Systemic Interactions between Inhaled Ultrafine Particles and Endotoxin

ALISON C. P. ELDER1*, ROBERT GELEIN1, MITRA AZADNIV2, MARK FRAMPTON2, JACK FINKELSTEIN3 and GÜNTER OBERDÖRSTER1

1Department of Environmental Medicine, 2Department of Pulmonary and Critical Care Medicine, and 3Department of Pediatrics, University of Rochester, Rochester, NY, USA

INTRODUCTION

Epidemiological studies consistently demonstrate an association between morbidity and mortality in susceptible populations and low ambient particle concentrations. The most susceptible are individuals >65 years old with cardiopulmonary disease (Schwartz and Dockery, 1992; Burnett et al., 1995; Schwartz and Morris, 1995). However, the lack of both specificity and plausible mechanisms have brought into question the validity of these associations. Although epidemiological studies focus mainly on the mass concentration of fine- and coarse-mode particles, the size distribution of ambient air particles is trimodal and includes ultrafines, which have the highest number concentration by a factor of ~60000 compared with the largest particles (Pekkanen et al., 1997). Ultrafine particles also have high predicted fractional deposition in the alveolar regions of the lung (International Committee on Radiological Protection, 1994). Furthermore, data from our laboratory suggests that ultrafine as compared to fine-mode inert surrogate particles (TiO2) have a greater potency per mass for inducing lung inflammation.

One factor apparently involved in the susceptibility of the elderly to particles is a compromised cardiopulmonary system. Lipopolysaccharide (LPS) is a component of Gram-negative bacterial cell walls that is both ubiquitously present in the environment and released in vivo during infection. It induces the...
expression of numerous molecules involved in inflammation, oxidative stress, the acute phase response and coagulation (Albelda, 1991; Sonesson et al., 1994; Hara et al., 1997; Jourdain et al., 1997; Wong and Wispé, 1997; Shingub et al., 1998).

There are several changes that occur during the normal aging process that can impact the cardio-pulmonary systems. Our own data, combined with what has been reported by other investigators, suggest that, as they age, inflammatory cells from the blood and lungs lose their ability to maintain an appropriate oxidant/antioxidant balance. In addition, the alveolar wall becomes less distensible, alveolar-capillary integrity, surface area and compliance are decreased, and lung injury becomes exaggerated (Evans et al., 1977; Cabral-Anderson et al., 1977; Martin et al., 1977; Braga et al., 1996; reviewed in Ricci et al., 1997). These factors may help to explain the susceptibility of the elderly population to the effects of airborne particulate matter.

Given the likelihood of exposure to ambient ultrafine particles and their distinct biology, we hypothesized that they could be causally related to the adverse effects described by epidemiologists. Part of our hypothesis is that ultrafine particles will be more inflammatory in the elderly because of their potential for exaggerated response. Lastly, we proposed that systemic LPS will create a compromised cardiovascular system, which could exacerbate the lung and systemic responses to inhaled particles. We administered LPS systemically in rats that were already in a state of greater fragility due to their age (23 months), and measured end points related to lung and blood inflammatory cell oxidant stress, the acute phase response, lung inflammation and coagulability.

**MATERIALS AND METHODS**

Male Fischer 344 rats (Harlan; Indianapolis, IN) were aged in-house to 23 months. Animals were housed individually in wire-bottom cages and given free access to food and water in a room with temperature and humidity control and a 12 h light–dark cycle. Groups of five rats were distributed among four treatment groups: (i) injected saline, inhaled air; (ii) injected saline, inhaled particles; (iii) injected LPS, inhaled air; and (iv) injected LPS, inhaled particles.

LPS was purchased from Sigma Chemical Co. (L-9143, 5 × 10^6 units/ml; St Louis, MO) and diluted in 0.9% sterile, pyrogen-free saline prior to i.p. injection. Rats were injected with either LPS (2 mg/kg body wt) or saline alone immediately prior to the beginning of the exposures to particles or filtered air in compartmentalized, horizontal flow whole-body chambers. Ultrafine carbon particles (175 µg/m^3; 6 h; count median diameter = 35 nm) were generated according to methods described previously (Elder et al., 2000).

Rats were euthanized 24 h after exposure (pentobarbital overdose); citrated blood was collected, and the heart and lungs were removed en bloc. A small amount of blood was also collected to determine the number and percentage of peripheral blood neutrophils. Lungs were extensively lavaged as described previously (Elder et al., 2000). Bronchoalveolar lavage (BAL) cell viability (trypan blue exclusion), number, differential analysis (Diff-Quik®; Baxter Scientific, Edison, NY) and chemiluminescence were determined. Total protein concentration and lactate dehydrogenase and β-glucuronidase activities were measured in the BAL supernate using commercially available kits (Pierce Chemical Co., Rockford, IL; Sigma).

Respiratory burst activity was measured in lavage cell suspensions under both resting and phorbol myristate acetate (PMA)-stimulated conditions according to previously described methods (Elder et al., 2000). Intracellular oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFDF, 20 µM; Molecular Probes, Inc., Eugene, OR) was measured in BAL and blood cells. Cells (10^6) were incubated with DCFDF with or without PMA (50 µM; Sigma) in Krebs–Ringer–phosphate buffer with Ca^{2+}, Mg^{2+} and glucose (pH 7.3); all oxidative reactions were stopped by fixing the cells in 2% paraformaldehyde. BAL and blood cells were stained with phycoerythrin-labeled anti-CD45 to aid in cell separation. Red blood cells were lysed with buffer purchased from Becton-Dickinson (San Jose, CA). Samples were analyzed for forward and side scattering properties as well as green and red fluorescence intensity using a FACScan flow cytometer (Becton-Dickinson). The green (DCFDF) fluorescence intensity of each sample was determined from a curve constructed with standardized beads (Flow Cytometry Standards Corp., San Juan, Puerto Rico).

Spun hematocrit and viscosity (Brookfield cone and plate viscometer; Stoughton, MA) were measured in whole blood and plasma (viscosity only). In addition, the concentrations of fibrinogen (heat precipitation method using standard from Sigma), TAT complexes (Dade-Behring, Newark, DE) and interleukin-6 (IL-6; BioSource International, Inc., Camarillo, CA) in plasma were assessed.

The results were analyzed for statistically significant effects by two-way analysis of variance (ANOVA) using SigmaStat. The factors were the presence or absence of LPS and particles. Data were appropriately transformed if an analysis of residuals suggested deviations from the assumptions of normality and equal variance. Differences between groups were further analyzed using Tukey multiple comparisons. All comparisons were considered statistically significant when P < 0.05.
RESULTS

Neither inhaled ultrafine carbon particles nor i.p. LPS caused a significant increase in BAL fluid neutrophils. Likewise, PMA-stimulated chemiluminescence of BAL cells, an indicator of extracellular reactive oxygen species (ROS) release, was not altered by particles or LPS. When individual lavage inflammatory cells were examined via flow cytometry, both ultrafine particles and systemic LPS had independent and significant effects on intracellular DCFD oxidation in the presence of PMA (an indication of ROS activity). These effects were in the opposite direction, however: particles decreased DCFD oxidation in alveolar macrophages (AMs) and LPS increased this oxidation. There was a significant interaction between particles and LPS such that the combined effect was to suppress intracellular oxidation relative to controls.

Both i.p. LPS and inhaled ultrafine carbon particles significantly affected the number of circulating polymorphonuclear leukocytes (PMNs) and, again, these effects were in the opposite direction: LPS caused an increase in blood PMNs and ultrafine particles decreased their numbers. The result of the significant interaction between the two components was to increase blood PMN number. The hematocrit was unaltered at treatment. Although their numbers were increased by i.p. LPS, the PMA-stimulated intracellular DCFD oxidation by blood PMNs was significantly decreased. Inhaled particles also had a significant impact on DCFD oxidation by blood PMNs, but the effect was an increase. Plasma fibrinogen was only affected by LPS, which significantly elevated its concentration; in addition, ultrafine particles and LPS interacted to increase even more its plasma concentration. The plasma concentration of IL-6 was only significantly altered (increased) by i.p. LPS. Whole blood and plasma viscosity were not affected by treatment and neither was the concentration of TAT complexes in plasma. All results are summarized in Table 1.

DISCUSSION AND CONCLUSIONS

In summary, the results from these studies demonstrate that inhaled ultrafine carbonaceous particles can independently decrease intracellular oxidation in lavage AMs and the number of circulating PMNs and increase intracellular oxidative reactions in blood PMNs. Systemically administered LPS independently increased DCFD oxidation in lavage AMs, the number of blood PMNs, and plasma fibrinogen and IL-6 concentrations, while it decreased DCFD oxidation in circulating blood PMNs. In addition, inhaled ultrafine particles and LPS were found to have significant interactions with regard to BAL cell intracellular oxidation, the number of circulating PMNs and the concentration of plasma fibrinogen.

Despite indications that the acute phase response had been activated (alterations in plasma fibrinogen, IL-6), the viscosity of the blood was not altered and neither was there any indication of on-going coagulation (TAT complex increases). Although there was evidence for particle–LPS interactions, these responses were largely driven by LPS. However, changes in end points that indicated oxidative stress in the lung and extrapulmonary compartments were driven by both the ultrafine particles and LPS.

These results suggest that inhaled ultrafine carbonaceous particles can independently induce systemic responses and that interactions between these particles and systemically administered LPS occur in both the pulmonary and extrapulmonary compartments.

The extent to which these effects are particle size- and composition-specific will be examined in the future, specifically in animal models of cardio-

---

**Table 1.** Exposure of old F-344 rats to i.p. LPS ± inhaled ultrafine carbon particles: summary of results

<table>
<thead>
<tr>
<th>End points</th>
<th>Carbon</th>
<th>LPS</th>
<th>Carbon + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% PMNs</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BAL cell DCFD oxidation (AMs) (stimulated)</td>
<td>P &lt; 0.001 †</td>
<td>P &lt; 0.001 †</td>
<td>P &lt; 0.001 †</td>
</tr>
<tr>
<td>BAL cell chemiluminescence (stimulated)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Blood parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of PMNs</td>
<td>P = 0.02 †</td>
<td>P &lt; 0.001 †</td>
<td>P = 0.05 †</td>
</tr>
<tr>
<td>Blood cell DCFD oxidation (PMNs) (stimulated)</td>
<td>P = 0.002 †</td>
<td>P &lt; 0.001 †</td>
<td>–</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Whole blood/plasma viscosity</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plasma fibrinogen</td>
<td>–</td>
<td>P &lt; 0.001 †</td>
<td>P = 0.008 †</td>
</tr>
<tr>
<td>Plasma TAT complexes</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plasma IL-6 concentration</td>
<td>–</td>
<td>P = 0.008 †</td>
<td>–</td>
</tr>
</tbody>
</table>

Results are from a two-way ANOVA, expressed as relative to controls. P values are shown only for significant main effects and interactions. A line indicates that no significant effect was found.
pulmonary disease, to aid in the prediction of outcome in humans exposed to ambient air particulate matter.

Acknowledgements—This work was supported by a STAR grant (R-82804601-0) to A.C.P.E. and a PM Center grant (R-827354) from the US Environmental Protection Agency.

REFERENCES