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Inhalation Toxicology

International Forum for Respiratory Research

ISSN: 0895-8378 (Print) 1091-7691 (Online) Journal homepage: https://www.tandfonline.com/loi/iiht20

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To cite this article: Lung Chi Chen, Chun Yi Wu, Qing Shan Qu & Richard B. Schlesinger (1995) Number Concentration and Mass Concentration as Determinants of Biological Response to Inhaled Irritant Particles, Inhalation Toxicology, 7:5, 577-588, DOI: 10.3109/08958379509014466

To link to this article: https://doi.org/10.3109/08958379509014466

NUMBER CONCENTRATION AND MASS CONCENTRATION AS DETERMINANTS OF BIOLOGICAL RESPONSE TO INHALED IRRITANT PARTICLES

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Particulate pollutants are mixtures of a variety of chemical species. Sulfuric acid aerosol is a highly irritating component of particulate matter less than 10 μm (PM-10) that can produce adverse health effects at current peak ambient concentrations in the United States. We hypothesized that, in addition to the mass concentration of sulfuric acid, the number of sulfuric acid droplets was also an important factor affecting lung injury. To test this hypothesis, guinea pigs were exposed for 3 h to either filtered air; inert carbon particles at 10^8 particles/ml; sulfuric acid at 350 μg SO_4^{2-}/m^3 layered on 10^8, 10^7, or 10^6 carbon particles/ml; sulfuric acid at 50, 100, 200, and 300 μg SO_4^{2-}/m^3 layered on 10^8 carbon particles/ml. Alterations in phagocytic capacity (PC), intracellular pH (pH_i), and intracellular free calcium concentration ($[Ca^{2+}]_i$) of harvested macrophages were used as indices of irritant potency. At a fixed number concentration of particles (10^8 particles/ml), there was a sulfuric acid concentration-dependent decrease in PC, pH_i, and $[Ca^{2+}]_i$. Furthermore, at a fixed mass concentration (350 μg SO_4^{2-}/m^3), sulfuric acid layered carbon particles at 10^8 particles/ml but not at other number concentrations decreased pH_i of macrophages. The number concentration of sulfuric acid layered carbon particles did not affect PC or $[Ca^{2+}]_i$. These results suggest that there is a threshold for both number concentration and mass concentration for the aerosols to produce a biological response, and that epidemiologic studies should consider other aerosol characteristics in addition to mass when attempting to relate health endpoints to ambient pollutant exposures.

Sulfuric acid (H_2SO_4) is an important component of the submicrometer particle mode of the atmosphere. It may exist in two forms: dissolved in aqueous droplets, and as a surface layer on solid particles. The first type of aerosol has been shown to produce alterations in a number of aspects of pulmonary physiology, biochemistry, and structure, and the alterations seem to be proportional to both exposure concentration and exposure duration [U.S. Environmental Protection Agency (EPA), 1989]. The second type of aerosol is formed by adsorption of H_2SO_4 onto particles with sufficient surface area, such as typical ambient carbonaceous, metal, or fly ash particles. These particles are very potent in altering various markers of exposure. For example, reductions in lung volumes and diffusion capacity, alterations in biochemical markers, and changes in lung tissue morphology have been reported in guinea pigs after exposure to ultrafine zinc oxide having a surface layer of sulfuric acid (Amdur & Chen, 1989). The induced respiratory

Supported by EPRI (RP2155-2), EPA (R819342), CIAR (92-04), and NIEHS (ES00260).

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Inhalation Toxicology, 7:577–588, 1995

0895–8378/95 $10.00 + .00

dysfunction is much greater than that due to H_2SO_4 aerosols in pure droplet form having similar size and concentration. A possible reason for this differential response is that the number concentration of particles in the exposure atmospheres is different, resulting in different numbers of deposition sites and local foci of reduced pH. At an equal sulfate concentration, sulfuric acid exists on many more particles when it is layered than when it is dissolved in aqueous droplets. Therefore, it is possible that the greater the number of particles containing sulfuric acid (as is the case with layered particles), the greater will be the number of cells affected after these particles deposit in the lung, and the more severe will be the overall biological response.

Since indirect evidence suggests that both total mass concentration of sulfuric acid and total number of acid particles in the exposure atmosphere may be critical parameters in determining the biological response, it was necessary to examine, in detail, the relationship between adverse health effects and these two exposure parameters. This article describes a system to produce a controlled aerosol of carbon having a thin layer of sulfuric acid on its particles' surfaces. These aerosols were used to investigate the roles of mass concentration and number concentration in the response to acid sulfates. When using this system, we can produce sulfuric acid aerosols with varying total sulfur mass concentration at a fixed particle size and number concentration, or with varying number concentration at a fixed total sulfur concentration and fixed size. Moreover, the generated aerosols simulate those found in ambient air, where sulfuric acid may be layered on cores of carbon particles. The carbon particles generated in this study are toxicologically important for a number of reasons. First, because of their submicrometer size (approximately 0.05–0.1 μm), they are difficult to remove from combustion effluents and, once emitted into the atmosphere, they have a long residence time. Second, these particles penetrate deep into the lung. Third, they are commonly enriched in trace metals, which react with SO_2 to form a layer of H_2SO_4 on their surface. Fourth, the coarse, spiculated surface of the particles, as visualized by electron microscopy, enlarges the surface area for acid retention.

EXPERIMENTAL DESIGN

Aerosol Generation System

Carbon aerosol having a thin layer of sulfuric acid on its surface was used in this study. The generation and characterization system is described in detail elsewhere (Chen et al., 1995). Briefly, a high-temperature silicon carbide furnace was used to thermally decompose acetylene in argon to produce ultrafine carbon particles. The carbon particles were then diluted and mixed with sulfuric acid droplets (produced by a Collison nebulizer) in a quartz tube maintained at 500°C to form a carbon aerosol with a thin

layer of sulfuric acid. The particle number concentration was controlled by varying the dilution ratio of the first ejector dilutor. The amount of sulfuric acid on the particles was controlled by the bypass flow rate of the nebulizer. Aerosols were collected on membrane filters (Millipore type FG, 0.2 μm pore size) and the sulfate concentrations were determined using ion chromatography (Dionex model 4000i). The number concentration was determined using a condensation particle counter (TSI model 3020, TSI Inc., St. Paul, MN).

Animals

Male Hartley guinea pigs (virus antibody free) weighing 275–300 g (Charles River Breeding Laboratories, Kingston, NY) were housed 2 per cage in a temperature- and humidity-controlled room (23°C, 30–50% relative humidity) and allowed free access to food and water.

Animal Exposures

A Canon 52-port nose-only exposure apparatus (Lab Products, Inc.) was employed in this study to prevent neutralization of sulfuric acid by ammonia, which can occur in whole-body exposures. The effect of number concentration on pulmonary response was investigated by exposing guinea pigs (n = 6 per group) to sulfuric acid layered carbon particles at 10^8, 10^7, or 10^6 particles/ml (total sulfuric acid mass concentration was maintained at 350 μg SO_4^{2-}/m^3) for 3 h. To investigate the effect of sulfuric acid mass concentration on pulmonary response, the animals were exposed to sulfuric acid layered carbon particles at 50, 100, 200, and 300 $\mu g/m^3$ (number concentration for all these exposure was fixed at 10^8 particles/ml) for 3 h. Separate groups of animals were exposed to filtered air or carbon particles alone (10^8 particles/ml) for comparison.

Bronchoalveolar Lavage

Animals were anesthetized by injection (im) of ketamine hydrochloride (Ketaset, Bristol Lab. Syracuse, NY) and xylazine (Rompun, Haver Lockhart, Shawnee, KS), 100 mg/kg and 15 mg/kg, respectively, 24 h after the end of the exposure. The animals were exsanguinated, the diaphragm was cut, and the lungs were lavaged in situ 4 times via a tracheal cannula with 7-ml aliquots of phosphate-buffered saline (37°C). The first two lavages were combined, an aliquot was removed for total and differential cell counts, and the remainder was immediately centrifuged (10 min at 400 × g). The supernatant was removed and assayed for total protein (BioRad, Richmond, CA) and lactate dehydrogenase (LDH) with commercially available kits (Sigma Chemical, St. Louis, MO). Protein was assayed in frozen samples, while LDH was measured in lavage samples held at room temperature due to the sensitivity of this enzyme to freezing. Total cell counts were determined by manual counting on a hemocytometer. Cell viability was determined by the trypan blue exclusion technique. Differential counts were made on Giemsa-stained

cells adhered to glass slides by cytocentrifugation. Data were expressed as the number of cells per milliliter and the number of each cell type recovered in the lavage.

The remaining two lavages were combined and centrifuged (10 min at 400 × g). The cell pellet was combined with the cells from the first two lavages, resuspended in RPMI 1640 (GIBCO, Grand Island, NY), and divided for the following measurements.

Assessment of Phagocytic Activity

The phagocytic activity of macrophages was evaluated using a suspension assay (Chen et al., 1992). Sterile polystyrene latex microspheres (3 μm, Duke Scientific, Palo Alto, CA) were suspended in RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (FBS), and adjusted to 5.0×10^8 particles/ml. A volume of the original macrophage suspension was centrifuged (400 × g, 10 min), resuspended in RPMI 1640, and adjusted to 5×10^5 viable cells/ml. One-milliliter aliquots of the cell suspension were added to sterile polypropylene culture tubes and preincubated for 15 min at 37°C in a shaking water bath. The phagocytosis assay was initiated by adding 50 μl of the opsonized latex particle suspension. Tubes containing the cells and particles were incubated for 60 min, after which time they were removed from the bath and cooled to 4°C. The particle–cell suspension was aspirated, smears were prepared on glass slides by air-drying, and the cells were fixed with phosphate-buffered, 2% glutaraldehyde (pH 7.2). The fixed smears were stained with Diff-Quik and placed in methylene chloride for 30 s to remove noningested particles. Three slides were prepared from each animal. On each slide, 100 macrophages were examined microscopically to determine the phagocytic capacity (PC). This measurement is an indication of the extent of phagocytosis, and was quantitated as the percentage of actively phagocytizing macrophages that had ingested four or more latex particles.

Measurement of Intracellular pH

Changes in intracellular pH (pH_i) were monitored by use of the pH-sensitive dye probe Carboxy-SNARF-1, AM acetate (SNARF-AM), according to the procedure described by Gillies and Martinez-Zaguilan (1991). Briefly, the pulmonary macrophage (PMϕ) suspensions in HEPES buffer (135 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 2 m*M* KH_2PO_4, 5 m*M* glucose, and 10 m*M* HEPES) were loaded with 10 μ*M* SNARF at 37°C for 40 min. After incubation, extracellular SNARF was removed by washing the cells three times with HEPES buffer. The cell pellets were finally resuspended in HEPES buffer at 5×10^6 cells/ml as the stock cell suspension for later use.

For the measurements of pH_i levels of PMϕ, aliquots of the cell suspension in HEPES (1.25×10^6 total cells) were centrifuged at 740 × g for 2 min, resuspended in 3.5 ml HEPES buffer in a cuvette, and held in the fluorometer at 37°C with gentle stirring throughout the experiment. Fluorescence intensities were recorded on a Spex Fluorolog (Edison, NJ) with an excitation

wavelength setting of 534 nm and emission wavelengths of 630 and 592 nm, with excitation and emission slit widths of 4.5 and 9.0 nm, respectively. The ratio of emitted intensities at the two different wavelengths ($R_{630/592}$) was used to calculate pH_i from a calibration curve.

The pH_i was calibrated using the K^+-nigericin technique (Thomas et al., 1979). Briefly, dye-loaded cells were resuspended in high-potassium solution (10 m*M* NaCl, 130 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 2 m*M* KH_2PO_4, 5 m*M* glucose, and 10 m*M* HEPES) and the pH of the medium was adjusted between 6.5 and 7.5 with KOH or HCl. An aliquot of the ionophore nigericin (1 μg/ml) was added to equilibrate pH_i to the pH of the medium (pH_0). The calibration curve was constructed by plotting pH_0 versus the corresponding emission ratio ($R_{630/592}$).

Measurement of Cytosolic Calcium

Changes in intracellular calcium ($[Ca^{2+}]_i$) were monitored by using a long-wavelength calcium fluorescent probe, Fluo-3/AM (Minta et al., 1989; Kao et al., 1989). Briefly, PMϕ were loaded with 5 μ*M* Fluo-3/AM (at cell density of 5×10^6 cells/ml) in HEPES buffer at 37°C for 40 min. After incubation, extracellular dye was removed by washing the cells three times with HEPES buffer. The cell pellets were finally resuspended in the same buffer at 5×10^6 cells/ml as stock cell suspension for later measurements.

For the measurements of $[Ca^{2+}]_i$ levels of macrophages in HEPES buffer, aliquots of the stock cell suspension (0.25 ml) were centrifuged at 740 × g for 2 min, resuspended in 3.5 ml of the same buffer in a cuvette, and held in the fluorometer at 37°C with gentle stirring throughout the experiment. Fluorescence intensity (F) was first recorded for 40 s with an emission wavelength setting of 525 nm and an excitation wavelength of 506 nm and excitation and emission slit widths of 4.5 and 9.0 nm, respectively. The maximum fluorescence intensity (F_{max}) was then measured following the addition of 40 μ*M* digitonin, and the minimum fluorescence intensity (F_{min}) was recorded after addition of 6 m*M* ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA).

The $[Ca^{2+}]_i$ levels were calculated according to the following equation:

$$[Ca^{2+}]_i = (400 \text{ nM}) (F - F_{min})/(F_{max} - F)$$

All chemicals used in this study were certified reagent grade. Standards used to calibrate the instruments were National Bureau of Standards (NBS) traceable. Distilled, deionized water with a resistance of 18 MΩ was used throughout the experiments. The fluorescent probes, SNARF-AM and Fluo-3/AM, were obtained from Molecular Probes (Eugene, OR) and dissolved in dimethyl sulfoxide (DMSO). Nigericin was purchased from the same source and dissolved in methanol for pH_i calibration.

Statistical Analysis

All data are expressed as the mean percent (±SEM) of filtered air control values. Statistical comparisons of PMϕ phagocytosis function and ion con-

centration data were made using a one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc test to identify any significant differences ($p < .05$) among group means.

RESULTS

While none of the exposure groups showed alterations in the biochemical parameters of lavage fluid, there were alterations in pH_i of PMϕs after exposures to sulfuric acid layered carbon particles. Carbon particles alone (10^8 particles/ml) produced a decrease in pH_i (0.052 pH unit). At a fixed number concentration of coated particles (10^8 particles/ml), a sulfuric acid concentration-dependent decrease in pH_i (for concentrations of acid above 100 μg SO_4^{2-}/m^3) was observed. For every 100 $\mu g/m^3$ increase in sulfate concentration (for concentrations of acid above 100 μg SO_4^{2-}/m^3), pH_i was decreased by 0.05 unit ($p = .0006$). The results are shown in Figure 1A. When animals were exposed to a fixed sulfate concentration (300 $\mu g/m^3$) of sulfuric acid layered carbon particles, there was a threshold for number concentration (10^7/ml) below which no decrease in pH_i was found.

As shown in Figure 2, carbon particles alone produced a 30.7% decrease in PC of PMϕs. However, sulfuric acid layered on the same number of carbon particles (10^8/ml) stimulated phagocytosis at low sulfate concentrations (63 and 100 $\mu g/m^3$), while sulfuric acid had no effect at higher concentrations (200 and 300 $\mu g/m^3$). Linear regression analysis showed that for every 100 $\mu g/m^3$ increase in sulfate concentration, there was an 8.6% decrease in PC of PMϕs ($p = .0005$). In contrast, at a fixed sulfate concentration (300 $\mu g/m^3$), different number concentrations did not produce alterations in phagocytosis.

As shown in Figure 3, at a fixed number concentration (10^8/ml) of acid-coated particles, there was no change in $[Ca^{2+}]_i$ after exposure to either carbon particles alone or sulfuric acid layered carbon particles at any sulfate concentrations used in this study. However, linear regression analysis showed that for every 100 $\mu g/m^3$ increase in sulfate concentration, there was a 9.6 nM $[Ca^{2+}]_i$ decrease in PMϕs. At a fixed sulfate concentration, different number concentrations of sulfuric acid particles had no effect on $[Ca^{2+}]_i$ of PMϕs.

DISCUSSION

By using an evaporation/condensation process, we produced a carbon aerosol with varying total sulfur concentrations, while maintaining the same particle size and number concentration. Using the same generation system, we also produced an aerosol with a fixed total sulfur concentration and fixed particle size while varying the number concentration. Because the particle size remains the same regardless of the number or mass (sulfur) concentrations, and because this type of aerosol is minimally hygroscopic, we did not create the problems associated with pure sulfuric acid droplets,

that is, differential growth. Furthermore, the sulfuric acid was layered on core carbon particles, and its characteristics resembled those found in the ambient air.

Alterations in macrophage phagocytic function were observed despite the lack of significant changes in biochemical parameters in the lavage fluid

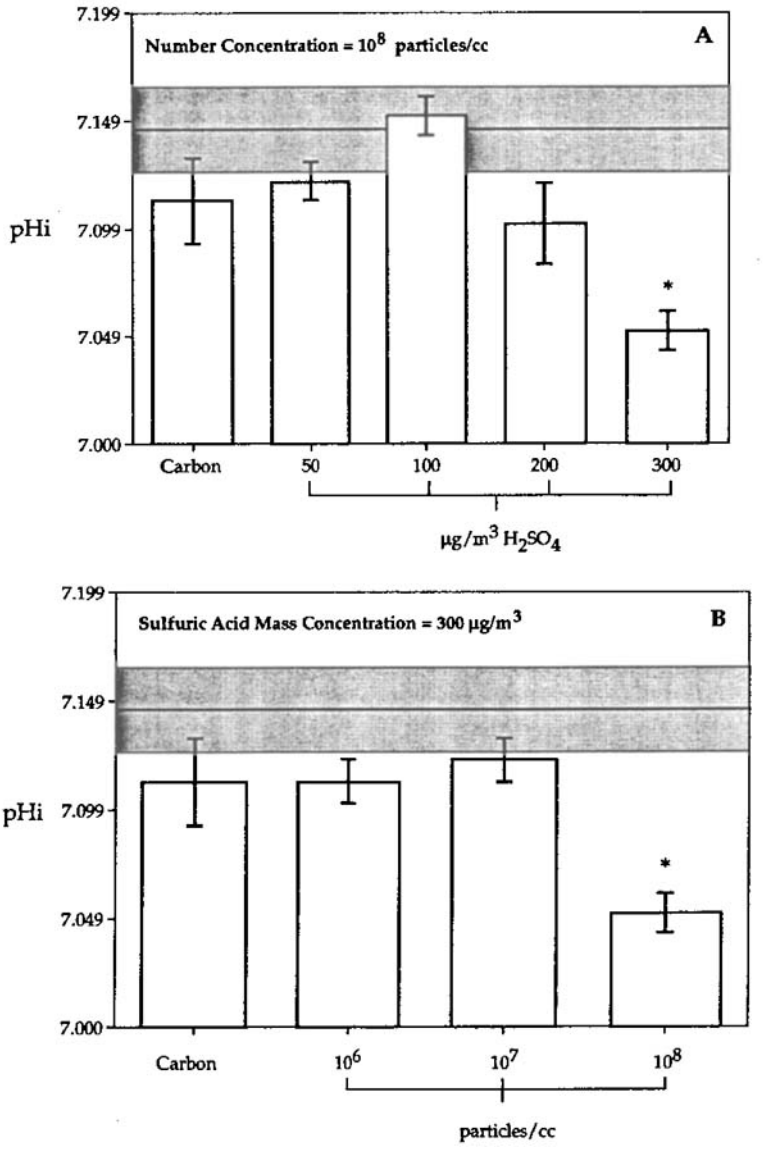

FIGURE 1. Effect of sulfuric acid layered carbon particles on pH_i of PMϕs. (A) Effect of total sulfate concentration at a fixed particle number concentration. (B) Effect of particle number concentration at a fixed sulfate concentration. Shaded area indicates pH_i of control (mean ± SE). Values were mean ● SE. Asterisk indicates significant difference from control ($p < .05$).

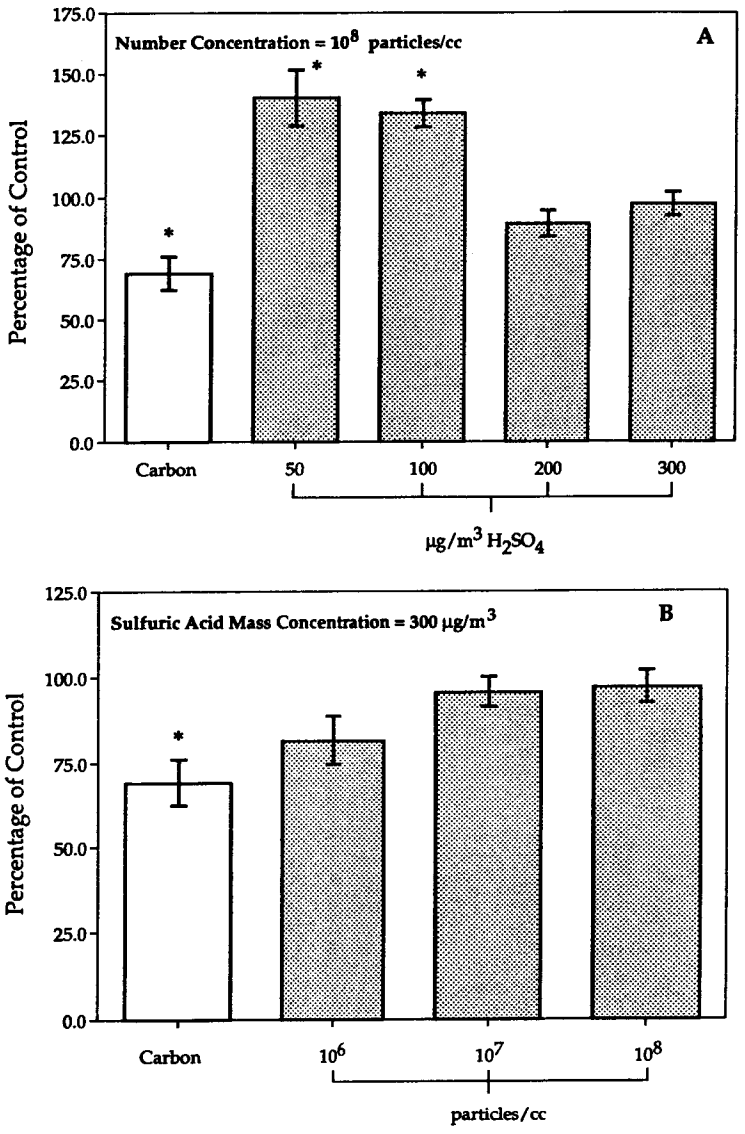

FIGURE 2. Effect of sulfuric acid layered carbon particles on phagocytic capacity of PMϕs. (A) Effect of total sulfate concentration at a fixed particle number concentration. (B) Effect of particle number concentration at a fixed sulfate concentration. Values were percentage of control (mean ± SE). Asterisk indicates significant difference from control ($p < .05$).

of animals exposed to sulfuric acid layered carbon particles. Because the regulation and control of many cellular events, such as phagocytosis, are proposed to be mediated by changes in intracellular ion concentrations such as H^+ and Ca^{2+}, we also measured pH_i and $[Ca^{2+}]_i$ of PMϕ. It appeared that alterations in these ion concentrations after exposure to sulfuric

acid aerosols depend not only on the mass concentration of sulfuric acid layered on carbon particles, but also on the number concentration of particles in the exposure atmosphere. Regression analysis showed that $[Ca^{2+}]_i$ decreased with increasing sulfate concentration (at a fixed number concentration) as well as number concentration of sulfuric acid layered carbon par-

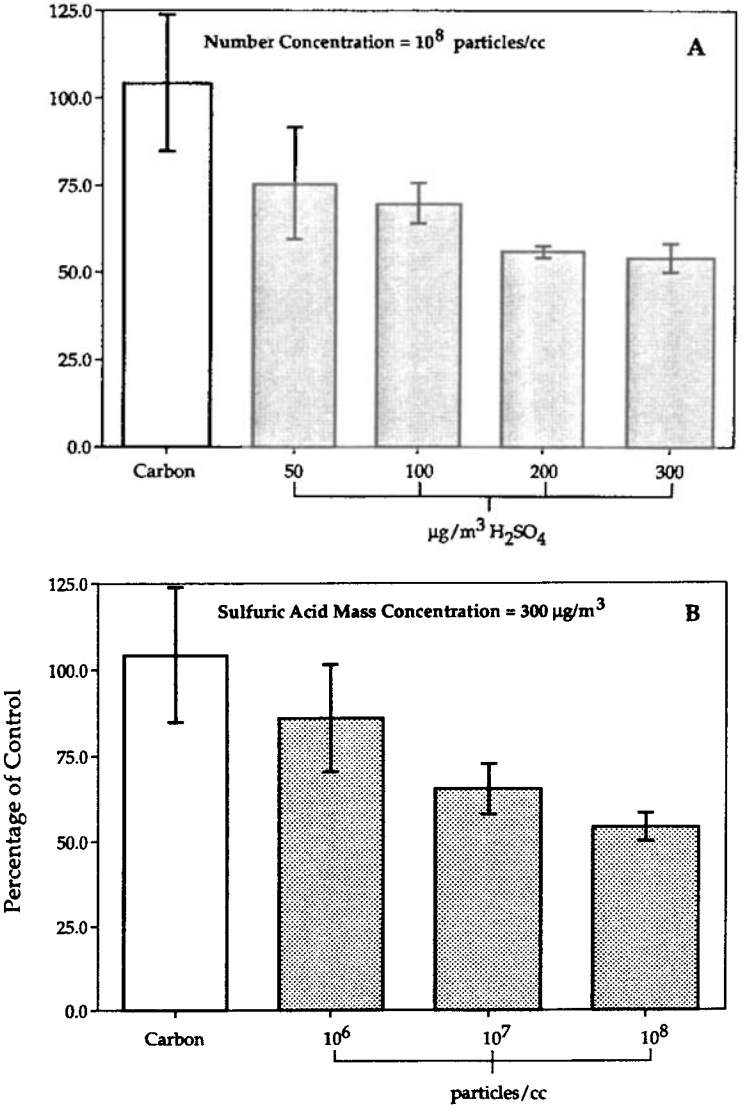

FIGURE 3. Effect of sulfuric acid layered carbon particles on $[Ca^{2+}]_i$ of PMϕs. (A) Effect of total sulfate concentration at a fixed particle number concentration. (B) Effect of particle number concentration at a fixed sulfate concentration. Values were percentage of control (mean ± SE). Asterisk indicates significant difference from control ($p < .05$).

ticles (at a fixed sulfate concentration). In addition, pH_i decreased with increasing sulfate concentration (at a fixed number concentration), while changes in pH_i did not occur until the number concentration of sulfuric acid layered particles was above 10^8/ml. Thus, there appears to be a threshold for both the mass concentration as well as the number concentration of sulfuric acid layered carbon particles below which no change in pH_i or $[Ca^{2+}]_i$ was observed.

The observation that the number concentration is a determinant of pulmonary response confirms, partially, the "irritation-signaling" model proposed by Hattis et al. (1987). They hypothesized that since particle number per unit mass concentration declines dramatically with increasing particle size, they will be fewer in number relative to their mass and therefore can deliver relatively fewer localized signals per unit of mass than do smaller particles. Indeed, as observed in this study, alterations in pH_i did not occur until the number concentration was above 10^8/ml, suggesting that number concentration is a determinant of pulmonary response. The "irritation-signaling" model also suggests that particles smaller than about 0.4–0.7 μm may not carry sufficient acid to cause damage. However, since the median particle size of the sulfuric acid layered carbon particles used in this study was 0.07 μm, the results of this study showed that particles smaller than 0.4–0.7 μm can induce alterations in PMϕs.

The importance of number concentration in eliciting pulmonary response found in this study also confirms our earlier finding that particle size, and therefore particle number, is critical in determining the ability of acid aerosols to alter macrophage function (Chen et al., 1992). In that study, different responses of PMϕs to two different sizes of pure sulfuric acid droplets (0.3 and 0.04 μm) were observed. Since the same mass concentration of sulfuric acid was used for both aerosol size, a 420 times greater number of 0.04-μm sulfuric acid droplets reached the alveolar region of the lung than 0.3-μm particles. Consequently, as shown in calculations in our previous study (Chen et al., 1992), each macrophage located in the lumen of the alveolar air spaces was bombarded by 10 acid droplets of 0.04 μm size during the course of a 3-h exposure, while only 1 out of 50 macrophages would be hit by a single 0.3-μm acid droplet. Therefore, as number concentration increases, more particles and thus higher doses are delivered to the macrophage population.

Although we did not compare the response of sulfuric acid layered carbon particles to that of pure sulfuric acid droplets, our previous studies (Amdur & Chen, 1989; Amdur, 1989) had shown that sulfuric acid layered on ZnO particles was more potent in producing adverse pulmonary effects than sulfuric acid presented as pure droplets. Reductions in lung volumes and diffusion capacity (DLCO), alterations of biochemical markers, and changes in lung tissue morphology were observed in guinea pigs after a single 3-h exposure to ultrafine ZnO with a surface layer of sulfuric acid at sulfate concentrations as low as 60 $\mu g/m^3$. The significance of these results is demonstrated by

the observation that it requires as much as 10 times more of a pure sulfuric acid droplet aerosol (same particle size) to produce the same magnitude of effect.

While animal experiments and human observational epidemiologic studies both suggest that acidic atmospheric environments can and do adversely affect humans, currently established clinical methods have generally failed to quantitate consistent lung function changes from exposures at or near ambient acid exposure levels in normals and in asthmatics exposed in controlled laboratory settings (U.S. EPA, 1989). It may be that consistent significant effects are not occurring in the groups tested. On the other hand, it is also possible that the techniques employed were not sufficiently sensitive to detect acid aerosol effects that may have occurred. A major gap in our knowledge of the human health effects of acid aerosols is related to the type of aerosol used in such controlled laboratory settings. Almost all of the published studies in this field have used aqueous aerosols of H_2SO_4 that have been produced by a variety of nebulizers. While these particles are generally of respirable size (less than 10 μm), they do not resemble the complex particles that are emitted from combustion processes used in the production of energy and which are a particular problem in the northeastern United States today.

The freshly generated combustion particles used in this study have the advantage of being similar to the combustion particles that occur in ambient air (Amdur et al., 1986), in contrast to the less realistic aqueous suspensions of larger carbon black particles recently tested by Anderson and co-workers (1991). In their study, aerosols of 100 $\mu g/m^3$ sulfuric acid applied to aqueous suspensions of carbon black aerosol did not elicit significantly different responses in normal subjects than did sulfuric acid alone by measures of specific airways resistance (SRaw), spirometry, and methacholine responsiveness. Because of their size and surface characteristics, freshly generated carbon particles, formed by thermal decomposition, are likely to be much more potent than the aerosolized slurry of carbon black particles and much more relevant to ambient acid aerosol pollution. In contrast, sulfuric acid layered either on carbon particles, such as the ones used in this study, or on metal oxide, such as that used in our previous study, resemble those produced in primary furnace effluent (Amdur et al., 1986). Because of their size and surface characteristics, freshly generated carbon particles formed by thermal decomposition are likely to be much more potent than the aerosolized slurry of carbon black particles and much more relevant to ambient acid aerosol pollution.

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