Inhalation Toxicology, 19(Suppl. 1):7–16, 2007

ISSN: 0895-8378 print / 1091-7691 online
DOI: 10.1080/08958370701490379

informa
healthcare

Comparative Toxicity of Size-Fractionated Airborne Particulate Matter Obtained from Different Cities in the United States

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Hundreds of epidemiological studies have shown that exposure to ambient particulate matter (PM) is associated with dose-dependent increases in morbidity and mortality. While early reports focused on PM less than 10 μm (PM_{10}), numerous studies have since shown that the effects can occur with PM stratified into ultrafine (UF), fine (FI), and coarse (CO) size modes despite the fact that these materials differ significantly in both evolution and chemistry. Furthermore the chemical makeup of these different size fractions can vary tremendously depending on location, meteorology, and source profile. For this reason, high-volume three-stage particle impactors with the capacity to collect UF, FI, and CO particles were deployed to four different locations in the United States (Seattle, WA; Salt Lake City, UT; Sterling Forest and South Bronx, NY), and weekly samples were collected

Received 29 January 2007; accepted 21 March 2007.

The authors gratefully acknowledge Steve Ferguson (Harvard School of Public Health) for technical advice on sampler operation, Martin Blaustein and Darlene Bowser (NYU) for sampler operation in New York, Drs. Tim Larson and Delbert Eatough for sampler operation in Seattle and Salt Lake City, respectively, Judy Richards, Jim Lehmann, Rick Jaskot (U.S. EPA) and Frank Weber (RTI) for technical assistance, and Drs. Aimen Farraj and Dan Costa for review of the manuscript.

This article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does the mention of trade names or commercial products constitute endorsement or recommendation for use.

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for 1 mo in each place. The particles were extracted, assayed for a standardized battery of chemical components, and instilled into mouse lungs (female BALB/*c*) at doses of 25 and 100 μg. Eighteen hours later animals were euthanized and parameters of injury and inflammation were monitored in the bronchoalveolar lavage fluid and plasma. Of the four locations, the South Bronx coarse fraction was the most potent sample in both pulmonary and systemic biomarkers, with a strong increase in lung inflammatory cells as well as elevated levels of creatine kinase in the plasma. These effects did not correlate with lipopolysaccharide (LPS) or total zinc or sulfate content, but were associated with total iron. Receptor source modeling on the $PM_{2.5}$ samples showed that the South Bronx sample was heavily influenced by emissions from coal fired power plants (31%) and mobile sources (22%). Further studies will assess how source profiles correlate with the observed effects for all locations and size fractions.

Epidemiological studies have demonstrated associations between acute exposure to elevated levels of ambient particulate matter (PM) and increased hospital admissions for cardiopulmonary disease (Milligan et al., 1998; Ostro et al., 1999, Dominici et al., 2006) and mortality (Dockery, 2001). Because these phenomena have been demonstrated in many cities and regions, no single component or group of chemicals has been implicated as being causal for these effects. Instead, a plethora of suspected agents including organic and elemental components of combustion emissions (Nel et al., 2001), transition metals (Pritchard et al., 1996; Costa & Dreher, 1997), secondary organic formation products (Doyle et al., 2004), and biogenic materials (Soukup & Becker, 2001; Becker et al., 2003) have all been proposed to contribute to health effects associated with PM.

Currently ambient PM is regulated according to daily mass averages for coarse (PM_{10}) and fine ($PM_{2.5}$) size fractions (U.S. EPA, 2006). Particles with a mass median aerodynamic diameter (MMAD) <10 μm (PM_{10}) have a daily standard of 150 $\mu g/m^3$, and mainly originate from accumulation of fine-mode particles and the physical dispersal of biogenic and crustal material (Wilson et al., 2002). Fine particles with an MMAD of <2.5 μm ($PM_{2.5}$) include products of combustion processes and are considered on a mass basis to be more toxic because they contain hazardous materials and more readily reach the respiratory region (Oberdorster et al., 1994). For these reasons, and the fact that $PM_{2.5}$ stay airborne longer (Wilson et al., 2002), the U.S. $PM_{2.5}$ standard of 35 $\mu g/m^3$ is more stringent than the PM_{10} standard.

While many of the original epidemiology studies used gravimetric measurements of particles less than 10 μm (PM_{10}), more contemporary reports focused on associations between mortality and morbidity and levels of $PM_{2.5}$. Additional studies have since demonstrated, however, that coarse particles with a lower and upper size cut ($PM_{2.5-10}$), and ultrafine particles (MMAD < 0.1 μm) may also contribute to the observed health effects (Lipsett et al., 2006; Peters et al., 1997). Unlike anthropogenic PM, which may become relatively homogeneous in a regional airshed, coarse PM is a heterogeneous mix of particles derived from abrasive processes such as tire and brake wear, sanding and grinding operations, release of biogenic components including pollen and vegetative fragments, and resuspension of crustal material (Wilson et al., 2002). Because of the larger size and mass, aerosolized coarse PM are less stable than $PM_{2.5}$ and are heavily influenced by air current strength and gravity. Thus exposures can be more variable in their nature and timing than the finer airborne PM, making it difficult to identify reproducible health effects.

Ultrafine particles (UF) are defined as having an MMAD of <0.1 μm and are regulated by the United States National Ambient Air Quality Standard (NAAQS) only insofar as they are gathered in both the $PM_{2.5}$ and PM_{10} fractions, although they contribute very little in the way of mass (Oberdorster & Utell, 2002). UF particles can be produced and emitted directly from combustion systems in the form of primary particles, or are generated via secondary processes in the atmosphere from gas-phase precursors. There is now evidence suggesting associations between ultrafine ambient particles exposure and cardiovascular health (Peters et al., 1997). One of the first laboratory studies to implicate ultrafine particles as a contributing factor for the health effects of PM reported that ultrafine titanium dioxide induced more inflammation on a mass basis, compared to fine particles composed of the same material (Ferin et al., 1992). This observation has been confirmed numerous times (Oberdorster et al., 1995) and extended to other materials such as carbon black (Li et al., 1999) and size-fractionated coal fly ash particles (Gilmour et al., 2004).

The objective of this study was to examine differences in chemical makeup of size-fractionated PM from various areas across the United States and to compare the relative toxicity of these samples in a murine pulmonary bioassay system. Following a 4-wk collection, period particle extraction, and chemical analysis, the samples were shared among several laboratories experienced with *in vitro* and *in vivo* toxicity testing of PM The results were first reported in 2005 in a workshop at the Society of Toxicology annual meeting in New Orleans, and later at the 2006 International Inhalation Symposium in Hannover, Germany. The study was designed as a complimentary approach to the "respiratory allergy and inflammation due to ambient particles" (RAIAP) project in Europe (Steerenberg et al., 2006). Particles were collected and extracted under the same experimental conditions and tested in several biological systems to determine the relative potency of size-fractionated material from locations with diverse source profiles.

METHODS AND MATERIALS

Animals

Pathogen-free BALB/*c* female mice, 8–10 wk old and weighing 20–22 g, were purchased from Charles River (Raleigh, NC). All of the animals were obtained in one shipment and were housed in AAALAC-approved animal facilities with high-efficiency particulate air filters and received access to food and water ad libitum. After a 2-wk rest period the entire study was conducted over 5 days. The study was conducted after approval

by the laboratory's Institutional Animal Care and Welfare Committee.

Particulate Matter Collection and Extraction

Coarse (MMAD 2.5–10 μm) , fine (MMAD $\leq$2.5 μm), and ultrafine (MMAD $\leq$0.1 μm) ambient PM samples were collected using a high-volume cascade impactor (Rupprecht & Patashnik, Co., Inc., Albany, NY) operating at a flow rate of 900 L/min. The 24-h samples were collected during 2003–2004 at four sites in the United States: Seattle (2/20/04–3/19/04), Salt Lake City (4/6/04-5/6/04), and two sites from New York taken simultaneously with replicate samplers (South Bronx and Sterling Forest [12/1/03–1/5/04]). The Seattle, WA, samples were collected from an urban site located in Snohomish County, one county north of Seattle. Salt Lake City, UT, samples were obtained at an urban site located south of Salt Lake City. South Bronx, NY, was an urban site sample located off the Cross Bronx Expressway, while the Sterling Forest, NY, material represented a rural site located about 50 miles northwest of Manhattan.

Coarse and fine particle fractions were collected onto polyurethane foam (PUF) substrates, while the ultrafine fraction was collected on a polypropylene fiber filter. Composites of the 4 weekly samples from each location were extracted in water under waterbath sonication, lyophilized to obtain a mass measurement, resuspended in water, and frozen at –80°C as described previously (Becker et al., 2003). Although filter weights for this series of runs were not obtained before and after extraction, subsequent testing has shown this technique to produce extraction efficiencies up to 80%. Particle suspensions were subsequently thawed, diluted in physiological saline to a final concentration of 2 mg/ml, and sonicated for 10–15 min prior to instillation. Blank filters were also subjected to the extraction procedure and tested for chemical makeup and toxicity following instillation in mice.

Chemical Analysis of PM and Receptor Modeling Analysis

Each PM dosing solution was vortexed and sonicated to ensure homogeneity, then aliquotted for chemical analysis of elemental, ionic, and carbon fraction content. Briefly, 1-mg aliquots were digested in 8% nitric acid at 60°C for 4 h, then assayed at the U.S. Environmental Protection Agency (EPA) for 30 elements by inductively coupled plasma-optical emission spectrometry (ICP-OES, using U.S. EPA method 200.7 rev4.4 protocol). A suspension of standard reference material (ambient urban atmospheric particulate NIST 1649a) was prepared in the same concentration as the study samples and used to track recovery of major elements, ions, and carbon content. The major elements iron, lead, sulfur (as sulfate), zinc, and total carbon were all within 15% of reference values with the exception of iron (40% recovery); the acid digest conditions used in this study were insufficient for total particulate dissolution. Aliquots of 150 μg were diluted in 5 ml of ultrapure water and analyzed by the Research Triangle Institute (RTI, Research Triangle Park, NC) for 11 ions using ion chromatography (IC, as described in McGee et al., 2003). Aliquots of 150 μg were analyzed at the U.S. EPA for elemental and organic carbon fraction content using a thermo-optical method based upon sequential pyrolytic vaporization and detection (McGee et al., 2003).

Sources impacting PM samples from each site were determined using the U.S. EPA chemical mass balance model (CMB8.2) as described by Duvall et al. (2007). Briefly, a combination of measured ambient fine PM speciated data, published source profiles, and profiles from the U.S. EPA SPECIATE Database (Speciate Version 4.0) were used as input for the CMB model. Source profiles for ultrafine PM were not available and the coarse PM profiles did not give reliable results and therefore ultrafine and coarse PM data were not run in the CMB model. A consistent set of fine PM source profiles was used across all sites. Elements selected as fitting species included elemental carbon, potassium, vanadium, copper, strontium, barium, and lead.

Involuntary Aspiration

Mice were anesthetized in a small plexiglas box using vaporized isofluorane. The mice were then suspended vertically by their front incisors on a small wire attached to a support. The tongue was extended with forceps and 50 μl saline (Sigma, St. Louis, MO) alone or saline containing either 25 or 100 μg of material was pipetted into the oro-pharynx. The nose of the mouse was then covered causing the liquid to be aspirated into the lungs. A separate group of mice received 2 μg of bacterial endotoxin (*Escherichia coli*, 011:B4, containing 10^6 U/mg material; Sigma) as a positive control to demonstrate maximal responsiveness to this well characterized inflammatory agent. Additional mice were instilled with blank extracts of the filters treated with the same sonication and extraction procedure as the ambient filters. These solutions yielded little in the way of chemical analysis, and no difference was seen in pulmonary inflammatory responses compared to saline-instilled control animals.

Bronchoalveolar Lavage (BAL)

After 18 h, 6 mice from each treatment group were euthanized with sodium pentobarbital and bled by cardiac puncture using a 1-ml syringe containing 25 μl sodium citrate to prevent coagulation. The trachea was then exposed, cannulated, and secured with suture thread. The thorax was opened and the left lung lobe clamped with a 1.5 inch building clamp. The right lung lobes were lavaged three times with a single volume of warmed Hanks balanced salt solution (HBSS; 35 ml/kg). This process was repeated another two times with fresh HBSS. The resulting lavage was centrifuged (717 $\times$ g, 10 minutes, 4°C) and an aliquot was stored at either 4°C (for biochemical measurement) or –70°C (for cytokine measurement). The pelleted cells were resuspended in 300 μl RPMI 1640 (Sigma, MO) containing 10% bovine serum albumin (BSA) (Invitrogen, CA). Total cell counts in the lavage fluid of each mouse were obtained with a coulter counter. Each sample (135 μl) was centrifuged in duplicate onto slides using a Cytospin (Shandon, PA) and subsequently stained with Diff-Quik solution (American Scientific, PA) for cell differentiation determination, with at least 200 cells counted from each slide.

Cytokine Measurements

Proinflammatory cytokines MIP-2, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) concentrations in BAL were measured by enzyme-linked immunosorbent assay (ELISA) using mouse quantikine kits purchased from R&D systems (Minneapolis, MN). The assays were carried out as per the manufacturer's instructions.

BAL and Plasma Biochemistry and CBC

All biochemical assays were modified to be assessed on a Konelab 30 clinical chemistry analyzer from Thermo Clinical Labsystems, Espoo, Finland. Activity for BAL lactate dehydrogenase (LDH) was determined using commercially available kits from Sigma, (MO). Total protein concentrations were determined with the Coomassie plus protein assay (Pierce Chemical, IL). For the plasma analyses, kits for creatine kinase (CK) amino-*S*-transferase (AST), lactate dehydrogenase (LDH), and controls were obtained from Thermo Electron, Melbourne, Australia. Kits and standards for alpha-2 macroglobulin (A2M), C-reactive protein (CRP), and fibrinogen (FIB) were obtained from DiaSorin, Inc., Stillwater, MN, except the standard for the CRP, which was a mouse reference serum standard with a known concentration (Kamiya Biomedical Company, Seattle, WA). Blood collected via cardiac puncture with 1-cm^3 syringes containing 25 μl of sodium citrate (3.2%) was aspirated into a Coulter AcT 10 hematology analyzer (Coulter Inc, Miami, FL), and hematology values including total white blood cells (WBC) and percent lymphocytes and red blood cells were determined. Additional markers included haemoglobin, hematocrit, mean cell volume, and platelets. Remaining blood was then further processed by centrifugation to plasma for subsequent biochemical analysis.

Endotoxin Measurements

Particles were diluted in endotoxin-free water at a concentration of 1 mg/ml and sonicated for 10 min. Endotoxin measurements were performed using the *Limulus* amebocyte lysate assay as per manufacturer's directions (Biowhitaker, Walkersville, MD).

Statistical Analysis

The data were analyzed in two steps using an SAS statistical package, version 8.02 (SAS, Inc., Cary NC). Step one employed a three-way analysis of variance (ANOVA) model. The three independent variables were sampling location, particle size, and PM dose (25 versus 100 μg). The single saline group was not included in this analysis because the significance of individual factors was of interest and such analysis was confounded by only one saline group. Subsequent to the overall analysis, pairwise comparisons were performed to assess the differences among the various location–size–concentration combinations. The second step used a one-way ANOVA model. The independent variable was formed by combining location, size, and concentration into a single group parameter. Each group value was compared to the lone saline value and the significance of the difference was evaluated. Significance was assigned to a statistic whose probability of occurrence was less than .05; however, statistics whose probability of occurrence was less than .01 were reported as such. No adjustment was made to the level of significance due to multiple comparisons.

RESULTS

Inflammatory Biomarkers in BAL Fluid

The effect of instillation of 25- and 100-μg size-fractionated PM from each of the four locations on BAL protein is shown in Figure 1 along with values of saline-instilled controls and animals instilled with 2 μg bacterial endotoxin (lipopolysaccharide, LPS). The Seattle CO fractions showed no dose-dependent effect, whereas results for the other locations were distinctly higher, with the Salt Lake City 100-μg dose being significantly different from the 25-μg sample and the saline control values. There were no other statistically significant differences in the other size fractions from any of the locations, and while the

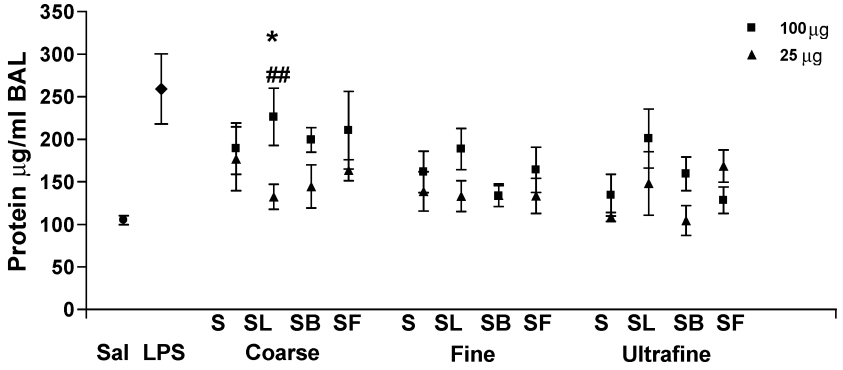

FIG. 1. Concentration of protein in BAL fluid of mice 18 h after instillation with 25 or 100 μg of coarse, fine, or ultrafine particles from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF). Data presented as mean $\pm$ SE ($n = 6$); asterisk denotes significant difference between 100- and 25-μg dose at $p < .05$. ## denotes significant differences from saline controls at $p < .01$.

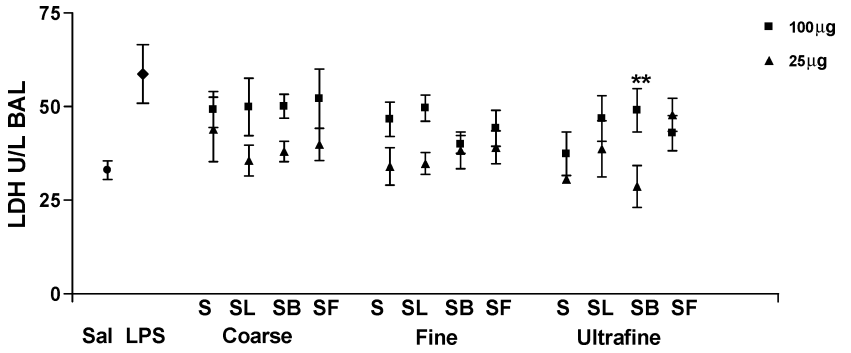

FIG. 2. Concentration of LDH in BAL fluid of mice 18 h after instillation with 25 or 100 μg of coarse, fine, or ultrafine particles from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF). Data presented as mean $\pm$ SE ($n = 6$); double asteriksk denotes significant difference between 100- and 25-μg dose at $p < .01$.

group means for the 100-μg dose were higher than for the 25-μg dose, the overall analysis did not show a statistically significant effect for size fraction, dose, or location. Responses from additional mice instilled with blank filter extracts were not significantly different from saline instilled animals. Instillation of LPS, on the other hand, showed a clear increase in BAL protein over saline controls. A somewhat similar pattern was seen with the BAL LDH samples, with evidence of a dose-response relationship in some but not all the samples (Figure 2). In this instance, the only significant difference was associated with the high dose UF fraction from the South Bronx.

Analysis of the PMN number in the BAL fluid showed a strong response in animals treated with the high concentration of CO samples from Seattle, Salt Lake City, and South Bronx but not Sterling Forest (Figure 3). The South Bronx data for the 100-μg dose were significantly different from those for the 25-μg dose and the saline controls as well as from those for the lowest value in the particle-treated groups. All the high doses of fine (FI) PM showed increased mean values over the 25-μg dose group, although these increases were not significant. The responses from the untrafine (UF) samples from Salt Lake City showed high variability because 1 animal out of 6 for each dose group had inflammatory responses 7 to 10 times the group mean. All the high-dose particles except SB and SF UF caused significantly greater PMN responses than the saline controls, and the effects were still evident for the low-dose coarse (CO) samples and Salt Lake City FI and UF samples. PMN responses for LPS were also significantly higher than the saline controls. No differences were seen in numbers of alveolar macrophages and lymphocytes between the various treatment groups.

The proinflammatory chemokine MIP-2 showed a very similar pattern of response to that of the PMNs, with the South Bronx CO sample being significantly different from the lower concentration as well as the other groups including saline (Figure 4). The Salt Lake City UF samples again showed high variability because of the same single animal in each group having a high response; however, no other differences were seen across sizes, doses, or locations. Again, LPS responses were vigorous and with relatively low variability, indicating that the mice were capable of responding in a consistent fashion.

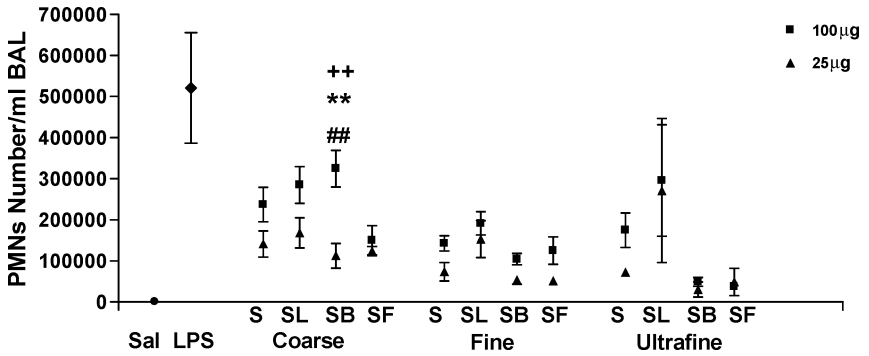

FIG. 3. Number of neutrophils in BAL fluid of mice 18 h after instillation with 25 or 100 μg of coarse, fine, or ultrafine particles from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF). Data presented as mean $\pm$ SE ($n = 6$); ** denotes difference between 100- and 25-μg dose at $p < .01$; ## denotes significant differences from saline controls at $p < .01$; ++ denotes significant differences from lowest value in particle data set at $p < .01$.

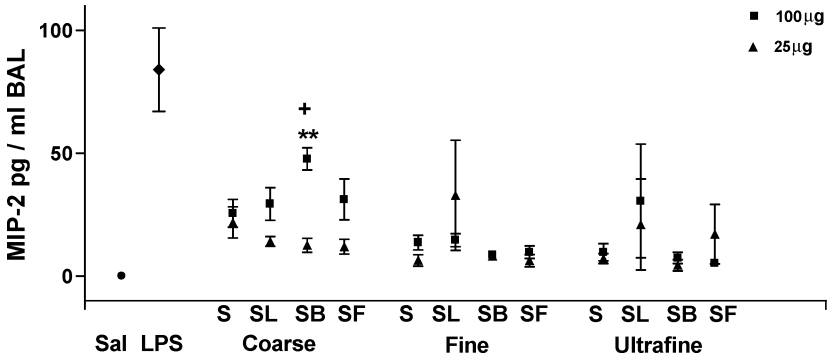

FIG. 4. Levels of MIP-2 in BAL fluid of mice 18 h after instillation with 25 or 100 μg of coarse, fine, or ultrafine particles from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF). Data presented as mean $\pm$ SE ($n = 6$); **denotes significant difference between 100- and 25-μg dose at $p < .01$; + denotes differences from lowest value in particle data set at $p < .05$.

Hematologic Markers in Whole Blood and Plasma

An extensive array of cellular and humoral markers of disease was monitored in whole blood and plasma. There were few significant effects by any of the particle treatments, although numerous changes were observed with the LPS-positive control instillation. We did, however, note that the South Bronx CO sample caused a significant increase in creatine kinase (Figure 5) and amino-*S*-transferase activity (data not shown) in the plasma, indicating at least some of these mice were experiencing systemic effects following instillation with this material.

Particle Characteristics and Receptor Source Analysis

The particle extracts were analyzed with several different chemical techniques, and a more complete source apportionment profiling analysis is being prepared for publication. For the purposes of this report, however, we were interested in identifying what features of the South Bronx CO sample were sufficiently different from the other locations which might explain the increased toxicity effects. LPS levels were highest in the Seattle and Salt Lake City CO fractions, and these locations also had higher LPS in the FI and UF fractions than the two New York sites (Figure 6). In contrast, total sulfate showed enrichment in the FI and UF fractions which was highest in the two New York locations (Figure 7). Zinc concentrations were highest in the South Bronx for each of the three size fractions with the highest level being measured in the UF samples (Figure 8). Total iron content was highest in the CO and FI fraction, with the most abundance being found in the CO South Bronx sample (Figure 9).

Results from the chemical mass balance model showed that the dominant PM sources in the South Bronx included secondary sulfate from coal-fired power plants (35% by mass) and mobiles sources (22% by mass), which incorporate gasoline, diesel, and brake wear. Sterling Forest was primarily dominated by secondary sulfate (48% by mass). Wood combustion contributions were higher in Salt Lake City and Seattle (34% and 39% by mass, respectively). Secondary sulfate from coal combustion was also

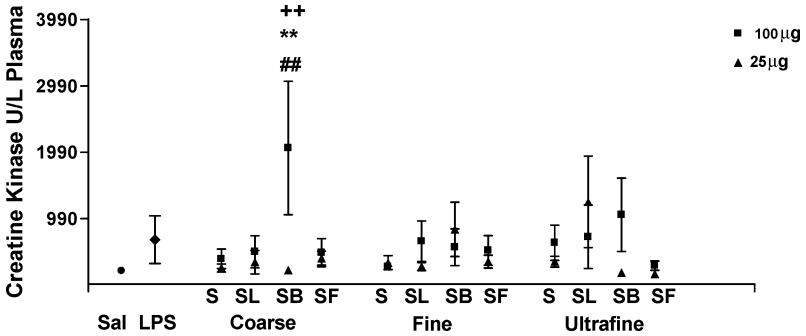

FIG. 5. Levels of creatine kinase in plasma of mice 18 h after instillation with 25 or 100 μg of coarse, fine, or ultrafine particles from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF). Data presented as mean $\pm$ SE ($n = 6$); double asterisk denotes significant difference between 100- and 25-μg dose at $p < .01$; ## denotes significant differences from saline controls at $p < .01$; ++ denotes significant differences from lowest value in particle data set at $p < .01$.

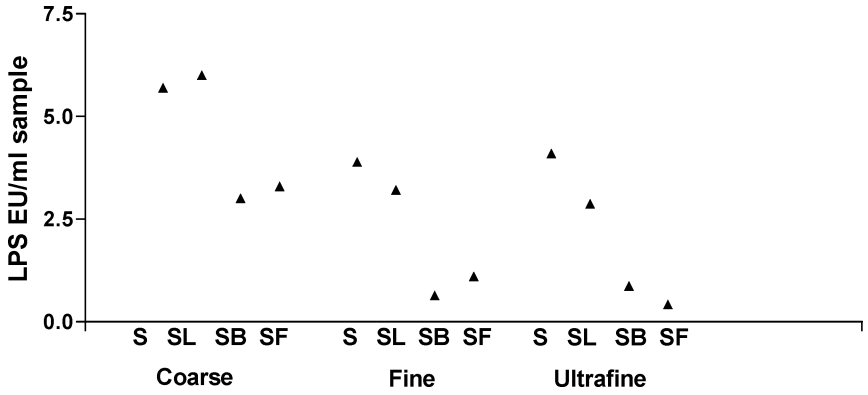

FIG. 6. Units of lipopolysaccharide (LPS) per milligram of coarse, fines or ultrafine particulate matter sample collected from Seattle (S), Salt Lake City (SL), South Bronx (SB)s and Sterling Forest (SF).

prevalent in Salt Lake City and Seattle (28% and 29% by mass, respectively). Other sources including residual oil combustion and soil dust also impacted the sampling sites, but had percent contributions to fine PM mass of less than 5%.

DISCUSSION

Despite compelling evidence reporting increased mortality and morbidity associated with PM exposure (Dockery, 2001; Dominici et al., 2006), the causal factor(s) and mechanisms responsible for these effects have not been identified. Multiple analyses in the United States and Europe have shown that the PM effect on mortality and morbidity is consistent across hundreds of different locations (Dominici et al., 2006, Medina et al., 2004), in the face of significant differences in the chemical make-up of airborne particulates. From these observations it could be argued that toxicity studies might not be able to shed light on the disparate components that may be causal to PM-induced health effects. Other investigators, however, using factor analysis in epidemiological (Laden et al., 2000), clinical (Huang et al., 2003), and animal toxicology studies (Kodavanti et al., 2005; Lippmann et al., 2005), have reported that certain sources emit chemicals that are inherently more toxic and cause greater effects than other sources such as crustal material. Although this assertion is intuitive, untangling the relative toxicity of chemicals in an ambient air mix has proven to be a complex problem. In addition, the question of particle size is a major factor to be considered since mass, which is regulated at present in the coarse and fine modes, differ greatly by size and particle number (Wilson & Suh, 1997).

In a similar fashion to previous experiments published by our laboratory (Dick et al., 2003; Gilmour et al., 2004) and others (Steerenberg et al., 2006; Seagrave et al., 2006) with both combustion and ambient air pollution samples, we conducted a comparative toxicity study using acute pulmonary PM instillations in mice and compared effects against known positive and negative controls. We found a tendency for a dose response in mice treated with 100 versus 25 μg of the coarse PM. However there was less of a discernible pattern with the fine and ultrafine fractions, or any pronounced effects produced by different locations. One possible exception was that the South Bronx CO particles produced the most apparent increases in pulmonary and systemic effects over saline controls and the other particles. Although an equivalent mass comparison approach was adopted for assessing relative toxicity, it should be noted that ultrafine

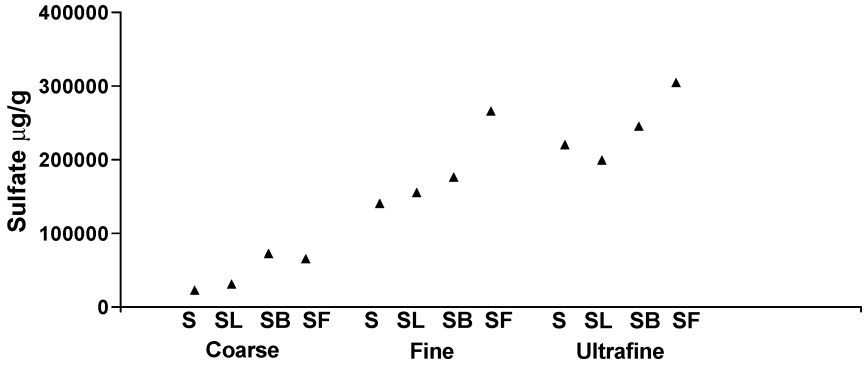

FIG. 7. Levels of sulfate (μg/g) in coarse, fine, or ultrafine particulate matter samples collected from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF).

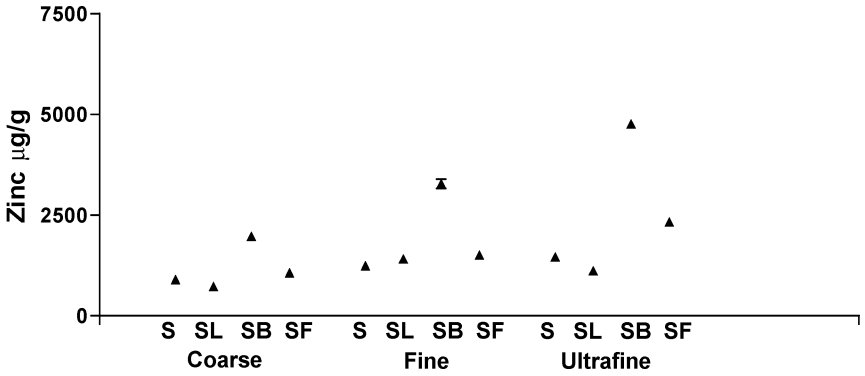

FIG. 8. Levels of zinc (μg/g) in coarse, fine, or ultrafine particulate matter sample collected from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF).

PM makes up a very small fraction (by mass) of total ambient particulates (Wilson et al., 2002), and therefore observed effects will likely be diminished in ambient PM because of the relative low abundance (by mass) of this subfraction. This is less of an issue for CO and FI fractions, which exist in approximately the same mass amounts (Wilson et al., 2002).

As in previously reported efforts from Europe (Steerenberg et al., 2006) and from another U.S. study comparing $PM_{2.5}$ from different locations (Seagrave et al., 2006), statistical analysis of this type of data set is challenging because of a combination of the large number of variables and appreciable error through either treatment application, animal responsiveness within a group, or assay accuracy. In addition, seasonality is an important factor as observed by Seagrave et al. (2006), who found no differences in toxicity of $PM_{2.5}$ between locations in the summer but reported increased toxicity from PM obtained from an urban industrialized area during the winter. Although we intend to employ more complex correlation methods to explore source-dependent associations in relation to seasonality and climatic conditions, we first chose to analyze and present the results in the most practical and simplest form to illustrate both the variability within groups, as well as the homogeneity of responses across treatments.

As expected, the LPS-treated animals exhibited large and significant increases in lung edema (protein), cell infiltration (polymorphonuclear leukocytes, PMNs), and cytokine release (macrophage inhibitory protein [MIP]-2), as well as many of the systemic markers, indicating that the mice were indeed capable of responding to a high degree in a consistent fashion. Similarly, the saline-instilled control animals showed very low responses, demonstrating that the anesthesia and involuntary aspiration of saline fluid were not factors. We did, however, observe substantial variation in responses within groups for many of the PM treated animals that indicated either a different dose received between animals and/or differences in the individual animals' response to the particular material. The largest variation within a group occurred with both concentrations of the Salt Lake City ultrafine treatment that each showed 1 animal out of 6 having PMN and MIP-2 responses up to 10 times higher than the mean cohort values. We have no specific explanation for this variability, but also had no good reason to omit the results from the data set. If these individual results were subtracted from the analysis, however, a general trend of coarse $>$ fine $\geq$ ultrafine would have been more apparent, and much of the coarse effects could be attributed at least in part to higher levels of LPS. Further studies with blocking agents to LPS (e.g., polymixin B) of in

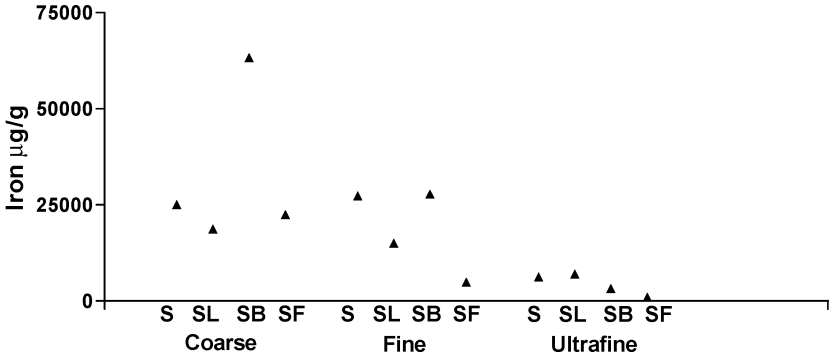

FIG. 9. Levels of iron (μg/g) in coarse, fine, or ultrafine particulate matter sample collected from Seattle (S), Salt Lake City (SL), South Bronx (SB), and Sterling Forest (SF).

LPS-resistant mice would demonstrate whether this is indeed the case.

In a manner similar to our previous investigations, the results show that PMN influx is a more sensitive marker of effect compared to biochemical and immune endpoints (Dick et al., 2003; Gilmour et al., 2004). Although the relative toxicity bioassay has proved to be a useful tool, a possible problem with the instillation approach is that extracted particles may agglomerate to different extents despite reasonable efforts to redisperse particles with sonication and repeated vortexing. These methods are therefore neither infallible nor exact, and serve as a strong reminder that instillations cannot replace inhalation exposures for true dosimetric assessment of toxicity.

Although we monitored a large number of pulmonary and systemic endpoints, we only highlighted the more apparent and statistically significant differences. Of particular interest was the increase in creatine kinase in the animals treated with the South Bronx CO sample. This enzyme, which catalyzes the conversion of creatine to phosphocreatine through the consumption of ATP, is a marker of muscle damage associated with myocarditis and myocardial infarction (Eriksson et al., 2006). This would suggest that in addition to having the highest pulmonary effects, the South Bronx coarse sample also had the greatest influence on systemic markers. Chemical analysis showed that the South Bronx CO material had the highest levels of iron which is associated with automobile brake wear (Sanders et al., 2003). Further speciation of the iron compounds in this sample and experiments to determine whether the effects can be reproduced with the individual compounds are ongoing.

In contrast to the variation in the biological responsiveness, the chemical analyses were very reproducible, with no more than 10% variation over the 4 replicate measurements. The results show that the size of the particle had a very profound effect on the overall chemical makeup in addition to clear differences between the locations. LPS levels in the PM samples were somewhat higher in the coarse particles, which have been reported to contain more biogenic material (Soukup & Becker, 2001; Becker et al., 2003). Interestingly, the LPS levels in the Seattle and Salt Lake City samples were higher at each size fraction level than the two New York sites, indicating a regional difference from west to east. Another clear pattern was that sulfate levels were higher in the FI and UF particles compared to the CO fractions. This is explained by the fact that elemental sulfur is a marker for fossil fuel (mainly coal) combustion aerosols that accumulate in these size fractions (Gilmour et al., 2004); however, it would appear that these factors did not drive the overall toxicity.

In conclusion, it seems either that this bioassay approach and form of analysis has limited sensitivity to detect size- and location-driven differences in biological effects, or that the generic and nondifferentiated responses reflect those of PM mass-based increases in health effects, with little influence of the noted differences in chemistry. However, if UF particles were assessed in terms of relative abundance by mass, it is possible that this size fraction would prove to be even less potent than the CO and FI PM. Further work, however, must prove that the collection and extraction processes do not affect chemistry and biological activity of these and other samples. With regard to assay sensitivity, other recent pulmonary bioassay experiments have likewise shown difficulty in demonstrating strong location-related differences (Seagrave et al., 2006; Steerenberg et al., 2006), despite the fact that the comparison of different combustion sources demonstrates quite obvious gradations in response (Gilmour et al., 2004; Singh et al., 2004). Thus, while this report supports the notion that ambient CO and FI PM as a complex mix can cause generic proinflammatory effects, further studies comparing the mass- and chemical-based toxicity of ambient and combustion PM from defined sources are warranted to quantify the relative potency of source materials within ambient PM.

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