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Health Effects of Ambient Particulate Matter - Biological Mechanisms and Inflammatory Responses to In Vitro and In Vivo Particle Exposures

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In this article, we review and analyze different modes of exposure to ultrafine particles in order to assess particle-induced inflammatory responses and the underlying mechanisms in vitro and in vivo. Based on results from monocytic cells cultured under submerged conditions, we discuss (1) the impact of particle properties such as surface area and oxidative potential on lipid metabolism as a highly sensitive regulatory pathway and (2) the interference of diesel exhaust particles with toll-like receptor-mediated inflammatory responses. Furthermore, new developments of air–liquid interface exposure used as an alternative approach to simulate cell particle interactions are presented. In addition to the in vitro approaches, animal exposure studies are described that apply selected mouse models to elucidate potential allergic and inflammatory pulmonary responses and mast-cell-related mechanisms after particle exposure. Long-term inhalation of ultrafine particles might lead to irreversible changes in lung structure and function. Clinical studies addressing the characteristics of inflammatory airway cells are a promising approach to understand underlying pathophysiological mechanisms in chronic obstructive pulmonary disease. Finally, a potential outcome of human particle exposure is chronic cough in children. Here, discrimination between asthmatic and nonasthmatic cough by means of immunological parameters appears to be an important step toward improving diagnosis and therapy.

Particulate air pollution has been associated with increase in morbidity and mortality (Peters et al., 1997, 2004). There is accumulating evidence from epidemiological studies that airborne particulate matter is involved in adverse health effects, including cough and wheezing, and is strongly associated with hospital admissions and emergency-room visits for treatment of respiratory and cardiovascular diseases. Particulate matter is a complex group of air pollutants that vary in size. Particles between 10 and 2.5 μm in diameter are classified as coarse (PM_{10}), particles between 2.5 μm and 0.1 μm in diameter are classified as fine, and particles with a diameter less than 0.1 μm are classified as ultrafine. However, it remains to be elucidated which of these fractions is responsible for the observed health effects and which particle features might play a role in initiating adverse mechanisms. Certain particle-associated parameters are considered to be determinants of pathophysiological effects. Among these are particle number, surface area, and surface reactivity, as well as reactive compounds adsorbed to the particles, like transition metals or polycyclic aromatic hydrocarbons (PAH), contributing to oxidative stress. After deposition in the lung epithelium, particles may be cleared or retained in the lung compartment (Kreyling et al., 2004; Semmler et al., 2004). Moreover, they may induce local pulmonary responses in healthy or diseased individuals (Gong et al., 2005), may be translocated to the capillary system (Kreyling et al., 2002; Oberdorster et al., 2002), and may cause systemic effects such as cardiovascular diseases (Nemmar et al., 2004b; Schulz et al., 2005) or neurological alterations (Calderon-Garciduenas et al., 2004).

In this article, we discuss the role of the particle surface area on lipid metabolism and the impact of ambient particles like diesel exhaust particles (DEP) on toll-like receptor (TLR)-mediated responses in cellular models. For studies on cell particle interactions in vitro under near-realistic conditions, new developments are presented allowing reliable exposures at the air-liquid interface. In moving from in vitro to in vivo exposure concepts, animal studies applying inhaled model aerosols are described in order to elucidate inflammatory pathways that may be triggered by ultrafine particles in a susceptible population. Moreover, in a perspective addressed to basic responses of innate immunity, the barely investigated responses of mast cells to ambient particles may direct investigators' efforts toward this promising topic of research. Alveolar macrophages (AM), relevant targets of inhaled particles, are known to play a major role in pulmonary disorders such as chronic obstructive lung disease. However, their impact on regulatory functions of AM and the potential consequences are still a matter of debate. One potential outcome of particle exposure in children, who are considered to be highly susceptible individuals, is chronic cough. Here, discriminating between asthmatic and nonasthmatic cough by means of immunological parameters seems to be an important step toward improving diagnosis and treatment.

WHAT CAN WE LEARN FROM IN VITRO EXPOSURES TO PARTICULATE MATTER?

In vitro studies with nanosized particles are performed for several reasons. Selected methods allow dissecting pathways of interactions under controlled conditions, in ways that are not feasible for in vivo tests. The use of cell systems as biological target allows study of the impact of particle reactivity, size, and composition on regulatory and functional pathways: induction of oxidative stress, immune responses leading to inflammation (Donaldson et al., 2005; Oberdorster et al., 2005a, 2005b), and apoptosis (Dagher et al., 2006).

Another objective for in vitro studies is the need to determine the dose-response relationship under standardized conditions, which enables a toxicological ranking of various nanoparticles. However, one should be careful and reserved with extrapolation of in vitro observations to the in vivo situation. Nevertheless, in vitro experiments are required for the preliminary understanding of mechanism of action, which should be validated by in vivo experiments (Dagher et al., 2006).

Parameters Defining Particle Reactivity

Undoubtedly, the severity of adverse reactions depends on the characteristics of nanosized particles. Parameters like oxidative potential, surface properties, functionalization, and particle dimensions are critical for cellular responses and are not independent from each other: For example, carbon black nanoparticles have innate oxidative capacity that may depend on the surface area (Donaldson et al., 2005; Koike & Kobayashi, 2006). The oxidative potential of particles as the driving force for oxidative stress results from the surface interaction with noncellular or cellular compounds. At a given mass of spherical particles the surface area is inversely related to the particle size. Because of this relationship, smaller particles might cause more oxidative stress compared to larger particles.

Oxidative Potential

The oxidative potential of particles is one of the major principles inducing biological responses. It might cause irreversible changes in the molecular structure to various lipid, protein, and DNA compounds. In a recent study we tested the hypothesis that the oxidative potential of ultrafine carbonaceous particles causes alterations in the homeostasis of eicosanoids. We specifically looked at the role of oxidative events triggered by ultrafine particles eliciting lipid mediator responses, which could have inflammatory implications. Various carbonaceous particles such as agglomerates of ultrafine particles (AUFP) of EC, Printex 90, Printex G, and diesel soot (SRM 1650a, NIST) were analyzed for induction of oxidative mechanisms in (1) a cell-free in vitro system by monitoring oxidation of methionine to its sulfoxide, and (2) a cellular system with canine AMs by quantifying 8-isoprostane formation as marker for oxidative stress.

Using the cell-free methionine/methionine sulfoxide system described by Beck-Speier et al. (2005), pronounced differences were found between the various particles (Figure 1A). While AUFP-EC exhibited a strong oxidative response, AUFP-Printex 90, AUFP-DEP, and AUFP-Printex G showed weak responses, i.e., less than 1% of that observed for AUFP-EC. These differences were confirmed by electron spin resonance (ESR) measurements, identifying a prominent ESR signal for AUFP-EC but not for the other particles investigated (Beck-Speier et al., 2005). With regard to the commercially available particles including Printex 90, Printex G, and DEP 1650A, our data on the induction of oxidative stress are in agreement with findings already known. As reported by several groups, various types of particles exhibit an innate oxidative capacity. This has been described as oxidation of dichlorofluorescein by ultrafine polystyrene particles (Brown et al., 2001), depletion of supercoiled plasmid DNA by ultrafine particles of carbon black (Printex 90) and various metal oxides (Dick et al., 2003), and hydroxyl-radical generation by fine and coarse particles occurring at rural and industrial sites (Schins et al., 2004).

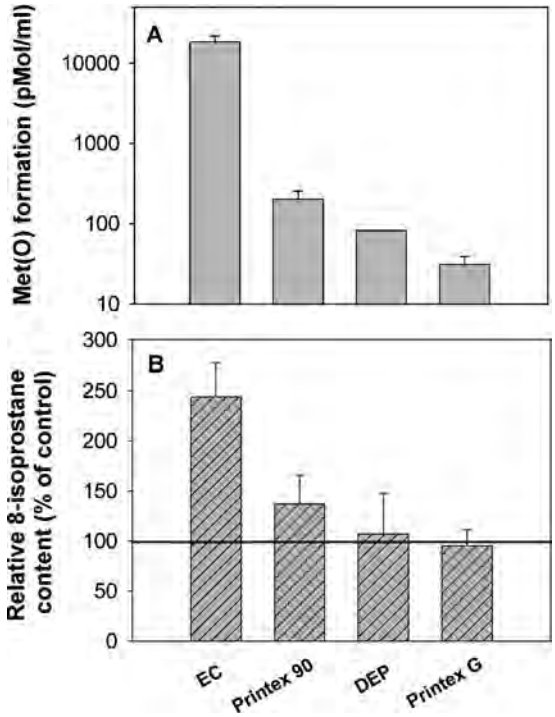

FIG. 1. Oxidative potential of ultrafine particles (AUFP) of EC, Printex 90, Printex G and DEP. (A) Oxidation of methionine to methionine sulfoxide in the cell-free system: The particles (100 μg/ml) were incubated with 0.5 m*M* methionine in distilled water for 2 h at 25°C. After centrifugation at 10,000 g for 10 min, the supernatant was analyzed for methionine sulfoxide by HPLC on a Spherisorb ODS-2 column (125 × 4.6 mm, 3 μm, Gram , Rottenburg, Germany) after precolumn derivatization by ortho-phthalaldehyde as described by Beck-Speier et al. (2005). Amino acid derivatives were detected by a fluorescence detector (Hitachi model F-1000) by excitation at 320 nm and emission at 450 nm. Peak areas were monitored using a Shimadzu integrator (C-R6A Chromatopac. (B) Formation of 8-isoprostane as marker for cellular oxidative stress in canine alveolar macrophages induced by ultrafine particles (AUFP) of EC, Printex 90, Printex G, and DEP with a constant mass concentration in μg/10^6 cells/ml after 1 h of exposure. Canine alveolar macrophages (1×10^6 cells/mol were incubated with the particles in PBS, pH 7.0, containing Ca^{2+}/Mg^{2+} and 0.1% glucose, for 60 min at 37°C. The incubation was stopped by centrifugation (400 × g for 10 min) and resuspended in PBS and deproteinized by adding an eightfold volume of 90% methanol containing 0.5 m*M* EDTA and 1 m*M* 4-hydroxy-2,2,6,6-tetramethylpoperidine-1-oxyl, pH 7.4. After storage at −40°C for 48 h the precipitate was removed by centrifugation at 10,000 × g for 20 min. Aliquots of the supernatant were vacuum dried, dissolved in assay buffer for determination of 8-isoprostane ($n = 5$; asterisk indicates significance at $p < .05$) with using a specific enzyme immunoassay from Cayman (Ann Arbor, MI).

In cellular systems, oxidative stress induced by particles can be assessed by quantification of 8-isoprostane, which is a marker for lipid peroxidation (Roberts & Morrow, 2000). Figure 1B shows that among the particles studied, only AUFP-EC induced a significant production of 8-isoprostane in AMs, which agrees with the in vitro oxidation of methionine (Beck-Speier et al., 2005) (see Figure 1A). Furthermore, particle-induced pulmonary oxidative stress was seen after exposure of healthy rats to airborne ultrafine EC particles at a dose that was equivalent to high ambient particle concentrations. This inhalation procedure not only induced 8-isoprostane formation but also evoked a mild inflammatory response in the rat lungs (Harder et al., 2005) (data not shown).

Specific Surface Area and Particle Size

Surface area itself is inversely related to the particle diameter. Considering spherical particles of different size, a decrease in diameter by a factor of 10 leads to a 10-fold increase in surface area and to 10^3-fold increase of particle number at a given particle mass (Table 1). Agglomeration of particles in aqueous suspensions might not cause a dramatic change of particle surface area. Under defined conditions, ultrafine carbonaceous particles released from spark discharge rapidly form agglomerates with an aerodynamic diameter of 90 nm, while the size of the primary particles ranges between 7 and 12 nm. In aqueous medium, these particles often form a flocculent suspension indicative of an ongoing agglomeration accompanied by a decrease of particle number. Therefore, it is more correct to relate particle effects to the surface area rather to the particle number when exposing submerged cultures to suspended particles.

To assess the impact of particulate surface area on biological responses, we primarily studied the surface area of ultrafine carbonaceous particles as a critical parameter for inducing biological responses (Beck-Speier et al., 2005). We could demonstrate a highly significant correlation ($r = 1.00$; $p < .0001$) of PGE_2/TXB_2 production with particle surface area but not with particle mass (Figure 2). When analyzing the role of particle surface area or particle mass on oxidative stress parameters, we also found high correlations between particle surface area and methionine sulfoxide formation or 8-isoprostane formation, respectively (data not shown). Particle toxicity is clearly closely associated with particle surface properties, while particle mass appears to be of secondary importance. Our observations are supported by findings of Brown et al. (2001) and Oberdorster

TABLE 1
Relationship between particle size (spheres) and surface area

Particle size (μm Ø)	Particle number (n)	Total mass ($d = 1$)* (μg)	Total surface area[a] (μm^2)
10.0	1	523	314
1.0	1000	523	3140
0.1	1,000,000	523	31,400
0.01	1,000,000,000	523	314,000

[a]Total surface areas have been calculated from constant mass (density 1) at various particle sizes.

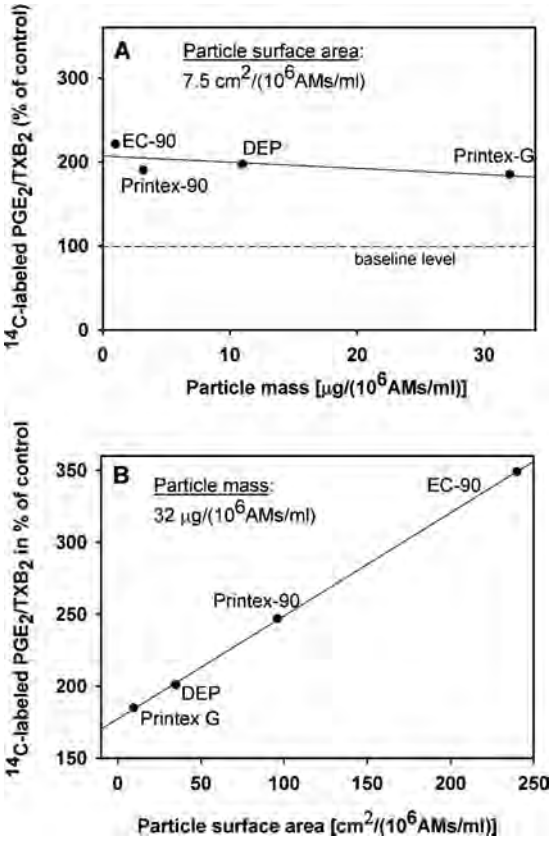

FIG. 2. Relation of PGE_2/TXB_2 synthesis of AM to particle mass concentration and particle surface area. Canine AMs (1×10^6 cells/ml), preincubated for 2 h at 37°C in RPMI medum containing penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 mg/ml), and 5% fetal calf serum, were labeled with [^{14}C]arachidonic acid (4 kB/1×10^6 cells) in fresh medium for 20 h. After removal of labeled medium and a preincubation for 30 min in PBS, pH 7, containing Ca^{2+}/Mg^{2+} and 0.1% glucose, cells were incubated with particles for 60 min at 37°C. The incubation was stopped by extraction of the metabolites. ^{14}C-labeled PGE_2/TXB_2 were separated by thin-layer chromatography and quantified by digital autoradiography as previously described (Beck-Speier et al., 2001). (A) Correlation between PGE_2/TXB_2 synthesis and the corresponding particle mass concentrations at a constant particle surface area of 7.5 $cm^2/(10^6$ AM/ml) and (B) correlation between PGE_2/TXB_2 and the corresponding particle surface areas at a constant particle mass concentration of 32 μg/(10^6 AM/ml). The Spearman rank correlation is 1.0 ($p < .0001$; $n = 5$). Reproduced from Beck-Speier et al. (2005).

et al. (1992) showing that lung injury by ultrafine particles of TiO_2 and polystyrene instilled into rat lungs correlates better with particle surface area than with particle mass concentration.

Interference With Regulatory Pathways

Inhaled particles might influence inflammatory pathways, e.g., by interacting with alveolar macrophages as relevant cells of innate immunity. However, the potential pathophysiological impact of nanoparticles on alveolar macrophages is poorly understood. Changes in the homeostasis of regulatory compounds might have consequences on basic mechanisms of inflammation. Among these compounds, arachidonic acid metabolites such as leukotrienes and prostaglandins are highly efficient mediators exhibiting pro- and anti-inflammatory characteristics (Bonnans & Levy, 2007; Levy et al., 2001; Vancheri et al., 2004). While leukotriene B_4 is involved in initiation of inflammation by exerting chemotactic activity toward neutrophils, prostanoids like prostaglandin E_2 attenuate inflammation, suppress respiratory burst of neutrophils and expression of inflammatory cytokines and have an antiallergic potential when acting via the EP2 or EP4 receptor (Largo et al., 2004; Ratcliffe et al., 2007; Takahashi et al., 2002). One important function of prostaglandin (PG) E_2 is its capability to trigger switching from proinflammatory to antiinflammatory mechanisms, which precludes activation of the 15-lipoxygenase pathway and formation of lipoxins as stop signals of inflammation (Levy et al., 2001).

Effects on Eicosanoid Pathways

Previous results have shown that short-time exposure (1 h) to agglomerates of ultrafine particles (AUFP) of elemental carbon (EC) and titanium dioxide (TiO_2) elicit the formation of arachidonic acid-derived lipid mediators such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) in alveolar macrophages (AM) (Beck-Speier et al., 2001). The supernatants of these particle-treated AM inhibit the respiratory burst activity of stimulated neutrophils, which was abolished when the AMs were pretreated with the unspecific cyclooxygenase (COX) inhibitor indomethacin. Our findings indicate that the COX-dependent PGE_2 synthesis of particle-treated AMs triggers the downregulation of the respiratory burst activity in neutrophils. Despite the particle-induced formation of pro-inflammatory LTB_4, the overall response of AMs to particles seems to be dominated by the immune-modulating PGE_2, which appeared to be more sensitive to the impact of AUFPs than LTB_4.

Freshly produced elemental carbon particles (EC, Pallas generated) exhibit a strong oxidative potential toward methionine (Beck-Speier et al., 2005). They induce an increased formation of PGE_2, LTB_4, and isoprostane from canine and human AMs at a given dose. However, aging for 24 h by incubating in aqueous suspension causes a marked change of the particle reactivity. The aged particles exhibit a significantly lowered oxidative potential. Interestingly, they do not trigger an increase in LTB_4 and 8-isoprostane formation compared to the non-aged particles at a given dose, while formation of PGE_2 remains unchanged. This differential response to non-aged and aged particles suggests that their oxidative potential is a stronger trigger for formation of LTB_4 and 8-isoprostane than for PGE_2. Other particles such as Printex 90, Printex G, and DEP from NIST showing a lower oxidative potential than freshly generated EC particles do not enhance formation of $LTB_4$and 8-isoprostane but have a significant effect on PGE_2, which confirms our observation with the aged AUFP-EC particles (Beck-Speier et al., 2005). Thus, the diversity of particle's oxidative potential might be crucial for the outcome of particle interaction with the biological targets.

Impact on TLR-Related Pathways

Here we wanted to investigate whether cell exposure to nanoparticles might induce enzymes involved in synthesis of lipid mediators like COX2 and whether those particles exhibit a costimulatory effect on the induction of COX2 by ligands of Toll-like receptors (Hofer et al., 2004). For these studies, we used Mono Mac 6 (MM6) cells as a model of the human monocytic lineage, part of the innate immunity. We selected standardized diesel exhaust particles (DEP; SRM 1650a, NIST) at a dose of 32 μg/ml given as suspension to the submerged cultures. After exposure to DEP for 1 h, lipopolysaccharide (LPS; 1 μg/ml) was added and cells were incubated for a further 2 or 4 h. Controls included untreated MM6 cells, and MM6 cells treated with LPS or DEP only.

Exposure of the MM6 cells with suspended DEP in the absence of LPS as biological stimulus did not increase baseline levels of COX2 mRNA. Although LPS only led to a nonsignificant increase of COX2 mRNA expression, postincubation of DEP-pretreated cells with LPS for another 2 h led to a significant enhanced formation of COX2 transcripts (Hofer et al., 2004). This finding suggests a costimulatory effect of DEP on COX2 induction via the toll-like receptor 4 (TLR4) (Figure 3A). Accordingly, COX2 protein in DEP-pretreated cells was significantly increased after 4 h postincubation with LPS compared to the LPS control (Figure 3B). The lack of a direct effect of DEP on COX2 expression indicates that (a) the particles are not contaminated by significant amounts of LPS and (b) DEP per se do not stimulate the cells via TLR4. Based on the finding that DEP enhances the expression of COX2 after stimulation with LPS as a prominent TLR4 ligand, we investigated whether ligands of other TLRs trigger a similar synergistic response. We observed indeed a comparable costimulatory effect of DEP with the TLR2 ligand Pam3Cys. In analogy to TLR4, there was no evidence that DEP stimulates MM6 cells directly via TLR2 for COX2 expression (Hofer et al., 2004).

Becker et al. (2002) recently reported the involvement of TLR2 and TLR4 in recognition of $PM_{2.5-10}$ sampled from ambient air. Gram-positive and gram-negative bacteria and their degradation products are found in PM of outdoor air in association with inhalable $PM_{2.5-10}$. Hence, loading environmental particles with bacterial products could explain an involvement of

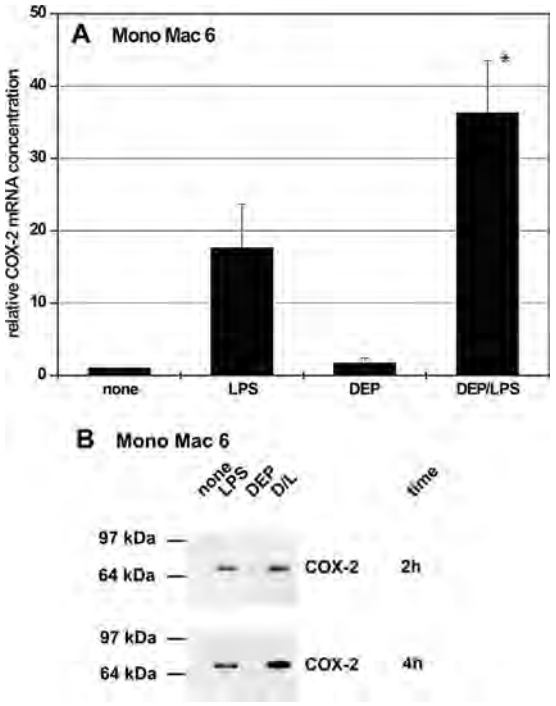

FIG. 3. (A) Effect of DEP and LPS on COX2 mRNA levels in Mono Mac 6 cells (MM6), a cell line with characteristics of mature monocytes. Cells were incubated for 1 h with diesel exhaust particles (32 μg/ml). (DEP, SRM1650, NIST, Gaithersburg, MD) or were stimulated for 2 h with lipopolysaccharide (1 μg/ml) alone (LPS, number L-6261, Sigma, Taufkirchen, Germany) or in combination with DEP (DEP/LPS). Semiquantitative PCR was performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany). As an internal control, the housekeeping gene alpha-enolase was amplified. Baseline level was set as 1 for untreated cells (none) ($n = 4$; $\pm$ SD; Student's t-test; asterisk indicates significant at $p < .05$). (B) Western blot analysis: effect of DEP and LPS on COX2 protein levels in MM6 cells. The groups are the same as depicted above. LPS stimulation (1 μg/ml) was performed for 2 h (upper panel) or 4 h (lower panel). Western blotting was performed on Novex 4–12% bis-tris gels; proteins were detected with primary human COX2 antibody (number 804-112-C050, Alexis, Grünberg, Germany) and visualized using an anti-IgG peroxidase-conjugated secondary antibody (number A-4416, Sigma) with the ECL system (number RPN2106, Amersham, Braunschweig, Germany), and the Hyperfilm ECL (number RPN3103, Amersham). Reproduced from Hofer et al. (2004).

TLRs in the response to the particles. The commercially available SRM1650a dust used herein has been made under laboratory conditions and is therefore less prone to environmental contamination.

The mechanism by which DEP particles enhance LPS-induced COX2 expression is not well understood. One possible explanation might be an increase of the intracellular free Ca^{2+} concentration, which has been shown to be triggered by several particle species. Stone et al. (2000) have shown that ultrafine carbon black particles induce Ca^{2+} influx in MM6 cells, and Donaldson et al. (2003) reported a rise of intracellular free Ca^{2+} by PM10. Ca^{2+} as a second messenger is generally required for activation of signal transduction pathways, e.g., activation of cytosolic phospholipase A_2 via mitogen-activated protein kinases. Beck-Speier et al. (2005) reported release of arachidonic acid from AMs after treatment with ultrafine particles such as AUFP-EC and SRM 1650a, indicating activation of phospholipase A2. Choi et al. (2003) found in microglia cells that influx of Ca^{2+} through store-operated channels is coupled to enhanced COX2 expression. Consequently, the impact of ultrafine particles and their agglomerates on Ca^{2+} homeostasis and on its role in immune responses needs to be studied in detail.

How to Perform In Vitro Exposures

In vitro exposures of cell cultures to ultrafine particles and their agglomerates are applied as part of a strategy to assess molecular mechanisms of cell–particle interaction. This approach is frequently used to evaluate the toxic impact of particulate matter (Nel et al., 2006; Oberdorster et al., 2005a), and the results obtained from those studies are fundamental to designing animal exposures to ambient particles.

Exposures of Submerged Cultures

In vitro exposures of cellular models to particles are usually performed in submerged cultures under standardized conditions. Readouts of responses either in the cells or in the supernatants provide fast information on the nature of cell–particle interactions, e.g., on particle toxicity. However, this routine mode of exposure is far from being realistic with respect to particle application and dosimetry. In the submerged approach, particles are added as a suspension to the culture medium, which differs substantially from deposition of airborne particles onto a confluent cell layer: (a) Particles may change their physical properties during collection and resuspension and (b) the fraction of suspended particles that interacts with the cells remains unknown. Because of this insufficiency, the direct delivery of a well-defined aerosol to cells cultured at the air–liquid interface is a promising alternative approach.

Exposures of Cells at the Air–Liquid Interface

Exposures of cells at the air–liquid interface simulate the impact of airborne particles on the respiratory epithelium and are thus more realistic than submerged exposures to particle suspensions. However, this technique is rather demanding, since the particle concentration and size distribution have to be monitored continuously and the deposited fraction has to be determined. Several techniques have been described to determine the deposited mass of an aerosol at the air–liquid interface. In one approach, an ultrafine aerosol of sodium fluorescein particles was

applied using the commercially available CULTEX system and Transwell membranes (Mülhopt et al., 2004; Ritter et al., 2003). After exposure, the membranes were extracted for quantitative analysis of the fluorescence intensity in solution. From these data, the fraction of deposited aerosol mass can be calculated and extrapolated to the cell exposure experiments. In the system described by Phillips et al. (2005), the deposition of cigarette smoke condensate on Transwell membranes was determined by washing the membranes with methanol and subsequent analysis of smoke constituents by high-performance liquid chromatography and fluorescence spectrometry.

A recently described in vitro exposure device is based on a modified perfusion unit from MINUCELL (Figure 4) (Bitterle et al., 2006; Tippe et al., 2002). The geometric design of the chamber permits the formation of a radially symmetric stagnation point flow. With this arrangement, 75- to 1000-nm particles are deposited spatially uniformly at a constant deposition rate of 2% (Tippe et al., 2002). Online analysis of particle number and particle size distribution together with the known deposition rate allows the calculation of the deposited particle mass over time. This exposure system was used to study the effects of freshly generated ultrafine carbon particles on confluent A549 cells at the air–liquid interface (Bitterle et al., 2006). The exposure system was operated with 47-mm membranes at an aerosol flow of 250 ml/min, which was well tolerated by the cells. Biological endpoints were cell viability, and transcription of interleukin-6 (IL-6), interleukin-8 (IL-8,) and heme oxygenase-1 (HO-1) measured after a 6-h aerosol exposure and a 1-h postincubation time under submerged conditions. For a mid-dose exposure a deposition of 87 ng cm^{-2} was calculated, which resulted in a significantly increased transcription of the antioxidant and stress response protein HO-1. Viability and transcription of IL-6 and IL-8 were not changed in comparison to clean-air-exposed cells.

The CULTEX exposure system is a patented method developed by Aufderheide and Mohr (1999) and distributed by Vitrocell Systems (Gutach, Germany). The application of this system for the exposure of pulmonary epithelial cells to different complex test atmospheres such as diesel exhaust fumes, whole cigarette smoke, or sidestream smoke was demonstrated in several studies (Aufderheide, 2005). These studies showed a depletion of the intracellular glutathione content in A549 cells after only 30 min of exposure to cigarette smoke. The CULTEX system has been employed by other research groups to study the effects of diesel exhaust fumes on A549 cells and rat alveolar macrophages (Seagrave & McDonald, 2004), or of cigarette smoke on A549 cells (Fukano et al., 2004).

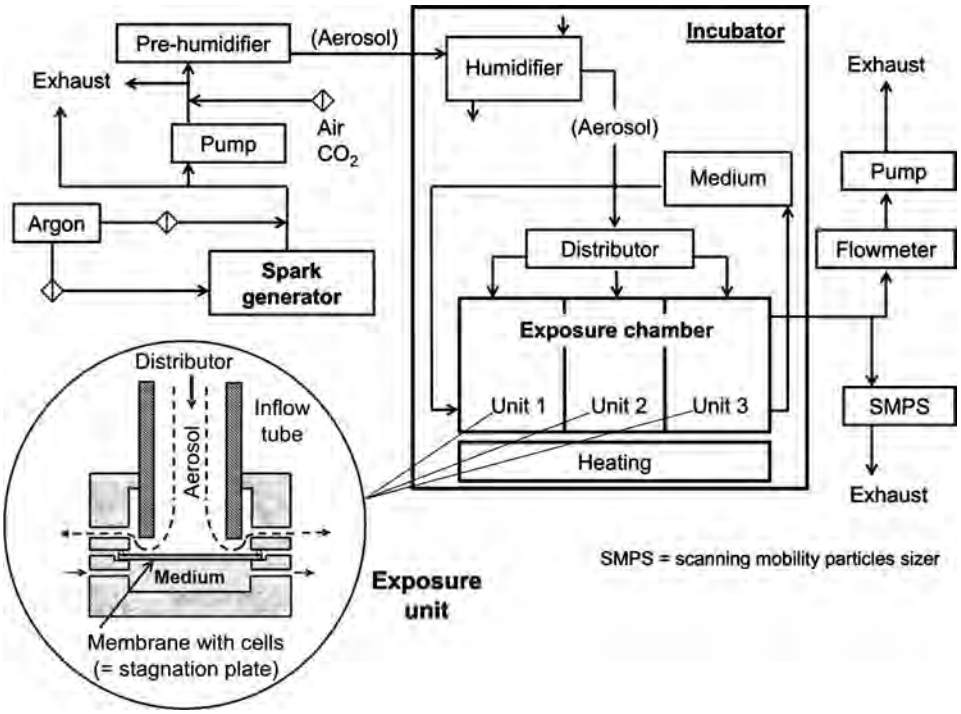

FIG. 4. Assembly of the equipment used for ultrafine carbon particle exposure and cross section of the cylindrical perfusion cell (insert). The ultrafine carbon particles were produced with a spark discharge aerosol generator (GFG 1000, Palas). To reduce particle coagulation the aerosol was diluted (1:1) with Ar directly after generation and then further diluted with clean air. Relative humidity was adjusted to 95.5% and the temperature to 36.5 $\pm$ 1°C. The 47-mm membrane that serves as support of the confluent cell layer separates the upper and lower compartments of the exposure chamber and is identical with the stagnation point plate. The dotted lines indicate aerosol streamlines with a volume flow of 250 ml min^{-1}. Modified from Bitterle et al. (2006).

Mühlopt et al. (2007) used a modified CULTEX system to study the effects of a fly ash model aerosol in BEAS-2B epithelial cells cocultured with THP-1 macrophages (Diabaté et al., 2004). The aerosol was generated by redispersion of the presized fly ash powder MAF02 in filtered air (Mülhopt et al., 2004). After humidification, the aerosol was directed into the exposure units described previously (Mülhopt et al., 2007) harboring the test cells grown on 24-mm Transwell membranes at 100 ml/min. This flow rate and the transportation to the exposure system were demonstrated to be well tolerated by the cells and showed no signs of acute cytotoxicity after exposure to clean air or fly ash aerosol for up to 6 h followed by a 20-h postincubation period under submerged conditions (Figure 5A). The observed increase of IL-8 in the culture medium after exposure to fly ash aerosol at 0.62 mg m^3 is therefore mainly attributed to the deposited particles (Figure 5B). Additionally, an increased expression of HO-1 protein in the MAF02-exposed cells was observed (Figure 5C). The responses to MAF02 aerosol increased time-and dose-dependently. The intracellular glutathione content was not affected by exposure to MAF02 aerosol (data not shown). In conclusion, in vitro exposure of lung cells at the air–liquid interface, although complex, can be used for screening the toxicological potential of unknown aerosols. This method would help determine rapidly whether exposure to particulate matter can raise the incidence of adverse health effects in humans and would facilitate to develop a dose-response relationship of particle toxicity in animal experiments.

Benefit and Limitations of In Vitro Exposures

Application of cellular models allows study of mechanisms of biological responses to ultrafine particles in detail, e.g., uptake and interference with inflammatory pathways. Macrophages, dendritic cells, mast cells, and epithelial and endothelial cells are targets to analyze cell specific mechanisms. Interactions with various receptors such as scavenger receptors, Fc receptors, or integrins provide information on the nature of particle-induced signal transduction. Uptake of particles, depending on their size, via phagocytic or endocytotic pathways might activate inflammatory pathways and trigger toxic mechanisms. Cellular systems allow studying mechanisms of oxidative stress as a major cause of particle toxicity. Furthermore, in vitro studies are suitable to elucidate the influence of nanoparticles on cell vitality, proliferation, necrosis, and apoptosis. Changes in these parameters might have a strong impact on tissue homeostasis.

Results obtained from cellular models should be considered carefully when extrapolating particle effects to the in vivo situation. Most in vitro studies are performed with monocellular systems that exclude intercellular communication. Signaling between cells is central to tissue and organ homeostasis. In addition, toxic effects in animal exposures are not restricted to the expression of signaling molecules such as chemokines or eicosanoids but include migration of inflammatory cells like polymorphonuclear leukocytes (PMNs), changes in the vascular compartment, tissue injury, and fibrotic alterations. Therefore,

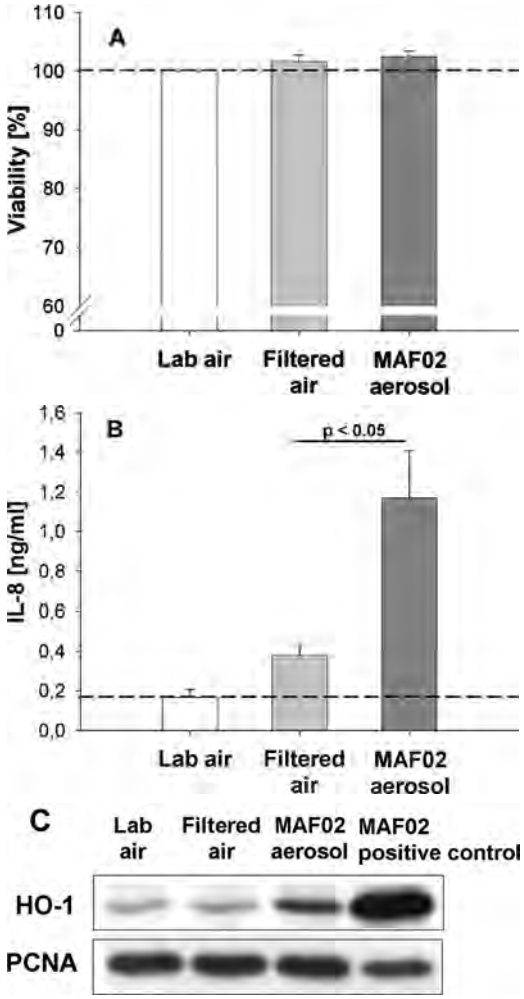

FIG. 5. Biological effects after exposure to aerosol at the air–liquid interface. Cocultures of BEAS-2B and differentiated THP-1 cells grown on Transwell membranes were exposed for 6 h to clean air without any flow (lab air), to filtered air, or to MAF02 aerosol of 0.62 mg m^{-3} at 100 ml min^{-1}. (A) The cells were analyzed for viability by the alamarBlue assay (Serotec) after a postincubation period of 20 h. The fluorescence intensity of cocultures exposed to lab air without any flow was set at 100%. Exposure to filtered air and MAF02 aerosol was well tolerated without loss of viability. (B) The medium supernatant was analyzed for the release of IL-8 (BD Biosciences); the dashed line indicates baseline IL-8 levels of cocultures kept submerged. The exposure to MAF02 aerosol resulted in a significant increase of IL-8 release compared to cells exposed to filtered air (mean ± SEM of three independent experiments, $p < .05$). (C) Western blots of the cell lysates indicating higher expression of heme oxygenase-1 (HO-1) in cocultures exposed to MAF02 aerosol compared to cells exposed to filtered air. A positive control of submerged BEAS-2B cells treated with 6.3 μg cm^{-2} MAF02 for 20 h is shown in the last lane. PCNA was used as a loading control. The Western blot shown is representative of three independent experiments.

in order to elucidate complex mechanisms of particulate-matter-induced injury, animal models are usually useful.

IN VIVO EXPOSURES TO PARTICULATE MATTER—STRATEGIES AND ANIMAL MODELS

Mouse models are becoming an increasingly accepted research tool to evaluate biological mechanisms caused by particle exposure. Animal models of disease are expected to yield valuable information regarding the molecular mechanisms of particulate-matter-driven health effects in susceptible populations. In addition, genetically modified mice have been engineered for the study of the role of specific cell types in the disease.

Adverse Effects of Particulate Matter in a Susceptible Population: Mouse Model of Allergic Disease

Epidemiological studies have shown that the prevalence of allergic diseases has increased worldwide in the last decades. The association between increased asthmatic disorders and ultrafine particle (UFP) number concentrations has been shown in epidemiological studies (Peters et al., 1997; von Klot et al., 2002). These studies suggest that people with allergic asthma are more susceptible to the short-term acute effects of fine and ultrafine particle exposures (Pope, 2000). Few experimental studies using various animal models of asthma have been used in order to investigate the detrimental effects of diverse fine and ultrafine particulate air pollutants in the elicitation phase of the allergic response (Alessandrini et al., 2006; Barrett et al., 2003; Gavett et al., 2003; Goldsmith et al., 1999). Microarray technology can be successfully utilized in order to screen for multiple gene regulation and help in gaining information on the molecular pathways implicated in various diseases. This technique has been employed both in the identification of differentially expressed genes in animal models of asthma (Kuperman & Lewis, 2005; Zimmermann & King, 2003; Zou & Young, 2002) and in screening gene regulation in lung injury induced by exposure to air pollutants (André et al., 2006; Nadadur & Kodavanti, 2002; Nadadur & Pinkerton, 2002; Nadadur et al., 2000).

In order to evaluate the effect of ultrafine particle exposure on gene expression in allergen-sensitized versus nonsensitized animals, we used a mouse model of allergic inflammation of the lung characterized by a long sensitization period and a short challenge protocol (Jakob et al., 2006). This model was designed to induce a mild inflammatory response and allowed us to evaluate potential enhancing effects of particle inhalation on allergen-induced inflammation of the lung. Allergen sensitization and challenge occurred as previously described (Alessandrini et al., 2006). Ten micrograms ovalbumin (OVA)/alum in phosphate-buffered saline (PBS) was injected intraperitoneally on days 0, 7, 14, 28, 49, and 77. Blood samples were taken on days 0, 14, 28, 42, 63, and 84. OVA-sensitized mice were characterized by high titers of OVA-specific immunoglobulin (Ig) E compared with nonsensitized controls (5555 ± 445 versus 9.2 ± 5.2 arbitrary units/ml). Challenge occurred on days 86 and 87 (2×15 min) with 1% OVA in PBS or PBS alone. A mouse whole-body particle exposure was used as described by Karg et al. (1998). Electric spark-generated ultrafine carbon particles were inhaled for 24 h (count median diameter 36.1 ± 1.5; number concentration 3.6×10^6 cm^{-3}; mass concentration 328 μg/m^3) 3 days after allergen challenge. Control animals were exposed to filtered air. Immediately after UFP inhalation the animals were sacrified. As read-outs we used characterization of cells obtained by bronchoalveolar lavage (BAL, $n = 6$/group) in order to evaluate lung cellular infiltrate as previously described (Alessandrini et al., 2006); gene expression data (NGFN Xpress, produced from the 20K cDNA mouse ArrayTAG set [LION Bioscience], see Beckers et al.] for details), were confirmed subsequently by real-time (RT) quantitative polymerase chain reaction (PCR) using the LightCycler (Roche), with mouse Hprt (NM_013556) and Pbgd (NM_013551) genes as normalization controls as described by Seltmann et al. (2005). In order to evaluate the localization of gene expression in the histological specimen, in situ hybridization was performed (Texogene International GmbH, Jena, Germany). We restricted this analysis on the specimen obtained with the lungs of sensitized mice with or without UFP exposure leaving out the lungs which underwent OVA challenge, in order to avoid the interference of potent lung inflammation caused by OVA challenge in the analysis of gene expression.

Our data show that UFP inhalation for 24 h in nonsensitized mice induced no significant alterations in the BAL cell population. Accordingly, no significant changes in gene expression could be seen due to particle inhalation in nonsensitized animals (data not shown). Similarly, no prominent alterations in BAL cell infiltrate were induced by UFP in sensitized and nonchallenged mice. On the contrary, challenge alone induced a significant increase in all BAL inflammatory cells in sensitized animals. Microarray analysis of the lungs from OVA sensitized and challenged mice revealed the following 9 genes to be induced more than 10-fold: immunoglobulin heavy chain constant gamma 1 (lghg1), resistin-like alpha and gamma (Retnla/Fizz1, Retnlg/Fizz3), chitinase 3-like 3 (Chi3l3), solute carrier family 26-4 (Slc26a4), serum amyloid A 3 (Saa3), C-C motif chemokine ligand 8 and 9 (Cccl8, Ccl9), and transthyretin/prealbumin (Ttr). All these genes are generally known to be induced under Th2-mediated inflammatory settings. Further details are given at the GEO database, ID: GSE6496 (http://www.ncbi.nlm.nih.gov/geo).

In sensitized and challenged mice, UFP exposure for 24 h induced a moderate but significant increase in BAL lymphocytes (62%) and neutrophils (33%) compared to filtered air. In order to identify genes that have been induced by particle inhalation in sensitized animals regardless of allergen challenge, we combined the available expression data obtained from lungs from sensitized and from sensitized and challenged animals and analyzed them according to exposure to UFP or to filtered air (GEO database ID: GSE6571). The results are shown in Table 2. We show five—but less than twofold—induced genes, and 10 (3.1- to 5.1-fold) repressed transcripts. Besides the weakest

TABLE 2
Genes regulated by particle inhalation in OVA-sensitized and OVA-sensitized and challenged animals

Symbol	Gene ID	Gene name	Fold change	Pathways (microarray expression data)
		Upregulated		
Crat*	MGI:109501	Carnitine acetyltransferase	1.9	Fatty acid metabolism (adipocytes, muscle, trachea)
Mylpf*	MGI:97273	Myosin light chain, phosphorylatable, fast skeletal muscle	1.6	Motor activity (trachea, adipocytes, muscle, bonemarrow)
S100g	MGI:104528	S100 calcium binding protein G	1.5	Vitamin D binding and induced (pulm. and intestinal epithelium)
Ier3	MGI:104814	Immediate early response 3	1.5	Radiation-inducible, cellular resistance to TNF-induced apoptosis (pulm. fibroblasts)
Timp3	MGI:98754	Tissue inhibitor of metalloproteinase 3	1.4	Metalloendopeptidase inhibitor (monocytes, mature dentritic cells, lymphocytes)
		Downregulated		—
Itih2*	MGI:96619	inter-alpha trypsin inhibitor, heavy chain 2	5.1	acute phase (liver)
Mb	MGI:96922	Myoglobin	3.8	Oxygen transport (muscle, trachea, lung)
Tnni3*	MGI:98783	Troponin I, cardiac	3.8	Muscle contraction (lung, trachea)
Plod1*	MGI:99907	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	3.6	Protein metabolism (lung, liver, adipose)
Tnnt2	MGI:104597	Troponin T2, cardiac	3.6	Muscle contraction (lung, trachea)
Dnase1	MGI:103157	Deoxyribonuclease I	3.4	DNA catabolism, apoptosis (intestine, kidney)
Snag1*	MGI:2137642	Sorting nexin associated golgi protein 1	3.4	Intracellular signaling, endosome-to-lysosome receptors sorting
K11Rik*	2210411K11Rik	RIKEN cDNA 2210411K11 gene (similar to TAO2)	3.4	Similar to Serine/threonine-protein kinase TAO2 (bone marrow, lung)
Atp5h*	MGI:1918929	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	3.3	ATP synthesis coupled proton transport (heart, brown fat)
Scn1b*	MGI:98247	Sodium channel, voltage-gated, type I, beta	3.1	Voltage-gated ion channel (neurons, muscle and pulmonary artery smooth muscle cells)

Note. Asterisk indicates genes that have been analyzed in addition by RT-PCR, and expressions are derived from microarray expression data (GNF1M Mouse Chip, http://genome.ucsc.edu).

induced gene tissue inhibitor of metalloproteinase 3 (Timp3), an essential factor for normal innate immune function, none of the upregulated transcript serves as an obvious marker for an inflammatory response. Myosin light chain (MLC-2/Mylpf) was also weakly upregulated. Although MLC and MLC-kinase are important factors in regulating epithelial cell barrier permeability, which can be influenced by particle exposure, we did not pursue this result or any of the upregulated genes because it is rather speculative to rate whether gene regulations below a factor of two are of biological relevance. In addition, it is rather difficult to identify/track the underlying "induced" molecular pathways of weakly induced genes. We focused therefore on the 10 downregulated genes. Four genes (Mb, Tnni3, Tnnt2, and Scn1b) are related to muscle contraction and are also known to be expressed in the airways and pulmonary blood vessels. Three genes (Plod1, Dnase1, and Atp5h) are supposed to be involved in cell metabolic processes. Two genes (Snag1, K11Rik) are involved in cell signaling, and one (ltih2) belongs to the anti-inflammatory acute phase reactants.

For RT-PCR we selected the following genes: inter-alpha trypsin inhibitor (ltih2), troponin l, cardiac (Tnni3), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (Plod1), and sorting nexin associated golgi protein 1 (Snag1) (Figure 6). Additionally, the genes interleukin-4 (IL-4) and resistin-like alpha (Retnla/Fizz1) have been implemented as positive controls because of their known expression in allergic inflammation. As expected, the expression of IL-4, the Th2 cytokine that mediates allergic pulmonary inflammation in asthma, increased three- to fivefold in the lungs of sensitized and OVA-challenged mice and further increased following particle exposure. Similarly, Fizz1 transcripts rose over 200-fold following allergen sensitization and challenge. All of the genes downregulated by UFP exposure in sensitized animals were confirmed by RT-PCR. In addition, Tnn3 and ltih2 were respectively downregulated ninefold and sevenfold by UFP exposure in sensitized and challenged animals.

Analysis of lung specimen by in situ hybridization has shown that the expression of Snag1 and Tnni3, related to cell signaling and muscle contraction respectively, was located mainly in the bronchiolar epithelium (Figure 7). The staining confirmed the data predicted by RT-PCR and showed decreased expression of both genes in sensitized animals exposed to UFP.

These studies stress the enhanced susceptibility of sensitized individuals toward particle exposure. Further studies are needed to follow pathways triggered by particle exposure in susceptible populations.

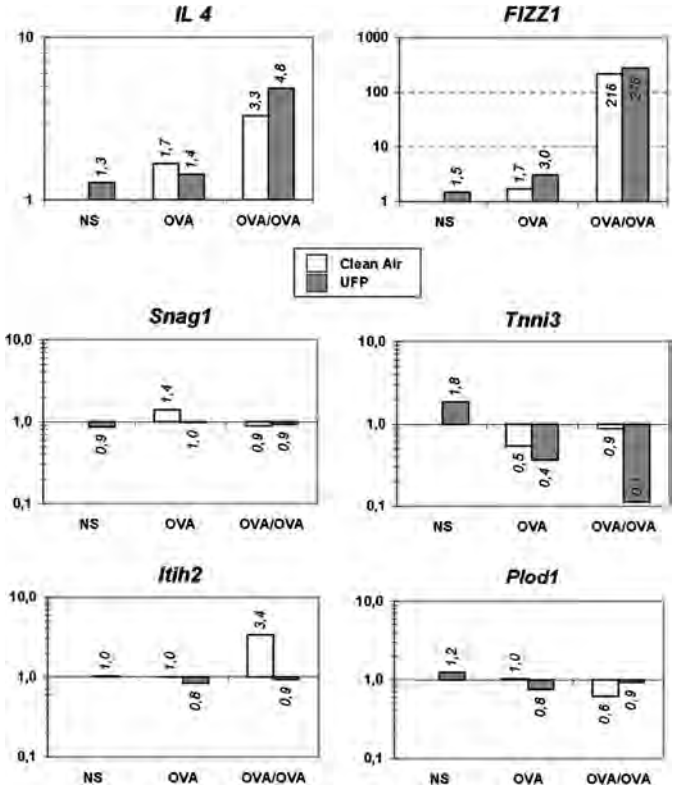

FIG. 6. Quantification of gene expression for selected genes by RT-PCR in the mouse allergic model. The expression of selected genes was determined by quantitative RT-PCR in lungs of nonsensitized (NS), ovalbumin-sensitized (OVA), or ovalbumin-sensitized and -challenged (OVA/OVA) mice exposed to filtered air (white bars) or to 328mg/m^3 elemental carbon ultrafine particles (gray bars) for 24 h. Mean relative expression levels of six mice per group were normalized to Hprt (housekeeping gene) and given as fold inductions relatively to the level obtained in NS mice exposed to clean air.

Role of Mast Cells in Particulate-Matter-Induced Injury: Use of Genetically Modified Animal Models

Mast cells occur on all potential entry sites for pathogens and are in close contact with the environment, namely, in the mucosa of the respiratory and digestive tracts and in skin. They are also found around blood vessels—especially near postcapillary venules, which are important sites of leukocyte emigration into the surrounding tissue (for review see Stassen et al., 2002). It has been recognized that mast cells fulfill a variety of biological functions due to their ability to respond to diverse stimuli, leading to the release of a broad variety of mediators (Mekori & Metcalfe, 2000; Metcalfe et al., 1997). In an early step of inflammatory responses, activated mast cells can initiate the recruitment of neutrophils via such mediators (Qureshi & Jakschik, 1988). This has been proven with a mast-cell-deficient mouse model (Kit^W/Kit^{W-v}). The Kit^W mutant allele encodes a truncated c-kit protein without a transmembrane region, and the mutant Kit^{W-v} allele has a point mutation within the cytoplasmic tyrosine kinase domain that leads to a strong decrease in the kinase activity of c-kit (Kitamura et al., 2000). By using this model, the role of mast cells can be elucidated in different biological scenarios.

As a proof of principle, mast-cell-deficient mice can be reconstituted with mast cells grown in vitro from bone marrow-derived

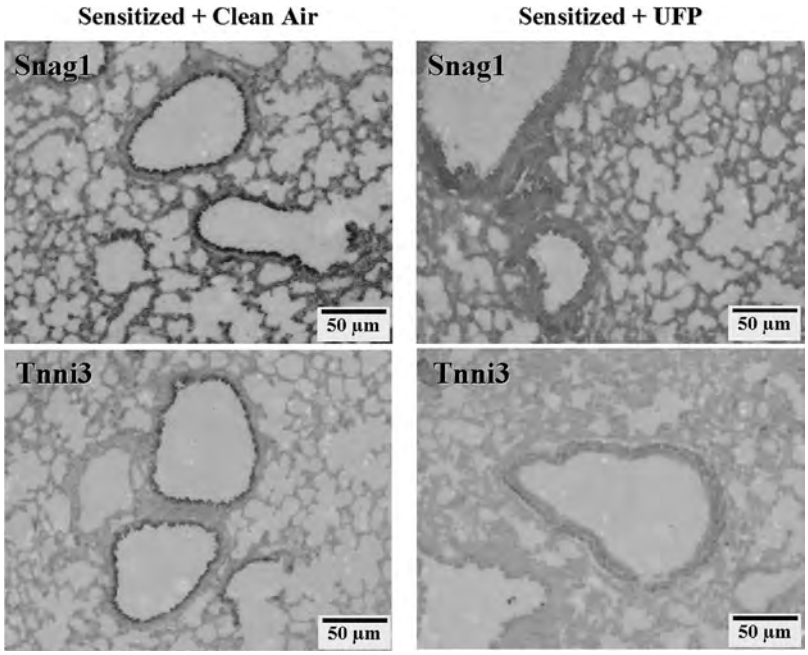

FIG. 7. In situ hybridization of Snag1 and Tnni3 in lungs of ovalbumin-sensitized mice. Gene expression was localized by mRNA in situ hybridization in the lungs of ovalbumin-sensitized mice either after 24-h exposure to clean air (left) or to 328 μg/m^3 elemental carbon ultrafine particles (right). Hybridization with Snag1 and Tnni3 antisense probes resulted in a strong staining of the bronchiolar epithelium of filtered air exposed lungs. Comparable tissue areas of ultrafine particles-exposed lungs exhibit clearly less signal.

precursors or embryonic stem cells in order to reverse the observed symptoms (Tsai et al., 2000). These mice show a delayed influx of neutrophils into the peritoneal cavities after local injection of thioglycollate in comparison with their congenic wild-type littermates. In addition, the time interval during which the neutrophils are elevated is shortened in Kit^W/Kit^{W-v} mice (Qureshi & Jakschik, 1988). Remarkably, the adoptive transfer of wild-type-derived mast cells in mast-cell-deficient mice compensates for the delay in neutrophil influx. Thioglycollate administration induced mast-cell degranulation, which is an early indication of mast-cell-induced inflammatory response.

In a mouse model for IgG immune complex-induced peritonitis, Ramos et al. (1990) described a delayed and retarded influx of neutrophils into the peritoneal cavities of Kit^W/Kit^{W-v} mice in comparison to their wild-type controls. Again, a substantial degranulation of mast cells was observed. Further studies revealed that mast-cell-derived TNF is responsible for the recruitment of neutrophils in immune complex-induced peritonitis in mice. In this study two peaks of TNF-alpha were evident in the lavage fluid of wild-type mice after challenge. The first peak within 5 min declined within 15 min, and a second wave of greater magnitude appeared 4 to 8 h after challenge. In Kit^W/Kit^{W-v} mice the early peak was missing, and the second peak was reduced by 60%. Obviously, the immediate release of TNF-alpha at the onset of an inflammatory response is due to the unique ability of mast cells to store preformed TNF-alpha in their granules.

The cross-linking of specific IgE bound on the mast cell membrane by challenging sensitized individuals with the respective antigen (allergen) elicits three types of associated responses. The acute allergic reaction, which develops immediately within seconds to minutes, is followed several hours later by more widespread late-phase reaction and, finally, the state of chronic inflammation, which can persist for years (Wedemeyer & Galli, 2000). Passive cutaneous anaphylaxis in mice, elicited by local id administration of hapten-specific IgE in the ear followed by systemic iv application of hapten–carrier conjugate, has been used to demonstrate that mast cells are indeed responsible for acute- and late-phase reactions in this model (Wershil et al., 1991). Cutaneous swelling, characteristic for the acute phase, and neutrophil infiltration, as a measure for the late phase, are severely impaired in mast-cell-deficient mice.

Since mast cells are located at entry sites for ambient pollutants, they may, for example, come into contact with fine/ultrafine particles or volatile chemicals. By interacting with these compounds, mast cells may alter their functional arsenal, and it is realistic to assume that degranulation mechanisms may be influenced. In a recent exposure study with WKY rats after inhalation of environmental combustion particles, Kodavanti et al. (2003) found in heart tissue decreased numbers of granulated mast cells, multifocal myocardial degeneration, and chronic active inflammation. However, the authors did not look for the nature of mediators being released from mast cells. Another

study dealing with the relationship between airway inflammation and thrombosis after intratrachel instillation of diesel exhaust particles in hamsters found clear evidence for mast-cell degranulation and histamine increase in bronchoalveolar lavage fluid and plasma (Nemmar et al., 2004a).

When looking for morphological effects of roadside air in rat lungs, Kato and Kagawa (2003) found infiltration of the airway epithelium by mast cells, invasion of the subepithelial space by particle-containing alveolar macrophages, and cell-to-cell contacts among bronchiolar-associated immune cells. Environmentally important metal and transition metal ions were recently shown to activate mast cells and to enhance allergen-mediated mast cell activation in mouse mast cell cultures. Thus, Al^{3+}, Cd^{2+}, and Sr^{2+} induced release of granule-associated *N*-acetyl-ß-D-hexosaminidase, and Al^{3+} and Ni^{2+} enhanced antigen-mediated release (Walczak-Drzewiecka et al., 2003). This may be one of the mechanisms mediating exacerbation of allergen-driven asthma symptoms by air pollution.

Another study performed in rats demonstrated that instillation of TiO_2 particles induced goblet-cell hyperplasia and expression of the Muc5ac gene, which is closely associated with mucus overproduction in the airways. This process was stimulated by increased production of the Th2-type cytokine IL-13 by mast cells (Ahn et al., 2005). In addition to IL-13, another TH2-type cytokine, IL-9, is critically involved in various facets of pulmonary inflammation such as in goblet-cell hyperplasia, pulmonary mastocytosis, pulmonary inflammation, and airway hyperresponsiveness (Kung et al., 2001; Townsend et al., 2000; Vermeer et al., 2003).

Mast cells are functionally linked to a variety of different cellular systems involved in tissue homeostasis and tissue remodeling such as endothelial cells and nerve cells. Studies designed to explore the adverse health effects and the underlying biological mechanisms of fine and ultrafine particles should consider evaluating the role of mast cells as integral part of a first-line alarm system coordinating and integrating inflammatory processes at strategic entry sites of the body for microbiological pathogens and inorganic noxes.

CHRONIC LUNG DISEASES AND THE POTENTIAL ROLE OF PARTICULATE MATTER

Alveolar Macrophages—Central Players in Chronic Inflammation

Alveolar macrophages may play a major role in lung disorders such as in chronic obstructive pulmonary diseases (COPD). Recently, two types of macrophages, which differ in size, have been identified in sputum samples of COPD patients and healthy control donors. A major portion of $CD14^{+}$ macrophages in COPD has lower forward scatter—i.e., they are small macrophages. While in control donors these small macrophages accounted for 6.9% of all macrophages, the percentage of these cells in COPD patients was increased to 45.7% (Frankenberger et al., 2004). Small sputum macrophages of both control donors and COPD patients showed higher level of constitutive tumor necrosis factor compared to the larger macrophages. Expression of $CD14^{++}$ and HLA-DR was high on these small sputum macrophages while the large sputum cells expressed low levels of these surface markers both in control donors and in COPD patients (Frankenberger et al., 2004). These data show that the small sputum macrophages are highly active inflammatory cells and may therefore play an important role in the pathogenesis of COPD.

Up to then, small sputum macrophages from either controls of COPD patients have not been available in sufficient quantity to perform in vitro exposures with nanoparticles. Therefore, for particle exposures studies, we used, in addition to the Mono Mac 6 (MM6) cell line, other primary cells such as peripheral blood mononuclear cells (PBMC), MACS-separated $CD14^{++}$ monocytes, and monocyte-derived macrophages (MDM). Pretreatment of these cells with DEP led to a costimulatory effect for COX2 mRNA expression after stimulation with LPS, shown for MDM in Figure 8 (for comparison, see Figure 3, A and B; Hofer et al., 2004). Since we consider MDM as a model of lung macrophages, we postulate that the impact of DEP on this cell population may also be relevant for alveolar or airway macrophages.

As the expression of COX2 is a bottleneck in the pathway of PGE_2 formation, we also looked for the effect of DEP on

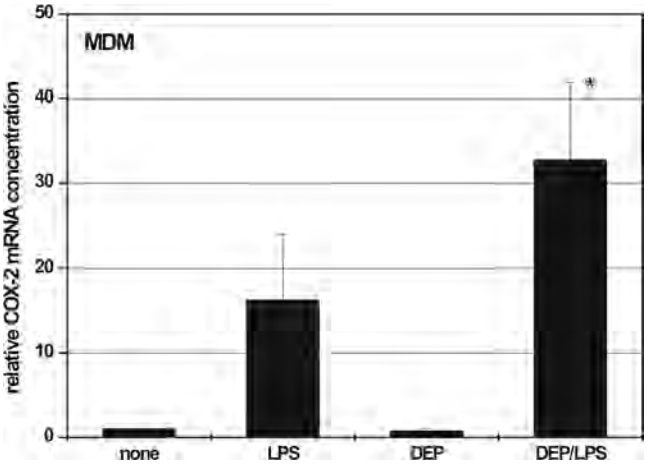

FIG. 8. Effect of DEP and LPS on COX2 mRNA levels in human monocyte-derived macrophages (MDM). MDM were generated from $CD14^{++}$ monocytes purified from PBMC by MACS separation (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions and subsequent 5-day incubation with 2% human serum. Semiquantitative PCR was performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany). As an internal control, the housekeeping gene alpha-enolase was amplified. The groups are the same as depicted in Figure 3. LPS stimulation was performed for 2 h. Baseline level was set as 1 for untreated cells (none) ($n = 3$; ±SD; Student's *t*-test; asterisk indicates significant at $p < .05$). Reproduced from Hofer et al. (2004).

PGE_2 production in MM6 cells. Untreated control cells and cells pretreated with DEP produced only small amounts of PGE_2, which may derive predominantly from the constitutively expressed COX1 enzyme. Stimulation of MM6 cells solely with LPS caused a marked increase in synthesis of PGE_2, while cells pretreated with DEP prior to LPS stimulation showed a twofold increase of PGE_2 (Chang et al., 2004). This "inducible" PGE_2 fraction seems to be related to an increased expression of COX2.

Looking at proinflammatory cytokines, DEP did not induce expression of tumor necrosis factor (TNF). LPS strongly induced TNF expression independently from the addition of DEP in MM6 cells (Hofer et al., 2004). However, the combination of DEP and LPS leading to a synergistic upregulation of the COX2 enzyme simultaneously triggered the downregulation of the chemokine MIP-1ß (data not shown). This indicates a modulatory effect of the inflammatory response induced by DEP, which, however, may be restricted to this cytokine. Whether the enhanced release of PGE_2 plays a central role in downregulation of MIP1ß has to be clarified in further studies. Recent reports have shown a suppressing action of PGE_2 on inflammatory cytokines. Activation of the PGE_2 receptors downregulates the expression of MCP-1 induced by IL-1ß in synovial fibroblasts (Largo et al., 2004) and of TNF release from human alveolar macrophages (Ratcliffe et al., 2007). In this regard, the release of endogenous PGE_2 might provide a signal to counteract an exaggerated or prolonged inflammatory response to physiological stimuli.

TH1/TH2-Mediated Mechanisms—Immunological Aspects of Pulmonary Disease

Cough is one of the most common symptoms in infancy, and chronic cough is a frequent symptom of asthma or nonallergic bronchitis, either of which may be induced or aggravated by particulate matter in ambient air (Gehring et al., 2002). The diagnostic differentiation between allergic asthma and nonallergic bronchitis may be difficult, and the standard clinical and laboratory parameters are of limited value, especially in young children. However, particulate matter may have different effects on both diseases. In addition, selection of patients who will benefit from a more extensive evaluation and/or antiasthmatic treatment is crucial. For these reasons it is important to improve diagnostic tools for the evaluation of chronic cough of unknown origin. Asthma is believed to be a T-helper type 2 (Th2) cell-dominated disorder. Selective trafficking of T cells to peripheral sites is controlled by adhesion molecules and through the interaction of chemokines with their counterpart receptors. Accordingly, a number of chemokine receptors are differentially expressed on lymphocytes in an organ- or disease-specific manner. CCR4 (Kim et al., 2001)is a chemokine receptor with high selectivity for T-helper type 2 cells and is attracted by its ligands TARC and MDC. $CXCR3^+$ cells are attracted by the interferon (IFN)-γ inducible chemokines, ITAC, IP-10 and Mig. Amplified frequencies of $CCR4^+$ lymphocytes (Nouri-Aria et al., 2002; Panina-Bordignon et al., 2001) and increased production of MDC and TARC (Berin et al., 2001; Bochner et al., 2003; Panina-Bordignon et al., 2001) as well as ITAC (Bochner et al., 2003) were observed in bronchoalveolar lavage fluid (BALF) from allergen-challenged asthmatics. In nonchallenged asthmatics, levels of MDC were increased in BALF (Lezcano-Meza et al., 2003), and TARC was found to be increased in induced sputum from adults (Sekiya et al., 2002).

Increased plasma levels of the CCR4-ligand TARC were found in asthmatic children (Leung et al., 2002) but not in adults (Hijnen et al., 2004). Other studies showed an increased production of TARC or MDC by peripheral naive T cells from asthmatic adults (Hirata et al., 2003). These findings led us to ask whether a differential expression of Th1/Th2 associated chemoattractants and cells bearing their respective chemokine receptors could be used for the differentiation of asthmatic versus nonasthmatic children with chronic cough. To address this question, BALF levels of TARC, MDC, IP-10, ITAC, and Mig together with IL-4 and IFN-γ were quantified in children with asthma, in nonatopic, nonasthmatic children with chronic cough, and in healthy controls. The frequencies of $CXCR3^+$, $CCR5^+$, $CCR4^+$,and $CCR3^+$ $CD4^+$ and $CD8^+$ pulmonary T cells were determined by flow cytometry in BALF.

We could demonstrate that TARC and MDC in BALF were increased in asthmatic children (Figure 9A), whereas ITAC and IFN-γ were elevated in nonatopic, nonasthmatic children with chronic cough (Figure 9, B and C) (Hartl et al., 2005). These findings were accompanied by elevated frequencies of BALF $CCR4^+CD4^+$T cells in children with asthma, whereas $CXCR3^+CD8^+$ lymphocytes were more frequent in children with chronic cough as compared to asthmatic or healthy children (Figure 10). Positive correlations between levels of TARC and MDC with $CCR4^+CD4^+$T cells ($r = .9$; $p < .001$ and $p < .01$, respectively) and IgE levels ($r = .7$, $p < .01$) were present in asthmatic children, but not in the corresponding control groups. More importantly, $CCR4^+CD4^+$T cells correlated inversely with FEV1 in the asthmatic children ($r = -.7$, $p < .01$). In contrast, levels of ITAC correlated with $CXCR3^+CD8^+$ BALF T cells in children with chronic cough.

As expected from the results of other studies, IFN-γ and IL-4 were not helpful in the differentiation of asthma versus nonasthmatic chronic cough, since they were almost undetectable (IL-4) or showed substantial overlap (IFN-γ) among the patient groups. In contrast, levels of TARC and MDC and, to a lesser degree, ITAC had a better discriminatory capacity. The same applies to the frequency of $CXCR3^+CD8^+$ and $CCR4^+CD4^+$BALF cells. In contrast to previous studies where increased levels of TARC or MDC were essentially observed after allergen challenge, this study revealed that both chemokines are also increased in nonchallenged asthmatic children. One study also reported increased levels of MDC in nonchallenged asthmatic adults (Lezcano-Meza et al., 2003), levels that were in the range of the MDC levels observed in this study. Although levels of MDC and TARC were clearly lower than those reported for

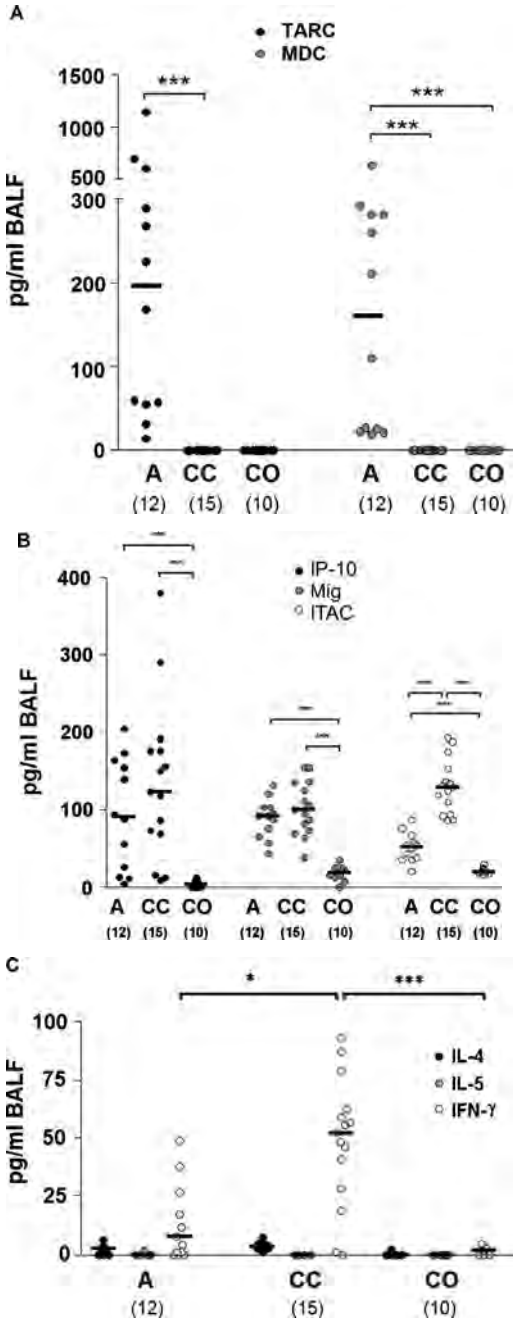

FIG. 9. Levels of the CCR4 (T_H2) chemokines TARC and MDC (A); the CXCR3 (T_H1) chemokines IP-10, Mig, and ITAC (B); and IL-4, IL-5, and IFN-γ (C) in BALF of 12 pediatric asthmatic patients (*A*), 15 patients with chronic cough *(CC)*, and 10 control patients *(CO)*. Chemokines were quantified by enzyme-linked immunosorbent assay (ELISA; R&D Systems Minneapolis, MN). For quantitation of cytokines a multiplex, particle-based assay (Bioplex, Bio-Rad Laboratories, Hercules CA) was used. Median values are shown by horizontal bars. Differences between the patient groups were tested with the Mann–Whitney *U*-test (asterisk indicates significant at $p < .05$; double asterisk, significant at $p < .001$). Reproduced from Hartl et al. (2005).

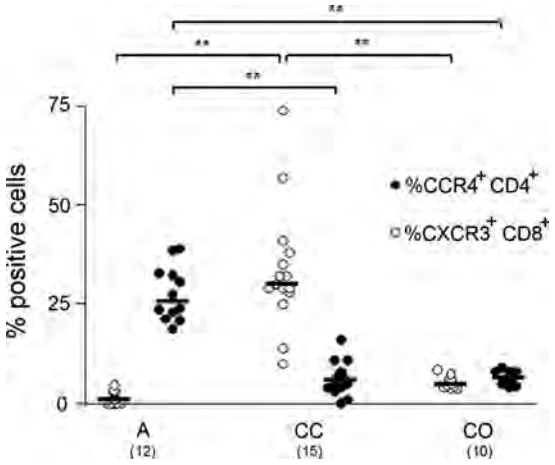

FIG. 10. Percentages of $CXCR3^+CD8^+$ (T_H1) and $CCR4^+CD4^+$ (T_H2) double-positive cells in BALF of 12 pediatric asthmatic patients (*A*), 15 patients with chronic cough *(CC)*, and 10 control patients *(CO)* as determined by flow cytometry (FACSCalibur; Becton-Dickinson, Heidelberg, Germany). CD4-allophycocyanine mouse IgG1, CCR4-pycoerythrin mouse IgG1 (both from BD Pharmingen, Heidelberg, Germany), CD8-phycocyanine 5 mouse IgG1 (Immunotech, Marseille, France), CXCR3-fluorescein isothiocyanat mouse IgG1 and (R&D Systems, Wiesbaden, Germany) were used together with appropriate isotype controls. Median values are shown by horizontal bars. Differences between the patient groups were tested with the Mann-Whitney *U*-test (double asterisk indicates significant at $p < .01$). Reproduced from Hartl et al. (2005).

allergen-challenged individuals, these "asthma baseline" levels seem to have a biological relevance since they were accompanied by increased frequencies of $CCR4^+CD4^+$ T cells. TARC and MDC are produced by airway epithelial cells (Berin et al., 2001; Panina-Bordignon et al., 2001) and attract Th2 lymphocytes. Our finding that TARC and MDC showed a significant positive association with serum IgE levels further supports their contribution to the pathogenesis of asthma. In the majority of the children with chronic cough, an infection with a viral agent must be suspected, since cystic fibrosis, foreign bodies, immune deficiencies, bacterial infections, microaspirations, tracheobronchomalacia, dyskinetic cilia syndrome, chemical exposures, or nicotine abuse could be excluded as triggers for chronic cough. Type 1 T (Tc1) lymphocytes are the main effector cells for viral infections and pulmonary $CXCR3^+CD8^+$ positive cells produce IFN-γ (Saetta et al., 2002). This suggests that in chronic cough of nonallergic origin, the bronchial epithelial cells release the IFN-γ-inducible chemokines, IP-10, Mig, or ITAC. These, in turn, predominantly attract $CXCR3^+$ effector T cells. Our finding that IFN-γ and ITAC correlated strongly with $CD8^+CXCR3^+$ in the patients with chronic cough further supports this hypothesis.

We speculate that the markers described here may help in the discrimination of asthma versus chronic cough. To substantiate these initial observations, prospective studies need to be conducted in children with chronic cough of unknown origin. Since BAL is a comparatively invasive procedure especially in children, induced sputum should be tested as a potential alternative to obtain material for the quantification of the markers described here. Furthermore, direct functional analyses of the cells present in BALF will further help to clarify their precise role in asthma or chronic cough in children. Data from the present study will serve as a basis to elucidate whether fine and ultrafine particles influence not only the clinical picture of asthma or chronic cough in childhood but also migratory patterns of pulmonary inflammatory cells as indicated by Fahy et al. (2000). Exposure to diesel exhaust particles favors Th2 cell recruitment by mononuclear cells and alveolar macrophages from allergic patients by differentially regulating macrophage-derived chemokine and IFN-γ-induced protein-10 production (Fahy et al., 2002). Other reports discussed that diesel exhaust particles downregulate Th1 cytokines like IFN-γ and cause a shift of the Th1/Th2 balance in favor of a Th2 response (Finkelman et al., 2004; Ohtani et al., 2005). Thus, it remains to be clarified whether children with asthma or chronic cough are more susceptible to particulate air pollutants.

FINAL REMARKS

On the basis of in vitro models utilizing monocytic cells, we have discussed the clear evidence showing that ultrafine particulate matter triggers inflammatory mechanisms that may play a role in chronic pulmonary inflammation. Particle surface area and the innate oxidative potential of UFP appear to be critical parameters for initiating and directing inflammatory events on the level of lipid mediators. COX2 acts as a central player in a regulatory network, including recognition pattern-induced signaling, which is markedly modified by the presence of UFP. DEP-related synergisms with LPS-induced TLR2- and TLR4 responses amplify the release of PGE_2 as a potent immunoregulator.

A better understanding of the processes involved in cellular systems requires the use of near-realistic exposures to airborne particles. Studies on interactions between cells and particles at the air–liquid borderline provide realistic data regarding particle dosimetry as well as response sensitivity. Use of coculture systems as a future strategy for in vitro exposures to airborne particles will be a promising tool to assess particle toxicity.

Aside from the in vitro concepts, animal studies focusing on specific mechanisms in chronic diseases like allergy are indispensable. In an allergic animal model using OVA-sensitized and -challenged mice, exposures to UFP led not only to an increased recruitment of inflammatory cells to the lungs but also to altered expression of genes involved in various physiological processes. This observation is a challenge for further research to describe pathways being affected by UFP exposures in a susceptible model. The mast cell as potential target for particulate matter is swiftly moving into the focus of research on nonallergic and allergic diseases. Therefore, the role of mast cells in influencing pathological developments related to ambient particles by sensing them at their site of entry needs to be investigated on an extended level of research using animal models.

There is accumulating evidence that airborne particles contribute substantially to the development of chronic inflammatory disorders like COPD. Since the central mechanisms of COPD-related inflammation seem to be mediated by AM, a thorough analysis of regulatory and functional implications arising from their interaction with fine and ultrafine particles is strongly recommended. Particulate air pollutants such as diesel exhaust are suspected of enforcing chronic cough, which is a frequent symptom of asthma or nonallergic bronchitis. Accordingly, future clinical studies should seek to understand the mechanisms of how ambient particles trigger or modify TH1 and TH2-like inflammatory responses in the lung in order to improve diagnosis and treatment.

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