Sample Characterization of Automobile and Forklift Diesel Exhaust Particles and Comparative Pulmonary Toxicity in Mice

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Two samples of diesel exhaust particles (DEPs) predominate in health effects research: an automobile-derived DEP (A-DEP) sample and the National Institute of Standards Technology standard reference material (SRM 2975) generated from a forklift engine. A-DEPs have been tested extensively for their effects on pulmonary inflammation and exacerbation of allergic asthmalike responses. In contrast, SRM 2975 has been tested thoroughly for its genotoxicity. In the present study, we combined physical and chemical analyses of both DEP samples with pulmonary toxicity testing in CD-1 mice to compare the two materials and to make associations between their physicochemical properties and their biologic effects. A-DEPs had more than 10 times the amount of extractable organic material and less than one-sixth the amount of elemental carbon compared with SRM 2975. Aspiration of 100 µg of either DEP sample in saline produced mild acute lung injury; however, A-DEPs induced macrophage influx and activation, whereas SRM 2975 enhanced polymorphonuclear cell inflammation. A-DEPs stimulated an increase in interleukin-6 (IL-6), tumor necrosis factor α, macrophage inhibitory protein-2, and the T_H2 cytokine IL-5, whereas SRM 2975 only induced significant levels of IL-6. Fractionated organic extracts of the same quantity of DEPs (100 µg) did not have a discernable effect on lung responses and will require further study. The disparate results obtained highlight the need for chemical, physical, and source characterization of particle samples under investigation. Multidisciplinary toxicity testing of diesel emissions derived from a variety of generation and collection conditions is required to meaningfully assess the health hazards associated with exposures to DEPs. ***Key words:*** **automobile, diesel exhaust particles, forklift, mice, pulmonary toxicity, SRM 2975.** ***Environ Health Perspect*** **112:820–825 (2004). doi:10.1289/ehp.6579 available via *http://dx.doi.org/* [Online 22 December 2003]**

Observed increases in the incidence of respiratory allergy, cardiopulmonary mortality, and risk of developing lung cancer are associated with extensive or long-term exposure to fine particulate air pollution, which includes diesel exhaust emissions (Pope et al. 2002; Sydbom et al. 2001). For many years, the health effects of diesel exhaust particles (DEPs) have been investigated (Lewtas 1982); however, full identification of the chemical components responsible for the biologic effects and a detailed understanding of the underlying mechanisms remain incomplete (Mauderly 2001; Rosenkranz 1996). Comparisons among health effects studies of DEPs can be complicated by variability in the chemical composition of the particles, which is influenced by the age and type of engine, fuel composition, load characteristics, lube oil components, presence and efficiency of emissions control devices, and sampling procedures (Claxton 1983; Mauderly 2001; Rosenkranz 1996; Schuetzle 1983). Consequently, the biologic activities of samples generated and collected under different conditions are likely to be different. To facilitate an understanding of the relationships among biologically active constituents of diesel engine particulate emissions, specific mechanisms of toxicity, and relative potencies, individual DEP samples should be characterized chemically and physically before experimental and clinical toxicity testing.

Routinely conducted, comprehensive chemical analyses of complex mixtures such as DEPs are not practical and probably not feasible in most laboratories. However, descriptive and analytical data should be available for individual source samples to provide a sample profile and establish a physicochemical basis of comparison for the interpretation of existing and future DEP health effects data. In this regard, standard reference materials (SRMs) of DEPs (SRM 1650 and SRM 2975) have been certified by the National Institute of Standards Technology (NIST, Gaithersburg, MD, USA) for use in the development, evaluation, and certification of analytical methods for complex environmental diesel mixtures (Claxton et al. 1992). The use of these SRMs to compare and contrast with DEP samples collected and generated under various conditions has not been practiced widely or appreciated adequately. SRM 2975 is a well-characterized DEP sample with which other samples may be contrasted and compared, in terms of both chemical composition and biologic activity, but it does not represent all other DEP samples.

Most studies of the mutagenicity of DEPs have been conducted using NIST SRMs (SRM 1650 or SRM 2975) in *Salmonella* (Hughes et al. 1997); however, few have investigated the pulmonary effects of these samples (Lovik et al. 1997; Madden et al. 2000). In contrast, most studies of the effects of DEPs on pulmonary inflammation and allergic airways disease in laboratory animals have used an automobile-derived sample (A-DEP; Kobayashi and Ito 1995; Sagai et al. 1993) whose mutagenic activity has not been reported. Evaluation of the same DEP sample for pulmonary effects as well as mutagenicity has been relatively rare (Seagrave et al. 2002). In a companion article (DeMarini et al. 2004), *Salmonella* mutagenicity testing of SRM 2975 and A-DEP organic extracts and serial fractions showed contrasting profiles, suggesting significantly different activities of the two DEP samples.

The causative components of DEPs associated with pulmonary inflammation and aggravation of allergic asthma are not known definitively, despite extensive research. Although certain polycyclic aromatic hydrocarbons (PAHs) enhance the same proinflammatory and allergic responses induced by DEPs in human airway cells (Diaz-Sanchez 1997; Kawasaki et al. 2001; Tsien et al. 1997), studies of the bioactivity of particulate matter both *in vitro* and in laboratory rats indicate that the size, composition, and surface reactivity of

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particles may also play a role in these effects (Dick et al. 2003; Donaldson et al. 1996). In the present study, mice were exposed by involuntary aspiration to 0, 25, or 100 µg of A-DEPs or SRM 2975 or to a dose of an organic extract of these DEP equivalent to the proportional mass of the fraction present in a 100-µg dose of the particle sample. Pulmonary inflammation and lung injury were evaluated in the bronchoalveolar lavage fluid (BALF) at 4 and 18 hr after exposure to determine the relative potency and bioactivities of these two samples and to relate these data to their physical and chemical characteristics.

Materials and Methods

Generation and collection of DEPs. The generation and collection conditions of the A-DEPs have been described previously (Kobayashi and Ito 1995; Sagai et al. 1993). Briefly, DEPs were collected "cold" at a sampling temperature of 50°C onto glass-fiber filters (GD-100R, 203 × 254 mm) and on steel duct walls in a constant-volume sampling system fitted at the end of a dilution tunnel. The particles were generated using a light-duty (2,740 cc), 4-cylinder, 4JB1-type Isuzu diesel engine (Isuzu Automobile Co., Tokyo, Japan). The engine had a torque load of 6 kg/m generated by an ECDY dynamometer (Meiden-Sya Tokyo, Japan) and was run at 2,000 rpm.

SRM 2975 was purchased from NIST. These DEPs were generated by a heavy-duty forklift diesel engine and collected using a filtering system designed for diesel forklifts under "hot" conditions, without a dilution tunnel, by the Donaldson Company, Inc. (Minneapolis, MN, USA; personal communication). To our knowledge, no further information on run conditions (e.g., load, fuel) is available. The certified analyses of these particles are accessible online (NIST 2000).

Physical and chemical analyses of DEPs. Particles were suspended in sterile saline (Sigma, St Louis, MO, USA) to evaluate color and solubility. Uncoated particles were examined by scanning electron microscopy (SEM; JSM-6400; JEOL Ltd., Peabody, MA, USA); IMIX/IMAGIST, version 10 software, Princeton Gamma Tech, Inc. (Princeton, NJ, USA) at 500× and 5,000× magnifications. The pH of each DEP sample in aqueous solution was evaluated using a pH meter (model 440; Corning, NY, USA) with pH probe (Orion semi-micro model P/N 911600; Thermo Electron Corp., Beverly, MA, USA) at the same mass:volume ratio in sterile saline. Carbon analyses were performed on a Sunset Laboratory (Tigard, OR, USA) carbon aerosol analysis lab instrument using method 5040 found in the National Institute for Occupational Safety and Health (NIOSH) *Manual of Analytical Methods* (NIOSH 1994). Briefly, particulate samples were heated in an inert atmosphere to approximately 400°C to evolve organic carbon (OC) and from 400°C to approximately 900°C to evolve carbonate. After cooling, the sample was placed in an oxidizing atmosphere, and elemental carbon (EC) was combusted and detected at 900°C. The material comprising the remaining mass after these steps has been termed "remaining fraction" and likely consists primarily of inorganic noncarbon material.

Organic extractions and fractionation. DEPs were weighed and transferred to a 50-mL glass culture tube fitted with a Teflon-lined cap. Dichloromethane (DCM) was added at twice the estimated volume of the particles, and the mixture was vortexed 1–2 min. The tube was placed in a sonicating bath for 20 min and centrifuged at approximately 2,000 rpm for 10 min, and the solvent was transferred to another glass tube. This extraction was repeated two more times. The pooled solvent extract was concentrated under a stream of nitrogen, and the volume was normalized to 10 mL in DCM. The percentage of extractable organic material (EOM) was determined by gravimetric measurement of a 15% aliquot of the volume. The remaining extract (85%) was concentrated to 1 mL and readjusted to 5 mL with hexane.

Silica gel (10 g of grade 62, 60–200 mesh) was added to a 40 × 300 mm open column with a medium-porosity ground-glass frit. The silica was washed with DCM followed by hexane. The extract was added to the column, and the EOM was eluted serially with hexane, 50:50 hexane:DCM, DCM, and methanol. Each fraction was then concentrated under nitrogen, and the mass of EOM for each fraction was determined as described above for the whole extract. Based on the mass distribution of the original extract among the four fractions, calculations were made to determine the approximate mass of each organic fraction present in a 100-µg dose of whole particles. Additional preparations of serial elutions were made for both the A-DEP and SRM 2975 extracts according to the number of doses needed for animal exposures (n = 4–6 mice/organic fraction). The eluent of each fraction was evaporated to dryness, and the residues were resuspended in a vehicle of 0.5% ethanol in phosphate-buffered saline.

Chemical analyses of EOM. The EOM of both DEP samples was examined by a combination of gas chromatography/mass spectrometry (GC/MS), nuclear magnetic resonance (NMR; 500-MHz), and infrared (IR) spectrometry techniques. For GC/MS analysis, 2-µL samples of the extracts allowed full-scan screening and tentative identification of compounds by comparison of the resulting spectra with standard libraries. Subsequently, the GC/MS was configured for greater sensitivity using selected ion monitoring mode, and the samples were reexamined for several PAHs and nitropyrene compounds. For NMR and IR analyses, 1 g of each DEP was extracted using 10 mL of DCM, sonicated for 1 min, and centrifuged for 20 min. This extraction was repeated three times with three fresh volumes of DCM, and the supernatants were pooled and evaporated by water evaporator. The resulting residue was further evaporated under vacuum.

Animal exposures. Outbred, female CD-1 mice (6–8 weeks of age) obtained from Charles River (Raleigh, NC, USA) were housed in animal facilities approved by the Association for the Assessment and Accreditation of Laboratory Animal Care with high-efficiency particulate air filters, and the exposure protocol was reviewed and approved by the U.S. Environmental Protection Agency's Institutional Animal Care and Use Committee. Mice were fed rodent chow and water *ad libitum* and were maintained under a 12 hr light/dark cycle. Mice were selected randomly upon arrival and tested serologically. Sentinels were monitored throughout the study to demonstrate that they were free of Sendai virus, pneumonia virus, and a variety of other rodent viruses and *Mycoplasma* species.

Mice were anesthetized using vaporized halothane (Sigma) and exposed to 25 or 100 µg of either A-DEPs or SRM 2975 in 50 µL saline (Sigma) vehicle by involuntary aspiration for whole-particle exposures. In this delivery technique, the tongue is distended with forceps and a bolus of material is injected onto the oropharynx where it remains until the nares of the rodent are blocked and involuntary aspiration occurs. Intratracheal instillation, a nearly identical exposure method, has been validated as an approach for assessing the comparative pulmonary toxicity of a variety of inhaled materials (Driscoll et al. 2000), and these two methods have similar deposition and clearance profiles (Foster et al. 2001). Particles were sonicated in solution for 5 min before administration. Separate groups of mice were administered 10 µg bacterial endotoxin (Sigma) in 50 µL saline vehicle by the same route, as a positive proinflammatory control, or 50 µL saline only as a vehicle control. For exposures to fractionated organic extracts of DEPs, mice were administered the equivalent dose of each fraction present in 100 µg whole particles in a total volume of 50 µL using 0.5% ethanol in saline as the vehicle. After 4 and 18 hr (whole DEP exposures) or after 4 hr only (DEP extracts), mice were deeply anesthetized via intraperitoneal administration of 25 mg/kg sodium pentobarbital (in 0.2 mL). The lungs were then lavaged three times with a single volume of warmed Hanks balanced salt solution (35 mL/kg), and lavage samples were assayed for evidence of pulmonary inflammation and injury. Only the 4-hr data are presented because similar overall effects were seen at both time points.

BALF cell counts. BALF was centrifuged (1,500 rpm, 10 min, 4°C), and an aliquot of the supernatant was stored at either 4°C for biochemical analyses or –80°C for later cytokine detection. The pelleted cells were resuspended in 300 µL RPMI 1640 medium (Sigma) containing 10% bovine serum albumin (BSA). Total cell counts in the lavage fluid of each mouse were obtained with a Coulter Z1 counter (Hialeah, FL, USA). Each sample (100 µL) was centrifuged in duplicate on microscope slides using a Cytospin (Shandon Corp., Pittsburg, PA, USA) and subsequently stained with Diff Quik solution (American Scientific Products, McGaw, IL, USA) for enumeration of macrophages (MACs), neutrophils, lymphocytes (data not shown), and eosinophils (data not shown). At least 200 cells were counted from each slide.

BALF biochemical analyses. BALF supernatant was kept at 4°C and analyzed using commercially available kits adapted for automated analysis using a Cobas Fara II centrifugal spectrophotometer (Hoffman-La Roche, Branchburg, NJ, USA). Microalbumin levels were determined using a MALB SPQ II kit (INCSTAR, Stillwater, MN, USA) and a standard curve prepared with BSA. *N*-acetyl-β-D-glucosaminidase (NAG) was measured using a commercially prepared kit containing sodium 3-cresolsulfonphthaleinyl-*N*-acetyl-β-D-glucosinamide, which can be hydrolyzed by NAG, releasing 3-cresolsulfonphthalein sodium salt (3-cresol purple), and standards from Roche Diagnostics, Mannheim, Germany. Total antioxidant capacity was determined by adapting the method of Miller et al. (1993) to the Cobas Fara II autoanalyzer. The unit of measurement was the Trolox equivalent antioxidant capacity, which is the concentration of Trolox (standard) with an antioxidant capacity equivalent to a 1.0 mmol/L solution of the test sample.

BALF cytokine analyses. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and macrophage inhibitory protein-2 (MIP-2) concentrations in BALF were measured by enzyme-linked immunosorbent assay using mouse Quantikine M kits purchased from R&D Systems (Minneapolis, MN, USA). Interleukin-5 (IL-5) concentrations in BALF were determined using an IL-5 mouse Cytoscreen kit purchased from BioSource International (Camarilla, CA, USA). These assays were carried out per manufacturer instructions.

Statistical analyses of BALF measurements. The data were analyzed using an analysis of variance (ANOVA) model. The independent variable was exposure. Subsequent to a significant finding, a Dunnett's test was performed to assess the differences between the control and the other exposures. If the data did not fit the assumptions of either normality or equal variances, then a Kruskal-Wallis test was substituted for the ordinary ANOVA. The probability for significance was set at 0.05.

Results

Physical and chemical analyses of DEPs. Table 1 shows the carbon analysis, percent EOM, and PAH ratios of the two samples. For all these end points, A-DEPs and SRM 2975 DEPs were quite different. The A-DEP sample had approximately one-sixth the amount of EC, 10× more OC, and > 10 times more EOM than was found in the SRM 2975 sample. The ratio of PAHs differed between the samples, with a greater abundance of PAHs per mass of particle in the A-DEPs compared with the SRM 2975 (Table 1). The GC/MS chromatogram of the hexane/DCM-extracted fraction of A-DEPs showed predominantly an aliphatic hydrocarbon pattern (Figure 1), whereas the proportion of aliphatics in the SRM 2975 hexane/DCM-extracted fraction was negligible by comparison. The mass distributions of the fractionated extracts of the two DEP samples were diametrically opposed, with most of the A-DEP EOM eluting in the neutral hexane fraction and most of the SRM 2975 EOM eluting in the highly polar methanol fraction (DeMarini et al. 2004). This result was consistent with the photomicrographs and GC/MS demonstrating that much of the A-EOM was incompletely combusted fuel, possibly neutral alkanes and alkenes, that would be expected to elute in the hexane fraction. Although we did not perform single chemical quantitative identification analyses, the SRM 2975 certificate of analysis states that the material does contain 1-nitropyrene. We detected 1-nitropyrene in the extracts of both DEP samples by GC/MS. Although NMR analysis did not indicate striking differences between the two extracts in chemical composition, evaluation by IR confirmed that the

Table 1. Chemical analyses of DEPs.

Compound	A-DEP	SRM 2975
Carbon analysis (% of total mass)		
EC	9	60
OC	50	5
Carbonate	< 1	< 1
Remaining fraction	41	35
PAH ratios		
Phenanthrene:anthracene	1	> 20
Fluoranthene:pyrene	1	> 20
EOM (%)	26.3	2.0

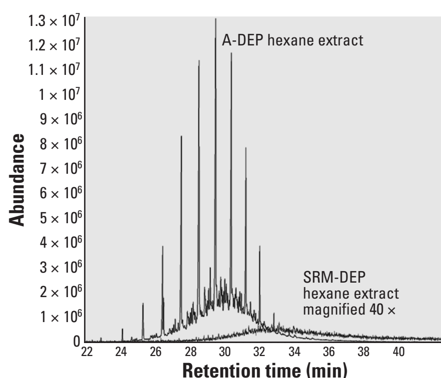

Figure 1. GC/MS of the hexane/DCM extract of A-DEPs and SRM 2975 illustrating relative amounts of aliphatic hydrocarbons in the two DEP samples (amplified up to 40× to fit scale).

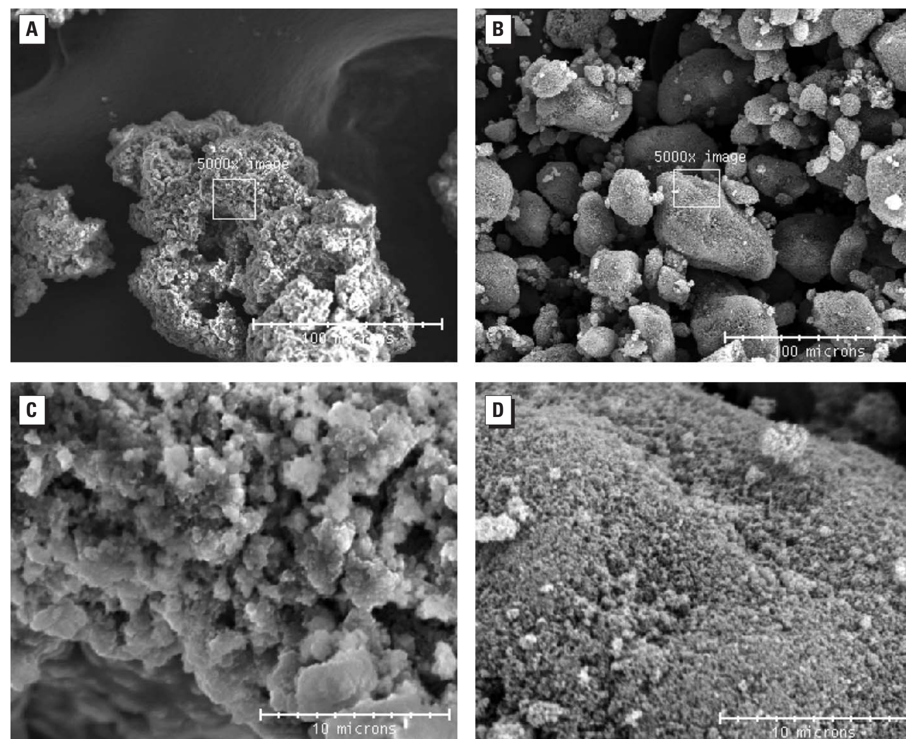

Figure 2. Scanning electron micrographs of DEPs processed by IMIX/IMAGIST: (*A*) A-DEPs and (*B*) SRM 2975 at 500× magnification; (*C*) A-DEPs and (*D*) SRM 2975 at 5,000× magnification.

amount of alkanes in A-DEP extract was much higher than in the SRM 2975 DEP extract (data not shown).

SEM images of A-DEPs and SRM 2975 at 500× magnification showed the presence of aggregated particles (> 50 µm) in both samples (Figure 2). However, SRM 2975 had a greater range of particle sizes (< 10 to > 50 µm) than did the A-DEPs (> 50 µm). At 5,000× magnification, A-DEPs showed more clumping than did the SRM 2975 particles. Compared with A-DEPs, the surface texture of the SRM 2975 particles was much finer and more porous, and the surface detail of individual particles comprising these large agglomerates was more apparent. In contrast, the A-DEP agglomerates showed less surface detail, consistent with being coated by amounts of semivolatile organic compounds. In addition, aqueous suspensions of the two DEP samples at identical mass:volume ratios were dramatically different in color: The A-DEP suspensions were yellowish gray and the SRM 2975 suspensions were black (not shown). Neither sample was completely soluble in saline; however, the SRM 2975 particles remained in suspension much longer than did the particulate phase of A-DEPs. Equal masses of each DEP sample had a pH of 3.3 in solution.

BALF cell counts. SRM 2975 (100 µg) significantly increased total polymorphonuclear cell (PMNs) in the BALF compared with saline 4 hr after instillation (Figure 3), and PMN counts remained significantly elevated at 18 hr (not shown). Endotoxin, which served as a positive control, produced the greatest numbers of PMNs at 4 hr, which then doubled at 18 hr (not shown). By contrast, A-DEPs produced significant increases in total numbers of MACs in the BALF at 4 hr, which reduced to control levels by 18 hr (not shown). Neither SRM 2975 nor endotoxin affected total counts of MACs in the BALF at either time point, and A-DEPs did not induce significant numbers of PMNs. However, for both DEP samples, increases in BALF MACs or PMNs were dose dependent. There were no significant changes in numbers of lymphocytes or eosinophils at these time points with either DEP or endotoxin exposures (not shown). Instillation of the organic fractions of each DEP sample present in the 100-µg dose of whole particles did not alter inflammatory cell influx to the lung (not shown).

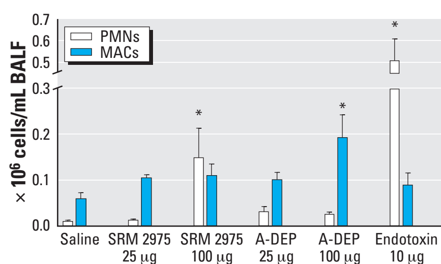

Figure 3. Pulmonary inflammatory cell counts measured in BALF after aspiration of saline, DEPs, or endotoxin in CD-1 mice. Total PMNs and MACs were counted in BALF of exposed mice (n = 5/group). Data are shown as group mean ± SE at 4 hr after instillation.

*Significant difference ($p < 0.05$) from the same time point in saline-exposed mice.

BALF biochemical analyses. Microalbumin concentrations were measured as an indicator of vascular leakage (edema) into the alveolar regions of the lung. SRM 2975 (only at 25 µg), A-DEPs (25 and 100 µg), and endotoxin (10 µg) significantly increased concentrations of microalbumin in the BALF 4 hr after exposure (Figure 4A). These results indicated that the two DEP samples induced comparable pulmonary edema. Endotoxin produced somewhat greater nonspecific lung injury by this measurement (Figure 4A). There were no significant differences in total antioxidant capacity in the BALF among the exposure groups compared with saline, although group means were notably lower in SRM 2975-exposed mice (Figure 4B). Only A-DEPs (100 µg) significantly elevated the levels of NAG, a marker of pulmonary inflammation and MAC activity (Metzger and Peterson 1988), compared with saline, and this increase was dose dependent (Figure 4C). The only end point in which a significant difference occurred between exposure groups after instillation of organic extracts of DEPs was microalbumin (not shown). A significant increase occurred with exposure to the DCM fraction of SRM 2975 organic extract, indicating increased microvascular permeability relative to the method blank in the same fraction (not shown). The method blank controls, consisting of residues from each organic solvent fraction after elution through a clean silica gel column and addition of vehicle (0.5% ethanol in saline), produced elevated responses for all the pulmonary end points compared with instillation of saline, the control used in whole-particle exposures. Possibly, either the concentration of organics extracted from 100 µg of either DEP sample was insufficient to stimulate any significant effects, or differences among the organic fractions were masked by the elevated baseline responses associated with the vehicle.

BALF cytokine analyses. Instillation of 100 µg of A-DEPs significantly increased the concentration of the proinflammatory cytokines IL-6 and TNF-α and the chemokine MIP-2 in BALF at 4 hr compared with instillation of saline (Figure 5A–C). SRM 2975 (100 µg) produced a significant increase in IL-6 only. The concentrations of TNF-α and MIP-2 were > 2 times greater in the BALF of A-DEP–exposed mice compared with SRM 2975–exposed mice. Observed increases in proinflammatory cytokines were dose dependent with exposure to either DEP sample. Concentrations of these cytokines were increased dramatically in mice instilled with 10 µg of endotoxin compared with mice exposed to saline or either DEP sample (Figure 5), demonstrating that the mice were responsive to a strong proinflammatory stimulus. Although both DEPs tended to increase IL-5 concentrations in the BALF compared with saline, only A-DEPs (100 µg) produced a statistically significant increase (Figure 5D), whereas endotoxin (10 µg) had no effect on IL-5 levels. There were no significant differences in levels of these cytokines in mice exposed to the organic fractions of either DEP sample (not shown).

Discussion

Physical and chemical features of DEPs. The differences between A-DEPs and SRM 2975 in size, texture, and color were not surprising considering the different types of engines used to produce the particles and the different collection methods used to obtain the samples. We have verified that large agglomerates such as those illustrated in Figure 2 occur for both filter-collected DEP samples and DEP samples collected from exhaust-duct surfaces that we have gathered ourselves from other diesel engines. These agglomeration effects were

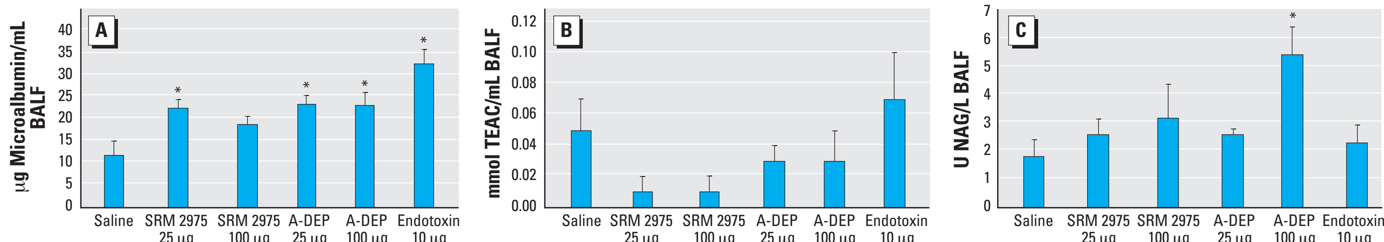

Figure 4. Biochemical analyses of BALF after aspiration of saline, DEPs, or endotoxin in CD-1 mice. (*A*) Microalbumin, (*B*) total antioxidant capacity, and (*C*) NAG concentrations. Measurements (see "Materials and Methods" for details) were taken from BALF samples of exposed mice (n = 5/group). Data are shown as group mean ± SE at 4 hr after instillation.

*Significant difference ($p < 0.05$) from the same time point in saline-exposed mice.

possibly caused by hydration or electrostatic attractions occurring on the collection surfaces. Thus, the large agglomerates seen in the SEM micrographs of the A-DEP and SRM 2975 particles were a consequence of the collection procedures and are not representative of primary DEP emissions. Particle size distributions of the emitted particles from other diesel engines currently under investigation show predominantly unimodal distributions, with volume mean diameters between 0.1 and 0.3 µm (data not shown). An understanding of the impact of collection conditions on the physical and chemical characteristics of DEPs is essential for the validation of existing and future exposure models.

Differences in percentages of EC and OC in the A-DEP and SRM 2975 samples may be explained by the sample collection methods, as well as by differences in engine design and operation. Although no information on particle emissions rates was available for either engine, we know that SRM 2975 was collected on filtration media at high temperatures, which would limit the condensation of semivolatile species on the particles or collection surfaces. Also, the hot combustion gases passing through the filter media could have revaporized any semivolatile species condensed on particle surfaces before collection. In contrast, A-DEPs were collected on relatively cool steel duct walls after sample dilution and cooling. This approach would promote diffusion and condensation of semivolatile species on particle surfaces and duct walls. In addition, the EC content of the DEPs may reflect differences in the design and operation of the automobile and forklift engines. Automobile engines are designed for high efficiency and low particulate matter emissions, optimized for on-road fuel economy, and fitted with control devices. In contrast, a forklift engine is designed for power applications for which fuel economy and exhaust emissions are of secondary concern. Therefore, different emissions profiles would be expected for these two types of engines. Identification of engine type and running conditions is vital to investigating and describing the relative bioactivities of different particulate exhaust emissions.

Biologic effects. A-DEPs and SRM 2975 produced similar levels of acute pulmonary injury and IL-6 but induced distinctly different cellular inflammatory responses. The increases in microalbumin and IL-6 levels may signify equipotent inflammogenicity of the two DEP samples, whereas the increase in PMNs by SRM 2975 and increase in MACs by A-DEPs suggest divergent mechanisms of cellular recruitment and activation. This distinction might be explained by the contrasting physicochemical properties of the two samples. The mass of SRM 2975 was composed predominantly of insoluble carbon particles, whereas A-DEPs were highly enriched in organic compounds with much less EC. Given these differences, it is possible that SRM 2975 was more readily phagocytosed by resident MACs, a process that is known to lead to increased chemoattractive mediators and rapid infiltration of PMNs (Granum and Lovik 2002). In contrast, the A-DEPs, having a reduced inorganic carbon matrix and higher organic content, may have activated pulmonary MACs directly, stimulating intracellular signals in the absence of substantial phagocytosis. The significant increases in NAG, indicating elevated MAC activity, and TNF-α in the BALF may have been evidence of this. Several studies have now shown that the amount of organics present in DEPs is directly associated with the generation of reactive oxygen species (ROS) and the magnitude of the cytokine response (Boland et al. 1999; Nel et al. 2001). Although the biomolecular targets and subsequent intracellular signaling pathways of DEPs are not known, new evidence has shown that ultrafine ambient air particles accumulate in the mitochondria of MACs and airway epithelial cells, and that the formation of ROS is directly associated with the levels of PAHs in the particles (Li et al. 2003). In the present study, the particle sizes of the DEPs instilled into the mouse lungs were not representative of real-world exposures to DEPs via inhalation; therefore, no conclusions can be drawn regarding cell-specific targeting and distribution in the lung from this study. Preliminary measurements of stable free radical concentrations on the surfaces of these two samples using electron paramagnetic resonance technology have indicated that SRM 2975 has significantly increased surface free radical concentrations compared with A-DEPs (data not shown). This suggests an alternative hypothesis that the higher levels of EC in SRM 2975 are associated with increased free radical activity. Data supporting the relative contribution of oxidative stress from organic and EC components of DEPs are required to clarify the source and activity of associated free radicals.

Allergic-adjuvant properties of the inorganic carbon core versus the soluble organic

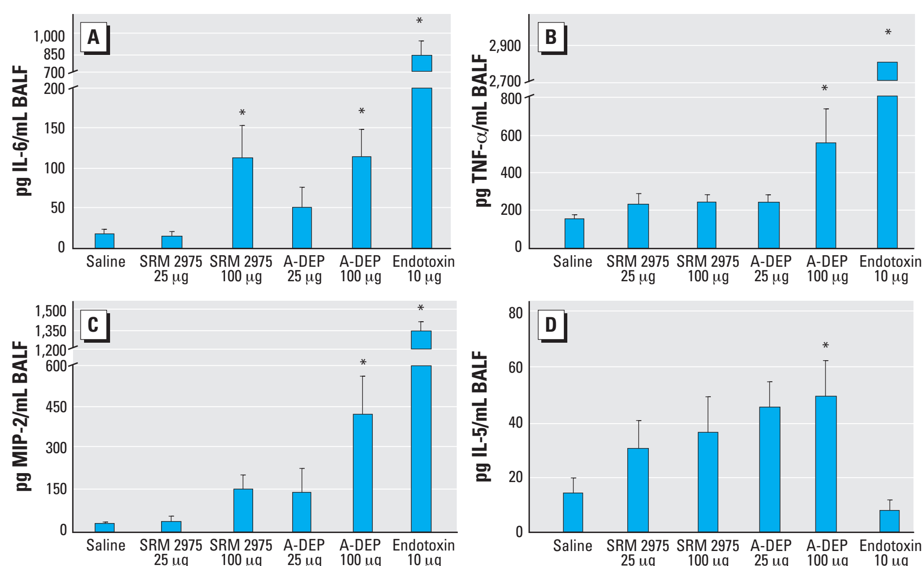

Figure 5. Cytokine concentrations in BALF after aspiration of saline, DEPs, or endotoxin in CD-1 mice: (*A*) IL-6, (*B*) TNF-α, (*C*) MIP-2, and (*D*) IL-5. Measurements were taken from BALF samples of exposed mice (n = 5/group). Data are shown as group mean ± SE at 4 hr after instillation.
*Significant difference ($p < 0.05$) from saline-treated mice.

Table 2. Summary of results.

Characteristics	A-DEP	SRM 2975
Physical/chemical		
Relative amounts of undercombusted fuel	↑	↓
Elemental carbon:organic carbon	9:50	60:5
Percent EOM	26.3	2.0
Relative amounts of organic carbon in EOM	10	1
Relative amounts of PAH	↑	↓
Fraction containing most of the mass	Hexane (55%)	Methanol (58%)
Pulmonary toxicity		
BALF inflammatory cell influx	Macrophages	Neutrophils
Significant increase in BALF NAG	No	Yes
Lung injury: vascular leakage of microalbumin	Yes	Yes
Proinflammatory cytokines increased in BALF	IL-6	IL-6, TNF-α, MIP-2
Significant increase in IL-5	No	Yes

Abbreviations: ↑, high; ↓, low.

components comprising DEPs have been discussed elsewhere in detail (Granum and Lovik 2002). Reports of the pro-adjuvant effects of DEPs and diesel exhaust in mouse models of pulmonary allergy have shown that these effects can be duplicated with the organic fractions of DEPs without the carbon core (Diaz-Sanchez 1997; Tsien et al. 1997). Specifically, PAH compounds extracted from DEPs have a demonstrated ability to reproduce both the proinflammatory (Kawasaki et al. 2001) and allergic adjuvant effects of DEPs (Diaz-Sanchez 1997). Likewise, carbon black has been shown to enhance allergen-specific IgE production in mice to similar levels seen with exposure to NIST SRM 1650 DEPs (Lovik et al. 1997). Other studies have demonstrated that the adjuvant effects of DEPs are greater with exposure to the insoluble carbonaceous component than to the organic extract (Heo et al. 2001). It appears that both the physical, particulate component and the organic constituents of DEPs play important roles in inflammation and the allergic adjuvant effects in the lung.

In the present study, we demonstrated that CD-1 mice exhibit robust inflammatory responses after aspiration of a known inflammogen, by using endotoxin as a positive control. However, endotoxin-exposed mice completely lacked an IL-5 response, despite significant lung injury, whereas exposure to DEPs increased production of IL-5. Studies have shown that mice exposed to diesel exhaust have increased IL-5 and total eosinophil counts in the lung when also challenged with antigen, yet exposure to diesel exhaust alone does not induce these responses (Miyabara et al. 1998; Takano et al. 1997). In contrast, an increasing trend in IL-5 mRNA production in bronchial tissue of healthy human volunteers exposed to diesel exhaust has been reported, suggesting that such exposures can stimulate T_H2 type responses in the absence of antigen (Salvi et al. 2000). Our data support the notion that DEPs alone may preferentially polarize the immune response to a T_H2 type with regard to the cytokine response but not in relation to eosinophil influx, which may require stimulation by antigen. Further work is needed to characterize the components of DEPs that do not depend on coexposure with antigen to possess proallergic activity.

Conclusions

Sample characterization of the DEP samples compared in this study established a basis for examining the association between biologic effects and physicochemical properties of each material and are summarized in Table 2. The disparate biologic activities of A-DEPs and SRM 2975 occurred as a consequence of their dissimilar chemical compositions that derived from the conditions under which they were generated and collected. These findings demonstrate the importance of characterizing a particular DEP sample before its use in biologic studies and also suggest the use of a broad range of biologic end points to provide a comparative basis for interpretation and hazard identification of diesel emissions exposures.

Recent attention has been placed on the possible health risks for children riding inside diesel-fueled school buses (Weir 2002), and there is new evidence that proximity to highways is associated with adverse birth outcomes (Wilhelm and Ritz 2003). Screening batteries similar to the one described here and to that proposed by Seagrave et al. (2002), involving pulmonary toxicology mutagenicity and serial fractionation of organic extracts of DEPs (DeMarini et al. 2004) should be applied to a wide variety of DEPs produced by various types of fuels, engines, and operating conditions so that the influence of these design and operating parameters can be determined. The notably different physical and chemical characteristics and biologic activities of the DEPs studied here illustrate the difficulty in comparing pulmonary toxicity studies, which have used mostly the A-DEPs, and mutagenicity studies, which have used mainly the NIST SRMs. Collectively, the results reported here and by DeMarini et al. (2004) argue for an integrated, multidisciplinary approach to DEP health effects research.

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