common test for membrane damage and it has been used for testing particles of various origins.

METHODS

Dust

Settled indoor dust from a building without any special indoor air problems was collected on membrane filters (Gelman Sciences Versapor – 1200, 1,2 μm) using a vacuum cleaner (Electrolux Excillio Z5045). The samples were transferred to glass bottles (Duran, 50 ml) and radiated (10 Gy) to prevent growth of microorganisms and then stored at room temperature. The coarse fraction of the dust (human hair etc.) was separated in a fractionation process. Samples of dust (40 mg) were placed in teflon tubes with stoppers of glass wool and treated in an automatic thermal desorption unit (ATD-400, Perkin Elmer) with helium carrier gas. The samples were heated for 10 minutes at 200°C. The He-atmosphere gives rise to anaerobic pyrolysis of dust.

Preparation of dusts

Stock concentrations (2,0 mg/ml) of heated and non-heated dust and silica particles (0,5 – 10 μm, Sigma, Cat. No S 5631) (control) were prepared in F12K culture medium (Gibco BRL products). All stock solutions were rotated for 24 h in room temperature and sonicated to disperse the particles prior to testing. The stock solutions were counted in a Coulter Counter (9914591-B, Kebo Lab, Norway) in two groups: Between 4,00 and 10,0 μm and above 10 μm. The reproducibility of sample preparation was assessed by this procedure.

In vitro system

The human bronchoalveolar carcinoma-derived cell line A549 [6] (American Tissue Type Culture) was chosen for testing differences in the effects of heated and non-heated dust. A549 cells were maintained in F12K culture medium supplemented with 10% foetal bovine serum (Gibco BRL products) and penicillin-streptomycin (100 U/ml -100 μg/ml) (Kebo Lab, Norway). The cells were grown to confluence in plastic culture flasks (Nunc, Denmark) at 37°C in a humidified atmosphere of 5 % CO_2 and harvested using 0,1 % trypsin (Gibco BRL products). All experiments were conducted with subculture 4 through 9.

Growth inhibition

Release of LDH was chosen as a biologic endpoint after exposure of dust to A549 cells. If dust inhibits the growth and proliferation of A549 cells in culture, then the amount of cells that contribute to LDH-release will vary depending on the dosage and the type of dust. To test this the cells were plated onto 24-well plates (Nunc, Denmark) at a density of $3x10^4$ cells/well and incubated for 24 h. After preincubation the old medium was replaced by 1ml of the previously prepared dust solution (2 mg/ml) or medium. Plates were incubated at 37°C for 48 hours. Before exposure and after 24, 30 and 48 hours adherent cells were harvested and counted in a Bürker chamber.

LDH-assay

A549 cells were cultured in 24-well plates, 2,5 x 10^5 cells per well, at 37°C. After 24 h (subconfluence) the F12K medium with serum was removed and the cells were washed with fresh medium. One ml of the test solutions with the appropriate concentrations was added to each well. The medium was without serum to increase sensitivity as recommended in a similar study [7]. All experiments were carried out in 4 or 5 parallels with a negative

(untreated cells) as well as a positive control (cells treated with 1% Triton X-100 in medium). To assess the reproducibility of our method two parallel preparations of dust solutions were tested. The plates were incubated at 37°C for 24 h. The medium of each well were then drawn off into plastic tubes and centrifuged at 3000 rpm for 15 min to remove cell debris and particulate matter. Controls and dust solutions were prepared from cell free wells in the same manner. The LDH content of the supernatants was determined in 96-well microtiter plates (flat-bottomed, Nunc, Denmark) by using the cytotoxicity detection kit (Boehringer Mannheim, No.1644793) and reading the absorption at 492 nm in a microtiter plate reader (Titertek Multiskan Plus MK II). Due to the possible growth inhibition of cells exposed to dust, the protein content (proportional to the cell biomass) of adherent cells in each well was measured. Lysis of adherent cells was produced in 1% Triton X-100 solution and the cell solutions were centrifugated (3000 rpm, 15 min). The protein content of the supernatants was measured in 96-well microtiter plates at 570 nm using Coomassie brilliant blue (G-250) and bovine serum albumin as a protein standard (Bio Rad laboratories and Kebo-lab, Norway).
The protein content was used to adjust every reading from the cytotoxicity test. The LDH-release was calculated as percent of positive control.

Specificity of LDH-assay

The LDH-assay may be disturbed by particles interfering with optics during reading at 492 nm in the microtiter plate reader. Dust particles and components desorbed from the particles may also interfere by inhibiting LDH or react with constituent parts of the test solution in the cytotoxicity detection kit, e.g. the tetrazolium salt, the cofactor or the catalysator. To examine a possible interference, medium and dust solutions were screened for absorbance in the LDH-assay with and without L-LDH (99,99% purity, Medinor, Norway, No. 0127230).

RESULTS

Reproducibility of dust solution preparation

The differences between parallel preparations are shown in figure 1. For the silica solution the parallels are almost identical. This was expected since particle size of the silica has a narrow distribution range. The particle size of the non-heated dust has probably a wider distribution range than the heated dust and it also has the largest difference between the parallel solutions. The particle count above 4,00 μm is lower for the heated dust than for the non-heated dust solutions. The major part of the particle count for all solutions was between 4,00 and 10,0 μm (not shown). The A549 cultures are inhibited in the presence of dust (figure 2). The doubling time for control cultures was 18,6 hours, while doubling time for cultures with 2 mg/ml dust was 30,5 hours.
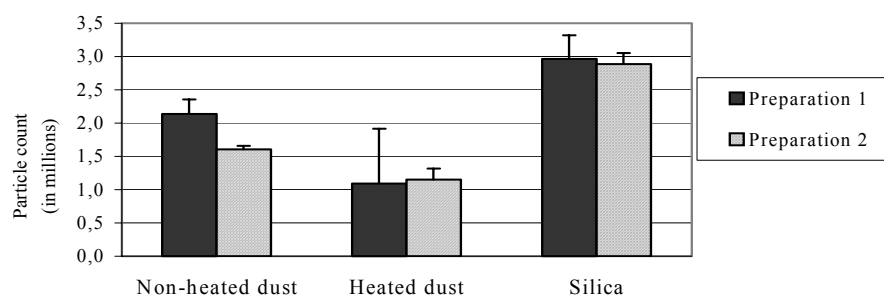
Figure 1. Particles per ml in stock solutions of 2 mg/ml heated dust, non-heated dust and silica. Values are expressed as mean + standard deviation (n=3). Effects of dust on growth and proliferation.
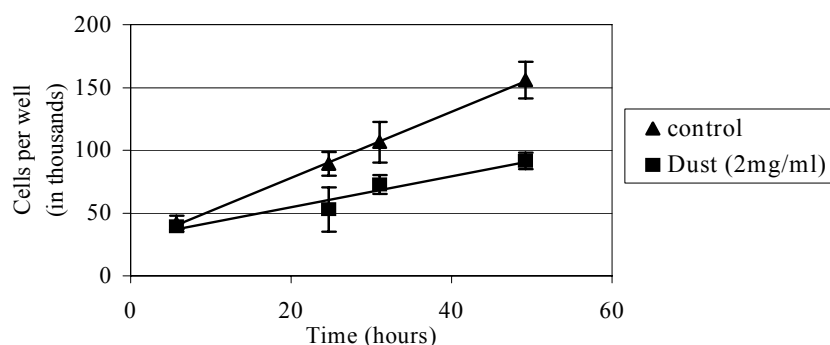

Figure 2. Growth inhibition of A549 cells exposed to dust. The vertical bars indicate standard deviation (n=3). The lines drawn were calculated by linear regression.

LDH-assay

Preliminary results of the LDH-assay of heated and non-heated dusts and silica control are shown in figure 3. The percent LDH-release produced by the two solution preparations for each test concentration differs in their mean values but they were not significantly different. Given the standard deviations, the effects of the heated and the non-heated dust were not significantly different. Silica showed a dose-dependent stimulatory effect on LDH-release. The heated and the non-heated dusts also seem to increase the LHD-release proportional to the increase in concentration. The difference in particle concentrations for preparation 1 and 2 were not reflected in the LDH-assay results. Optic interference or inhibition of the enzyme or the constituents of the test kit solutions in the LDH-assay was not detected for any of the dust solutions.

DISCUSSION

The A549 cells have provided a model for the study of differences in human health effects caused by heated and non-heated dust. This cell line has been used in testing other dust particles [8, 9, 10]. This cell type retains the main morphological features of human lung type II alveolar epithelial cells that may be exposed to respirable particles *in vivo*. Also, a

cell line is preferred to keep the variance of results low as compared to the expected greater variance in primary cultures. For the purpose of this study an *in vitro* system is adequate. The LDH-assay represents a single but non-specific cellular parameter to investigate the risk arising from heated dust. Injury to the A549 cell membrane might be caused by the physical properties of the dust particles or by components released from the dust surface into the medium surrounding the cells. This could be a suitable test for assessing differences between dusts as long as revealing the mechanistic action is not the main issue. The growth inhibition of A549 cultures caused by non-heated dust implicates the need for a correction of LDH-release to the biomass of cells in the test wells.
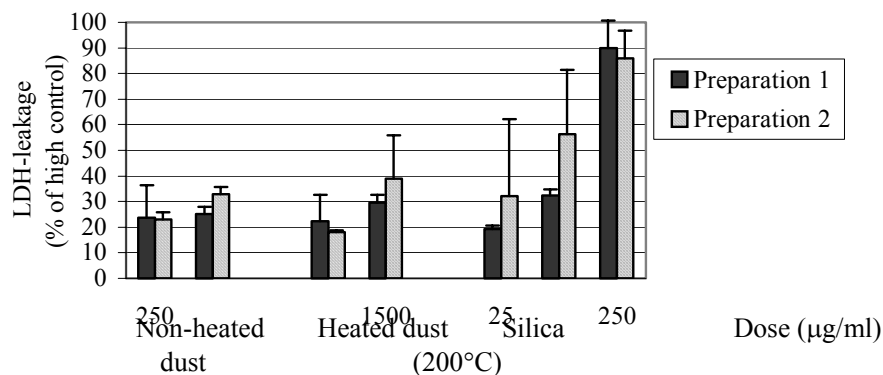

Figure 3. LDH-release after 24 h exposure of heated and non-heated dust and silica particles. The figure also shows the reproducibility of the LDH-assay. Vertical bars indicate standard deviation (n=4 or 5).

The LDH-assay in A549 cultures seemed appropriate because of the increase in LDH-release due to both heated and non-heated dusts. There are two major concerns about the method, however; reproducibility and sensitivity. The former, the difference in particle counts and LDH-release for the parallel preparations of dust solutions in our study, might be caused by the heterogeneity of the dust samples. The different particle concentration for heated and non-heated dust solutions of equal concentration based on weight might indicate a change in particle size during heating to particles smaller than the lowest threshold for counting. In order to reduce this variation the dust could perhaps be more extensively fractionated or dosage should be based on particle number instead of mass.

LDH-release from A549 cells has been shown to be a suitable cytotoxicity test for particles like crocodolite and chrysolite [9]. The silica particles are potent toxicants in the same way and have a considerable effect at the lowest dose. Cell membrane injury in A549 cultures from wool dust and grain dust assessed by measuring ^{51}Cr release showed that wool dust had a significant lytic effect. The grain dust did not produce a lytic effect even at 100µg/well. Indoor dust from our source proved to have an effect on A549 cells, but obviously the potency is low. Detecting any possible difference between heated and non-heated dust seems to demand more sensitive assays. On the other hand particles from buildings with indoor air problems might show a higher toxicity in this in vitro system.

The preliminary results have not demonstrated that heated dust would impose a greater biological effect than the non-heated dust. However, improving the reproducibility and

conducting studies with more sensitive methods still remains before a definite conclusion can be drawn.

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Paper II

Heating of indoor dust causes reduction in its ability to stimulate release of
IL-8 and TNF• *in vitro* compared to non-heated dust

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Paper III

Emissions from heated indoor dust: an approach for sample
preparation and in vitro toxicity testing

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Paper IV

Emissions from indoor dust inhibit proliferation of A549 cells and TNF•-
release from stimulated PBMCs

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