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Effects of diesel exhaust particles on human lung epithelial cells: An in vitro study

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Summary
Atmospheric particulate matter (PM), an ingredient of urban pollution matter, is a mixture of solid and liquid particles differing in origin, dimension and composition. There is big concern about inhaled PM in urban areas, especially due to its adverse effects on the respiratory system. Diesel exhaust particulate (DEP), which constitutes the major part of PM, is characterized by a carbonic mixture composed of approximately 18,000 different high-molecular-weight organic compounds. Diesel engines release 10 times the amount of NO_2 aldehydes and breathable PM compared to unleaded gasoline engines and more than 100 times that produced by catalysed gasoline engines; these data gain great significance when taken into account the fact that diesel-powered vehicles are becoming more and more popular. DEP polyaromatic hydrocarbons (PAH), once deposited on airways mucous surfaces easily pass through epithelial cells (ECs) membranes, bind themselves to cytosolic receptors and then affect cell growth and differentiation. Human lung epithelial cells and macrophages engulf DEP, this resulting in increased proinflammatory cytokines release (IL-6, IL-8 and GM-CSF). We investigated the biological effects of DEP-PM on the human lung EC line A549. Light microscopy analysis suggested the presence of cell wall alterations, and provided evidence of PM internalization and cytoplasmic vacuolization. Following PM stimulation, nuclei also were seen undergo clear gross morphological modifications. Immunocytochemistry was used to detect intracytoplasmic IL-6 and IL-8 expression.
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Introduction

Particulate matter (PM) is an air pollutant consisting of a mixture of particles, that can be solid, liquid or both, are suspended in the air and represent a complex mixture of organic and inorganic substances, with particles varying in size, composition and origin. Their diameter is critically related to PM biological properties. According to the size, PM is classified as PM_{10} and $PM_{2.5}$ (particles with a diameter less than 10 and 2.5 μm, respectively[1,2]). Inhaled PM_{10} easily can reach even the lower respiratory tract. Particles bigger than 5 μm and lower than 10 μm normally do not reach the alveoli and are most likely to be wiped away by mucociliary clearance.[1,2] $PM_{2.5}$, but also smaller-sized particles, can reach human alveolar parenchyma, where part of particles can be internalized by pneumocytes and macrophages, as a general rule. There is big concern about urban atmospheric PM pollution, for PM being a component of atmospheric pollution strongly correlated with adverse effects on the respiratory system. The so-called "dust" pollution is associated with increased mortality by respiratory and cardiovascular diseases, but also with exacerbations of allergies, asthma, chronic bronchitis, respiratory infections. World Health Organization (WHO) reports that PM inhalation is responsible for 500,000 deaths annually.[3] Adverse events to the respiratory apparatus, according to WHO guidelines, have been observed at levels of PM atmosphere concentration usually considered "safe". Seaton et al.[4] hypothesized that small size PM from urban areas can penetrate deeply into the airways and there cause alveolar inflammation, and suggested a role for inflammation mediators in the pathogenesis of acute respiratory distress episodes; furthermore, the same authors indicated PM as capable to induce blood coagulation alterations as well as other cardiovascular upsets. To explain the acute respiratory effects caused by inhaled PM, the same authors suggest that transition metals (iron in particular) present in PM would damage airways through the induction of increased free radicals formation, which would then determine adverse respiratory effects. The presence in urban PM of other transition metals such as chromium, cobalt, copper, manganese, nickel, titanium, vanadium and zinc, in fact, also was shown to correlate with free radicals increase and related adverse effects on lungs in animal experimental models.[5,6]

Diesel exhaust particles (DEPs) represent the greatest part of PM (up to 90%) in the atmosphere of the largest cities of the world.[7,8] DEP is characterized by a carbonic nucleus, in which some 18,000 high-molecular-weight organic compounds are adsorbed. Although, diesel motors produce a lesser quantity of carbon monoxide than gasoline motors, they release 10 times the amount of nitrous oxide, aldehydes and PM compared to unleaded gasoline engines, and over 100 fold more than catalysed unleaded gasoline engines.[7] DEP-PM acts particularly through polyaromatic hydrocarbons (PAHs), which deposit on the mucosa. Due to their hydrophobic nature, PAH can easily cross cell membranes, to then bind to a complex of cytosolic receptors: such binding would be responsible for DEP-PAH effect on cell growth and differentiation.[9] Acute exposure to DEP is known to cause headache, fatigue and nausea, irritation of nose and eyes, alteration of the respiratory function, while chronic exposure is responsible of cough, sputum production and respiratory function impairment.[7,10]

Primary biological targets of inhaled DEP seem to be cells of the pulmonary epithelium as well as resident macrophages.[11] Part of inhaled DEP normally gets entrapped by surfactant and only part of this quote would reach pulmonary epithelial cells (ECs),[12] on which PM would then exert its effects often leading to less or more severe lung diseases.[13–17] Part of inhaled PM can also be internalized by resident alveolar macrophages.[18] The effects of environmental PM on alveolar macrophages have been the subject of numerous in vitro studies. The exposure of rat, but also human alveolar macrophages to urban pollution particulate has been shown to cause various cytotoxic effects, mostly caused by the production of oxygen radicals and the release of a series of proinflammatory cytokines.[19–22] Human alveolar ECs and macrophages are known to engulf DEP, a phenomenon that is followed by increased release of proinflammatory cytokines like IL-6, IL-8 and GM-CSF.[8,10]

All experimental data on the effects of DEP-PM are of particular interest, especially if taking into account the fact that diesel engine-powered vehicles are becoming progressively more popular in industrialized countries. This rises the interest towards the potential effect of PM on alveolar ECs.[23,24] On this basis, we employed the use of the human lung EC line A549.[25] In particular, we wanted to evaluate on these cells the cytotoxic effects and the proinflammatory functional responses exerted by small size DEP-PM particles. A549s show high proliferative potential and, unlike primary type I ECs, respond to PM stimuli exhibiting the typical transformations normally observed I primary type II ECs as signs of inflammatory response, thus representing a suitable model for evaluating PM cytotoxicity. In particular, we investigated the effects induced on cell surface, cytoskeleton and growth. We also aimed to clarify whether $PM_{1.0}$ particles (thus sizing 1.0 μm or less) could be taken up by ECs and, as in macrophages,[26] would eventually affect proinflammatory cytokine production.

Materials and methods

Epithelial cell culture

We used the well established and widely used "A549"cell line, derived from human lung non-small cell adenocarcinoma. These cells have been described to exhibit a closely matched type II alveolar cell phenotype, and to share many properties with human primary alveolar ECs, including cytokeratin expression and growth parameters.[25] A549s were grown as follow: briefly, they were cultured in RPMI-1640 (Sigma, Poole, UK) supplemented with 10% foetal bovine serum (FBS) (Sigma) and 10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 mg/mL amphotericin B (Sigma), grown at 36.5 °C in humidified atmosphere containing 5% CO_2, upon confluence. At this stage, A549 monolayers were gently detached using ready prepared Trypsin solution (Sigma), cells were rinsed and pelleted; pellets were resuspended in serum-free RPMI-1640 medium only supplemented with antimicrobial agents and plated onto "Multi-G-Lass" chambers (Iwaki Asahi Techno Glass); incubation took place at 36.5 °C in humidified atmosphere containing 5% CO_2 for 48 h, where cells normally reached semiconfluence. At this time (our "starting time") three

different concentrations of $PM_{1.0}$ (0.1, 0.2, 0.4 mg/mL) were added to cell cultures; *Escherichia coli*-derived lipopolysaccharide (LPS, 100 μg/mL) was added to positive control cell samples. Negative control cell samples only received fresh medium. ELISA assays and immunocytochemistry experiments were performed at: starting time, 24, 48, and 96 h following exposure to $PM_{1.0}$. Experiments always were performed as triplicates.

Immunocytochemistry

An anti-(α-) cytokeratin (IgG) monoclonal antibody (Ab) (Sigma) was used to confirm the epithelial-like nature of the A549 cell line in the first place. Slides from "Multi G-Lass" chambers were fixed for 10 min with 4% buffered formalin pH 7.4; some slides were stained with haematoxylin-eosin, others with Toluidine Blue. Light microscopy observations were performed at 100×, 400×, 630× and 1000×.

Intracytoplasmic cytokines assessment

Detection of intracytoplasmic IL-6 and IL-8 was performed as follows: endogenous peroxidase activity was blocked by 10 min incubation with peroxidase blocking reagent (Dako). Cells were incubated for 32 min at 40 °C with either α-IL-6 and α-IL-8 primary Abs diluted 1:50 in phosphate-buffered saline (PBS). Streptavidin-biotin peroxidase complex (Dako) was added for 30 min, followed by 5 min incubation in 3,3′ diaminobenzidine tetrahydrochloride in PBS. Nuclei were counterstained with Mayer's hematoxylin. Entelann (MERCK)-mounted slides were analysed for intracellular IL-6 and IL-8 positivity on a LEIKA IMM-500 image analyser.

Proinflammatory cytokines detection in cell supernatants

A549 cells were exposed to $PM_{1.0}$ at the final concentrations of 0, 0.1, 0.2, 0.4 mg/mL. After 0, 24, 48, and 72 h of treatment, supernatant aliquots were taken. IL-8 and IL-6 were measured using commercially available enzyme-linked immunoabsorbent assay (ELISA) kits (Cell Com IM 2237 and IM 1120, respectively). ELISA analysis was performed according to manufacturer's instructions. Experiments always were performed as triplicates.

Particulate sampling

According to EU regulations (Directives 91/441/CE, 94/12/CE and 98/69/CE), Diesel PM is sampled by filtering air-diluted diesel car exhaust fumes at temperatures not higher than 52 °C. A dilution tunnel connected with a NOVA-MMB Messtechnik PDP-CVS unit has been used to produce the air/diesel combustion gases mixture; this configuration (Fig. 1) cleverly resembles conditions at which diesel exhaust PM is released from vehicles into the atmosphere.

PM was collected at the exhaust of a primary brand automobile equipped with a 1400 cm³ turbo-diesel engine, compliant to Euro IV emission regulations (Euro IV emission limits for cars not exceeding 2.5 tonnes laden being reported in Table 1). The car was tested over the New European Driving Cycle (NEDC) chassis dynamometer.

Diesel particulate is composed of elemental carbon particles, which agglomerate and adsorb other species to form structures of complex physical and chemical properties. PM has a bimodal size distribution, with particles in nuclei mode and in accumulation mode, as shown in Fig. 2.

PM nuclear particles are very small with diameters between approximately 7 and 40 nm and they constitute the majority of particle number (about 90%), although they only represent a small part of total PM mass. Accumulation mode particles normally size less than 1 μm, with particle diameter most often ranging from 30 to 500 nm with a maximum concentration around 0.1–0.2 μm.

Particulate sampling filters were conditioned, then weighted; after sampling, filters were weighted again, and 5.5 mg of DEP-PM were placed in a beaker containing HPLC-grade Et-OH (Aldrich CHROMASOLV®). The suspension so obtained was sonication-treated for 30 min on a Misonix XL-2020 device, at a frequency of 20 kHz. Most Et-OH was subsequently removed from sample using a vacuum rotary evaporator, in order to get stock samples with PM concentrated to 900 mg/mL. Aliquots so prepared were stored at 2 °C upon usage.

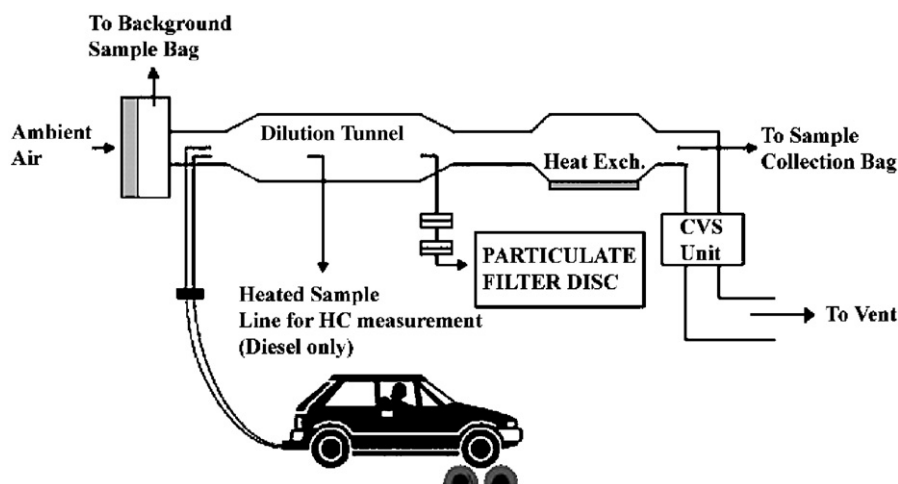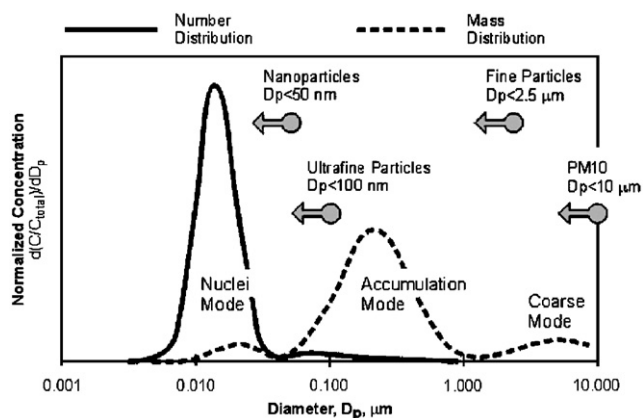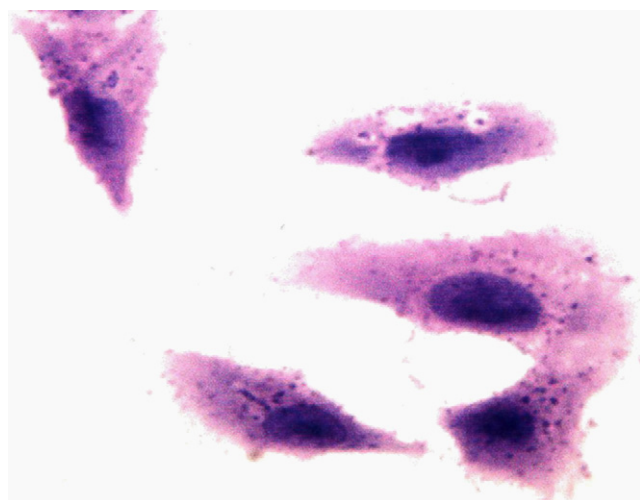**Figure 1** Schematic representation of PM sampling device.

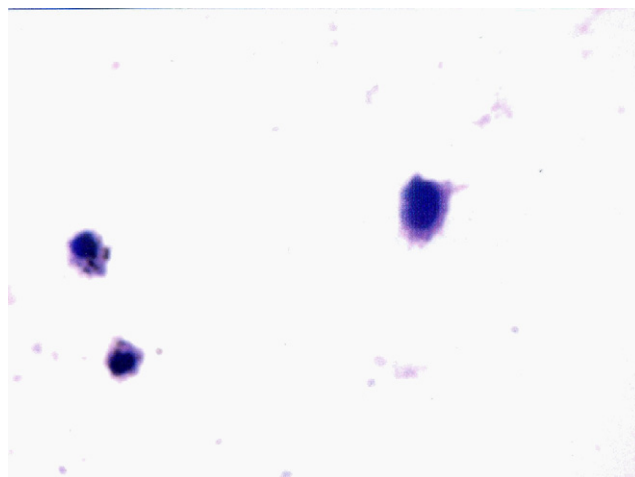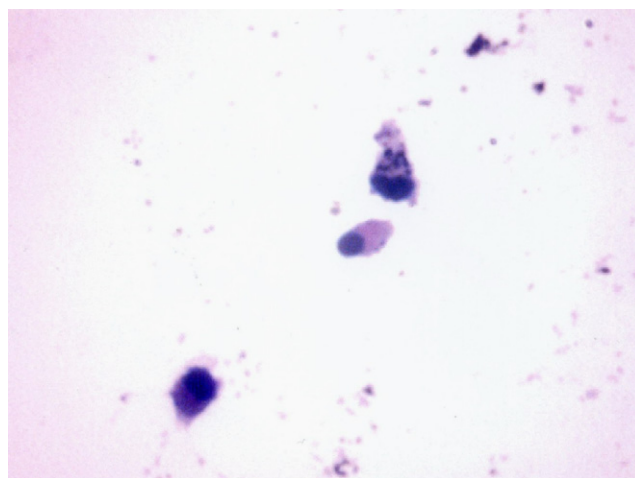**Table 1** Euro IV emission limits for cars not exceeding 2.5 tonnes laden.

Fuel	Directive	Limit values (g km)				
		CO	HC	NO_x	$HC+NO_x$	PM
Gasoline	98/69/EC	1.00	0.10	0.08	—	—
Diesel	98/69/EC	0.50	—	0.25	0.30	0.025

Figure 2 Schematic drawing showing typical PM composition.

Figure 4 PM$_{1.0}$-stimulated (24 h) A549 s. H/E staining. Original magnification: 1000 × .

Figure 3 Unstimulated A549 cells. H/E staining. Original magnification: 630 × .

Figure 5 PM$_{1.0}$-stimulated (24 h) A549s. H/E staining. Original magnification: 1000 × .

Results

Unstimulated cells appeared moderately large and exhibited different kind of shapes, yet most often vaguely hexagonal or spindle-like (Fig. 3). They also always appeared nicely and neatly separated from one another. High magnification light microscopy observations unveiled further, finer morphological detail. Cytoplasmic compartment showed granular inclusions with a diameter of 1–2 μm; Most commonly, nuclei appeared hyperchromic, with a well defined nuclear wall and prominent nucleoli.

At a magnification of 1000 × , cells stimulated with LPS were of small size and sometimes very thick, with pleomorphic appearance. Cells stimulated with PM$_{1.0}$ [0.1 mg/mL] appeared moderately big with clearly atypical nuclei, acidophilic cytoplasm containing inclusions with a diameter of 2–3 μm (Figs. 4 and 5); scarce or none particulate was visible in culture medium.

LPS addition to culture medium generally resulted in a 20% overall cell loss whereas PM$_{1.0}^{[0.1\,mg/mL]}$ stimulation resulted in a 66% cell loss.

We simply cannot present any data, nor pictures of cells stimulated with PM$_{1.0}$ at higher concentrations (0.2 and 0.4 mg/mL) as these concentrations proved highly cytotoxic and cells basically died within few hours from stimulation.
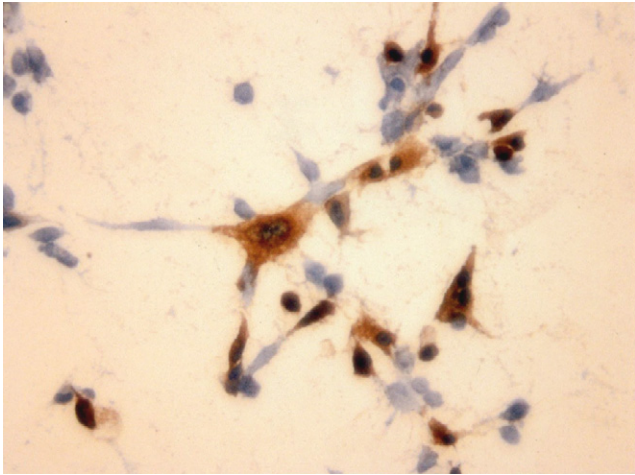
Figure 6 Immunostaining of LPS-induced intracytoplasmic IL-8 (brown areas). Time point: 24 h after stimulus. Original magnification: $630\times$.
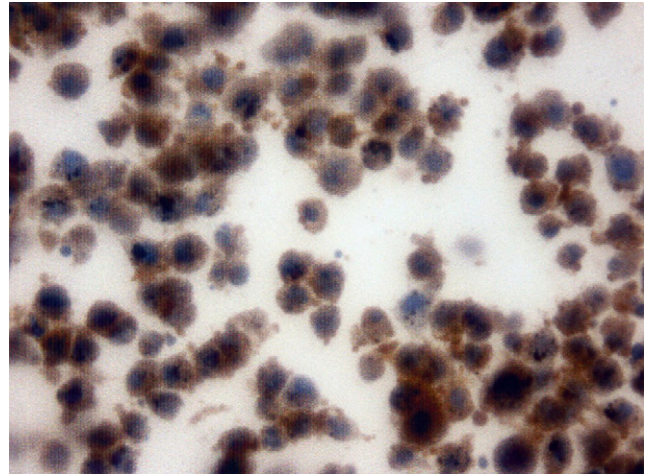

Figure 9 Immunostaining of $PM_{1.0}$-induced intracytoplasmic IL-8 (brown areas). Time point: 48 h after stimulus. Original magnification: $1000\times$.
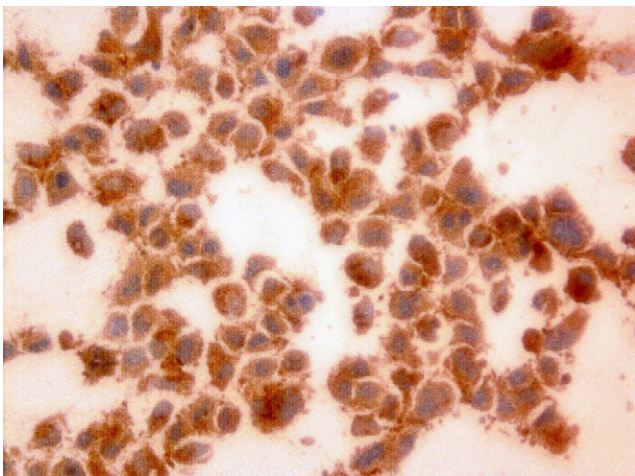

Figure 7 Immunostaining of LPS-induced intracytoplasmic IL-8 (brown areas). Time point: 48 h after stimulus. Original magnification: $630\times$.
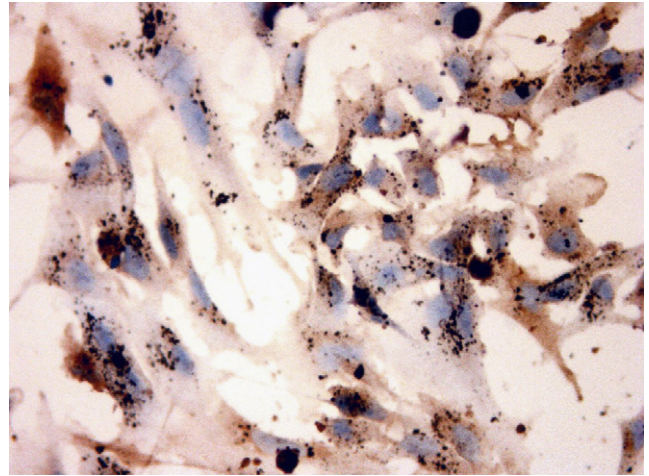

Figure 10 Immunostaining of $PM_{1.0}$-induced intracytoplasmic IL-8 (brown areas). Time point: 72 h after stimulus. Original magnification: $1000\times$.
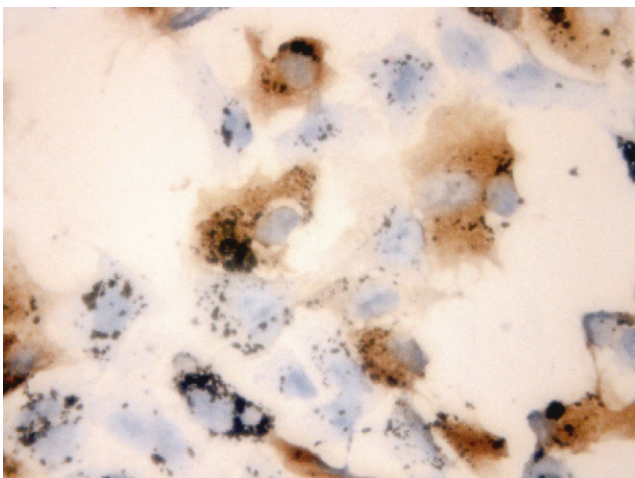

Figure 8 Immunostaining of $PM_{1.0}$-induced intracytoplasmic IL-8 (brown areas). Time point: 24 h after stimulus. Original magnification: $1000\times$.
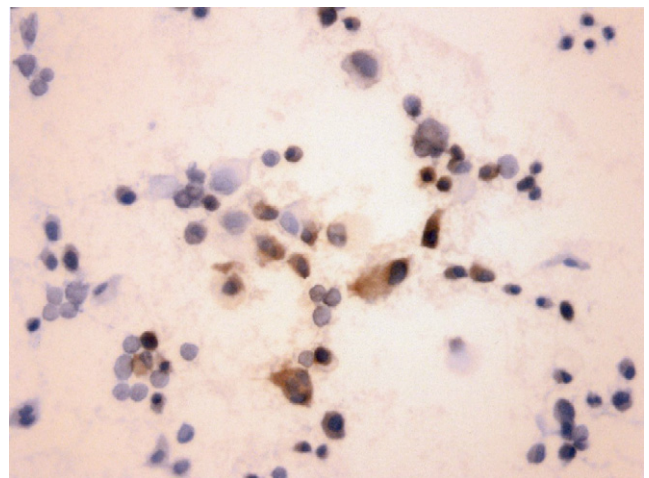

Figure 11 Immunostaining of LPS-induced intracytoplasmic IL-6 (brown areas). Time point: 24 h after stimulus. Original magnification: $630\times$.
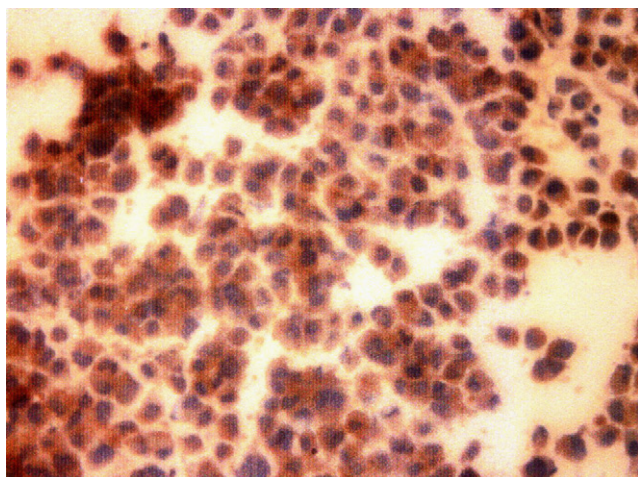
Figure 12 Immunostaining of LPS-induced intracytoplasmic IL-6 (brown areas). Time point: 48 h after stimulus. Original magnification: $630 \times$.
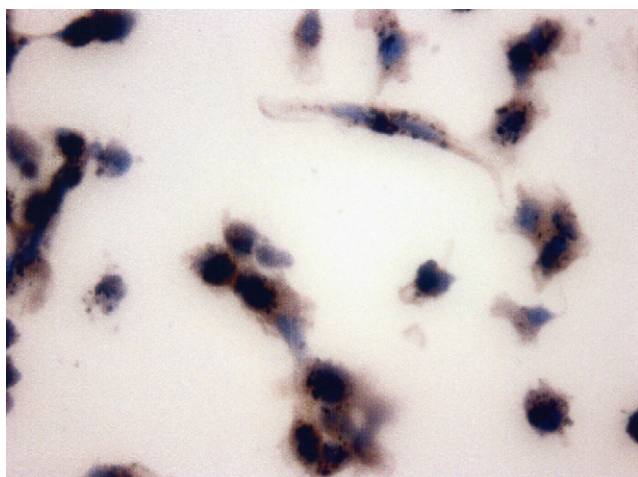

Figure 13 Immunostaining of $PM_{1.0}$-induced intracytoplasmic IL-6 (brown areas). Time point: 48 h after stimulus. Original magnification: $1000 \times$.

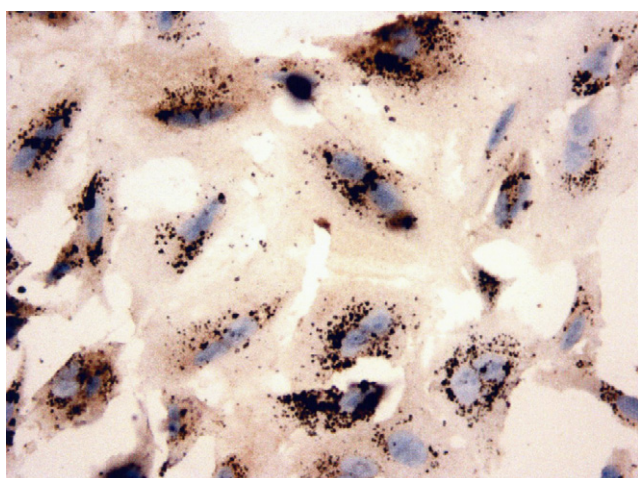**Figure 14** Immunostaining of $PM_{1.0}$-induced intracytoplasmic IL-8 (brown areas). Time point: 72 h after stimulus. Original magnification: $1000 \times$.
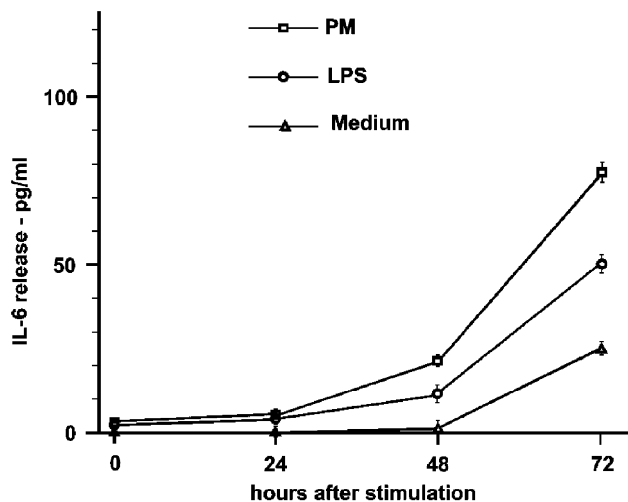

Figure 15 ELISA reading of LPS- and $PM_{1.0}$-induced IL-6 secretion. Time points: 0, 24, 48 and 72 h after stimulus.
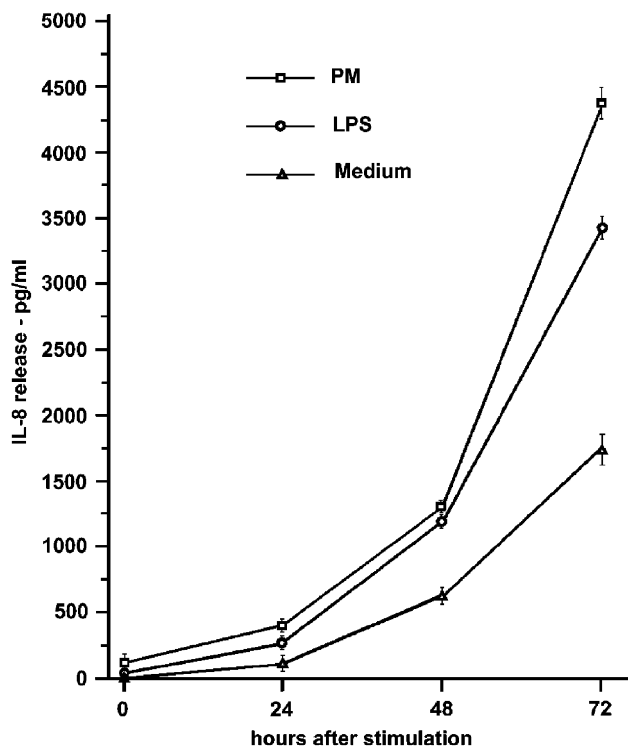
Figure 16 ELISA reading of LPS- and $PM_{1.0}$-induced IL-8 secretion. Time points: 0, 24, 48 and 72 h after stimulus.

Immunocytochemistry revealed that at 24 h from LPS stimulation, 81% of A549s would express Intracytoplasmic IL-8, and 98% both at 48 and 72 h. $PM_{1.0}$ stimulation led to IL-8 intracytoplasmic expression in 25% of A549s at time 24 h, and 98% both at 48 and 72 h. LPS-treated cells appeared oval-shaped, small in size, with eccentric nucleus and granular cytoplasm (Figs. 6 and 7); $PM_{1.0}$ stimulation led to the appearance of medium- to large-sized cell elements, often characterized by a "starry"-like shape (Figs. 8–10).

Normally, an increase in intracytoplasmic IL-6 expression was noticed in about 80% of total cells stimulated with LPS,

whereas about 85% of A549s stimulated with $PM_{1.0}$ responded increasing IL-6 expression (Figs. 11–14).

We also employed ELISA to actually measure the amounts of IL-6 and IL-8 produced by A549s (and by these released into the medium) following stimulation with either fresh medium alone (neg. control), or fresh medium complemented with LPS (pos. control), or fresh medium containing $PM_{1.0}$ [0.1 mg/mL]. Typical readings gave us the following values: at 24 h, LPS stimulation resulted in the production of about 4 pg of IL-6/mL or 270 pg of IL-8/mL; at the same time point, $PM_{1.0}$ treatment led to the release of about 5 pg of IL-6/mL or 400 pg of IL-8/mL; at 48 h, LPS stimulation resulted in the production of about 10 pg of IL-6/mL or 1100 pg of IL-8/mL; at the same time point, $PM_{1.0}$ treatment led to the release of about 20 pg of IL-6/mL or 1200 pg of IL-8/mL; at 72 h, LPS stimulation resulted in the production of approx. 50 pg of IL-6/mL or over 3000 pg of IL-8/mL; at the same time point, $PM_{1.0}$ treatment led to the release of about 50 pg of IL-6/mL, and over 4000 pg of IL-8/mL (Figs. 15 and 16).

Discussion

Many studies have shown that DEP can induce the production of inflammatory mediators and cytokines like IL-6, IL-8, GM-CSF and ICAM-1 from bronchial ECs in vitro.[28,29] These effects appear to be greater in light atopic asthmatic subjects than in non-asthmatic subjects, or controls.[30] Leila A. and Otto MP.[27] had similar results using $PM_{2.5}$ derived from diskette filters obtained from urban air pollution control devices displaced in areas characterized by high vehicular gases content. Of course, DEP-PM only accounted for part of total PM, though.

We used $PM_{1.0}$, where particles have a size of 1.0 μm or less, at a concentration of 0.1 mg/mL and derived from filters applied to diesel engine exhausts: basically, virtually 100% of PM employed in our study was DEP-PM.

IL-8 is a strong chemotactic factor for neutrophils, eosinophils and T-lymphocytes and is known to play an important role in bronchial hyperresponsiveness. Takizawa and others[31] provided evidence that suspended exposure of cells to DEP PM may lower the expression of the IL-8 encoding gene. Our results indicate that sub-micron DEP PM are capable of inducing striking increases of the level of IL-8 mRNA in cultured lung-derived alveolar ECs of the Type II pneumocyte class. This effect is likely to involve transcriptional factors like NF-κB. This has been described to be chronically up-regulated in the macrophages and bronchial ECs of asthmatic subjects, thus playing an essential role in the regulation of many cytokines and adhesion molecules. NF-κB can be activated by oxidative stress. According to recent studies, DEP organic extracts stimulate free radical production in macrophages and in the bronchial ECs, thus activating the promoters of cytokines and chemokines of allergic inflammation through the protein-1 activator and through the nuclear κ-B signal network.[31]

In the last 30 years both vehicular traffic and the rate of aerial pollutants and emissions have also increased considerably. During these three decades the prevalence of asthma and allergic illness have also increased in frequency, so it is plausible that these two events may be related. Epidemiological studies show some links between the variation of aerial concentration of inhaled dust and reactivations of asthma.[29,30]

DEP can induce sensitization in vivo and may make easier IgE synthesis in vitro. DEP also increases the hyperresponsiveness of airways in animal models of asthma. Studies on humans showed acute inflammatory responses in healthy individuals exposed to high environmental concentration of DEP. Other studies showed some effects of DEP on the expression of inflammatory cytokines in the nasal mucosa.[32] However, the mechanism by which DEP can influence the immunologic response to aerial allergens is still uncertain.

Our data suggest a clear cytotoxic effect for sub-micron DEP-PM particles, as proved by the high cell death ratio we always obtained in our experiments. Yet, our experimental data also strongly support the idea that small size DEP-PM is capable of inducing a functional activation of lung alveolar ECs, also stimulating their production and release of proinflammatory cytokines. We may therefore confirm that ECs represent a primary target of air pollution-induced inflammatory responses; the strong chemotactic activity of IL-8, and its strikingly increased expression in $PM_{1.0}$-stimulated human lung Type II ECs strongly suggest a critical role for ECs in the cell/humoral interplay involved in the recruitment of inflammatory cells in the airways. In our system, ECs treated with $PM_{1.0}$ were also found to express considerable amount of another proinflammatory cytokine, IL-6, thus providing even stronger support to our hypothesis. Further investigations—possibly involving also different cell types—are needed in order to gather a better understanding of inflammatory mechanisms and pathways correlated to PM exposure. In vitro experiment results will—of course—also need to be correlated to studies performed in vivo, which would prove finally validating.

Acknowledgements

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