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INHALED PARTICLE-BOUND SULFATE: Effects on Pulmonary Inflammatory Responses and Alveolar Macrophage Function

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Acid sulfate-coated solid particles are a significant environmental hazard produced primarily by the combustion of fossil fuels. We have previously described a system for the nascent generation of carbonaceous particles surface coated with approximately 140 μg/m³ acid sulfate [$cpSO_4^{2-}$; 10 mg/m³ carbon black (CB) and 10 ppm sulfur dioxide (SO_2) at 85% relative humidity (RH)]. The effects of inhaled $cpSO_4^{2-}$ on pulmonary host defenses are assessed in the present work. Mice were acutely exposed (4 h) to either 10 mg/m³ CB, 10 ppm SO_2, or their combination at 10% or 85% RH in a nose-only inhalation chamber. No evidence of an inflammatory response was found following any of the exposures as assessed by total cell counts and differential cell counts from bronchoalveolar lavage fluid. However, alveolar macrophage Fc receptor-mediated phagocytosis decreased only following exposure to 140 μg $cpSO_4^{2-}$, significant suppression occurred after 24 h, maximal suppression occurred at 3 days postexposure, and recovery to preexposure levels required 7–14 days. Intrapulmonary bactericidal activity (IBA) was also suppressed only after exposure to 140 μg $cpSO_4^{2-}$; suppression was maximal at 1 day postexposure and recovered by day 7. To assess the effects of lower $cpSO_4^{2-}$ concentrations, mice were repeatedly exposed to 1 mg/m³ CB and 1 ppm SO_2 at 85% RH (~20 μg/m³ $cpSO_4^{2-}$ for 4 h/day) for up to 6 days. A significant decrement in IBA was observed following 5 and 6 days of exposure. These studies indicated that acute or repeated exposure to $cpSO_4^{2-}$ could alter pulmonary host defense mechanisms.

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Epidemiological studies from various cities in the United States have reported an association between elevated particulate concentrations and increased incidence of morbidity and mortality (Pope, 1989; Pope et al., 1991, 1992, 1995; Schwartz & Dockery, 1992; Schwartz, 1994). Acid sulfate aerosols represent a specific particulate chemical composition that has been associated with increased risk of mortality (Ozkaynak & Thurston, 1987; Thurston et al., 1989; Dockery & Pope, 1994; Ozkaynak et al., 1994; Pope et al., 1995). Toxicological studies investigating acid sulfate have reported a wide variety of detrimental effects. These include decrements in airway function (Amdur et al., 1978; Naumann & Schlesinger, 1986; Chen et al., 1991; Chen et al., 1992a), alterations in pulmonary morphology (Conner et al., 1982, 1985; Wolff et al., 1986; Gearhart & Schlesinger, 1988; Schlesinger et al., 1992), increased pulmonary inflammatory responses (Wolff et al., 1986; Conner et al., 1989; Amdur & Chen, 1989; Schlesinger et al., 1990; Chen et al., 1991), and abatement of pulmonary defense mechanisms including phagocytosis (Schlesinger, 1987; Schlesinger et al., 1992; Chen et al., 1995b).

Acid sulfate particles form by two prominent pathways in the urban atmosphere: homogeneous aqueous-phase oxidation of sulfur dioxide (SO_2) to form acid sulfate droplets (dSO_4^{2-}), and heterogeneous solid particle-surface catalyzed oxidation of SO_2 to produce a particle surface-coated with sulfate (pSO_4^{2-}). At present, the large majority of toxicological studies have focused on the detrimental effects of dSO_4^{2-}. However, comparative studies have reported that inhalation of pSO_4^{2-} particles caused decrements in pulmonary function and exacerbated pulmonary inflammation at one-tenth the concentration of inhaled dSO_4^{2-} (Amdur, 1989; Amdur & Chen, 1989; Chen et al., 1989, 1991, 1992b).

Few studies have examined the toxicological effects of a prominent pSO_4^{2-}, carbonaceous particulate matter coated with sulfate ($cpSO_4^{2-}$). Airborne carbonaceous particles are abundant in the urban atmosphere, comprising up to 50–80% of all ambient matter (Novakov et al., 1974; Hildemann et al., 1994). These particles are produced by combustion of fossil fuels and are emitted simultaneously with SO_2, allowing for direct interaction and formation of $cpSO_4^{2-}$ (Novakov et al., 1974; Brodzinsky et al., 1980; Chang et al., 1981; Benner et al., 1982; Hansen et al., 1991; Govindarao & Gopalakrishna, 1995). Previous work from our laboratory with this system (Jakab et al., 1996) showed depressed alveolar macrophage (AM) phagocytic capacity following exposure to $cpSO_4^{2-}$ that did not occur following exposure to either the carbon black particle vector or sulfate components alone.

The present study examined the lung response following inhalation of freshly generated $cpSO_4^{2-}$. The present work describes inhalation effects of $cpSO_4^{2-}$ particles on pulmonary inflammation and pulmonary defense mechanisms as indicated by inflammatory markers, alveolar macrophage

phagocytic function, and antimicrobial defenses mediated by alveolar macrophages.

METHODS

Animals

Outbred Swiss female mice (20–23 g, Hilltop Laboratories, Scottsdale, PA) were obtained and maintained for 1 wk in a 14-h light/10-h dark photoperiod before use. Mice were housed in plastic cages with wood shaving bedding. Mice were given food and water ad libitum. National Institutes of Health guidelines for the care and handling of laboratory animals were observed.

Acid Sulfate-Coated Carbon Black Particle Generation and Analysis

The particle generation system used in the present study was described and schematically presented in detail in a previous report from our laboratory (Hemenway et al., 1996). Briefly, carbon black (CB; 10 mg/m^3; Regal 660; specific surface area of 112 m^2/g; composition: 96.90% carbon, 0.30% hydrogen, 1.42% oxygen; empirical formula of $C_{910}H_{34}O_{10}$; generous gift of the Cabot Corporation, Billerica, MA) was generated with a Wright dust feed. The aerosol was generated with a house air compressor at a flow rate of 10 L/min and was mixed with a dilution airstream, making a total flow of 80 L/min. The house compressor air was dehumidified by passing through Drierrite cylinders and filtered by passing through an activated carbon cylinder to remove impurities. The total air flow was then directed through a humidification chamber [range 10–95% relative humidity (RH)], allowing the CB to absorb moisture. Sulfur dioxide (SO_2) was mixed with the humidified CB aerosol (10 ppm; 1.5% SO_2 in air; Matheson Gas Co., East Rutherford, NJ). A ballast tank allowed for a brief mixing time and chemical formation of cpSO_4^{2-}, which was then delivered to the nose-only animal exposure chamber. Carbon black aerosol levels of 1 mg/m^3 and SO_2 levels of 1 ppm at 85% RH were employed in low-level, repeated-exposure studies.

Surface formation of sulfate on CB was assessed by a modification of a previously published turbidimetric method (Clesceri et al., 1989; Hemenway et al., 1996). Briefly, particle aerosols were collected gravimetrically on DM-450 filters (Gelman Co., Ann Arbor, MI) held in electrically conductive filter units (Fisher Scientific Products, Pittsburgh, PA). Sulfate and particles were eluted from filters with deionized, distilled water; particles were removed from the eluent by ultracentrifugation. Acid sulfate was expressed as micrograms sulfate per cubic meter.

Laser Scanning Confocal Microscopy

Micrographs were generated using a Sarastro 2000 (Molecular Dynamics, Inc., Sunnyvale, CA) laser scanning confocal microscope (Opti-

phot-2, Nikon, Inc., Melville, PA) fitted with an argon-ion laser. Images were recorded through a 60× objective. Emission spectra >510 nm were diverted to a separate photodetector and used to image cells. Reflected light <510 nm was simultaneously passed to a separate optical path, and provided images of carbon black particles. Images of lung cells and carbon black particles were combined to depict internalization of particles within cells. To ensure images were generated from inside the cells, scans were performed only while cell nuclei were in focus.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed for measurement of pulmonary inflammation markers and retrieval of pulmonary cells for the alveolar macrophage (AM) phagocytic assay as previously described (Hemenway et al., 1996). Lungs were surgically removed, washed with phosphate-buffered saline, and lavaged by inserting a Pasteur pipet into the trachea and gently forcing in and withdrawing 1.5 ml of sterile phosphate-buffered saline [0.85% sodium chloride, 0.1% glucose, 0.1% ethylenediamine tetraacetic acid (EDTA), and 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)]. The first BAL fluid recovered was put aside so the supernatant could be used for total protein analysis. The lungs were gently massaged between lavages and a second 1.5-ml aliquot of BAL fluid was introduced. This procedure was repeated once more for a total of 3 lavages totaling 4.5 ml BAL fluid/lung.

Pulmonary cells were isolated from BAL fluid by centrifugation for 10 min at 500 × g (model 7R, International Equipment Corporation, Needham Heights, MA). The acellular supernatant was removed from the first lavage sample for each animal and saved for determination of total BAL fluid protein. Cell pellets from the two lavage tubes were combined in a total of 1 ml of tissue culture media (TCM). The TCM consisted of Roswell Park Memorial Institute (RPMI)-1640 tissue culture media (Life Technologies, Grand Island, NY), 10% newborn calf serum (Hyclone Co, Logan, UT), 100 units/ml penicillin/streptomycin/fungizone (PSF; Whittaker Bioproducts, Dulles International Airport, VA), and 20 mmol HEPES buffer (Whittaker Bioproducts, Dulles International Airport, VA), combined.

Analyses of Inflammatory Parameters

Total cell counts and viability (via trypan blue exclusion) from individual BAL samples were determined using a hemacytometer (Fisher Scientific Co., Pittsburgh, PA). The cell suspensions were subsequently diluted in TCM to a final concentration of 500,000 cells/ml. Two aliquots of 100 µl (total = 5×10^4 cells) from the already described cell suspensions were used for BAL differential cell analysis. The samples were cytocentrifuged (Shandon Cytospin II, Shandon Southern Instruments, Inc, Sewickley, PA) onto glass slides at 200 × g for 10 min. Two hundred cells from each slide were randomly counted from each sample and scored as either AM, polymorphonuclear leukocytes (PMN), or lymphocytes (LYM).

Total BAL fluid protein content was measured via a commercially available assay kit (BioRad Scientific, San Diego, CA).

Alveolar Macrophage Phagocytosis

Alveolar macrophage phagocytosis of opsonized sheep red blood cells (RBC, Becton-Dickinson, Franklin Lakes, NJ) was employed as a marker of AM function as previously described (Hemenway et al., 1996; Warr et al., 1979). Briefly, three 200-µl aliquots from each BAL cell suspension (1×10^5 total cells) were cultured on individual bovine serum albumin-coated 22-mm^2 coverslips in 35 × 10 mm petri dishes for 45 min (37°C, 5% CO_2, 95% RH). Macrophage monolayers were subsequently incubated with immunoglobulin G (IgG)-opsonized RBC suspension for 45 min (37°C, 5% CO_2, 95% humidity). Postincubation, fixed monolayers were stained with Wright–Giemsa (Fisher Scientific Products). The stained monolayers were examined microscopically (Zeiss Instruments) to quantify the percentage of AM containing RBC and the number of RBC ingested per phagocytic AM. A total of 200 cells from each individual aliquot was quantified. The product of the percentage of AM containing RBC and the average number of RBC per phagocytic cell is referred to as the phagocytic index. Values are expressed as percent of the cage control mean; an age-matched set of untreated controls was simultaneously assessed with each experimental set.

Intrapulmonary Bactericidal Activity

Intrapulmonary bactericidal activity (IBA) was assessed as previously described (Jakab, 1992; Jakab & Hemenway, 1993). The bacterial agent, *Staphylococcus aureus* (coagulase positive FDA strain 209 phage type 42D), was incubated in 200 ml trypticase soy broth (TSB; Difco, Detroit, MI) for 20 h at 37°C in a rotating shaking water bath. A previously described whole-body aerosol inhalation chamber was used to challenge mice with bacteria (Jakab & Green, 1972). During a 30-min exposure period, it has been estimated that approximately 3×10^5 staphylococci are deposited in each murine lung (Jakab & Green, 1972). Twenty-four hours following pollutant exposure, animals were exposed in the infectious exposure chamber for 30 min. Immediately following *S. aureus* challenge (0 h) and after 4 h, groups of 6 treated (pollutant-exposed) and 6 control (air-exposed) animals were sacrificed. The lungs were aseptically removed and homogenized in 3 ml TSB. A 1-ml aliquot of each homogenate was diluted 10-fold in phosphate-buffered saline (PBS) and cultured quantitatively in 5% NaCl-trypticase soy agar (Difco) in quadruplicate for 48 h. Individual bacterial colonies were quantitated with a colony counter (Artek Systems Corp, Farmingdale, NY) modified for Petri X-plates. Bactericidal activity was expressed as the percent of bacteria killed by dividing the number of colonies present at 4 h by the number at 0 h and subtracting from 100. Experimental group comparison was expressed as the percent of the untreated control mean IBA.

Statistical Analyses

The results are expressed as the mean ± standard error (SE). The effects of $cpSO_4^{2-}$ on inflammation, Fc receptor-mediated phagocytosis, and IBA were assessed by one-way analysis of variance (ANOVA; two-tailed). Student–Newman–Keuls analyses were used for post hoc comparison of means. Statistical significance was accepted at $p < .05$.

RESULTS

Particle analyses were described in detail in our previous study (Hemenway et al., 1996). Exposure conditions to produce $cpSO_4^{2-}$ are summarized in Table 1. The $cpSO_4^{2-}$ particle size range was 0.30–0.40 μm mass median aerodynamic diameter with a geometric standard deviation of 2.7–3.0. The size range did not significantly differ due to any of the exposure conditions described in the present work.

Confocal microscopic examination of BAL cells retrieved from lungs of $cpSO_4^{2-}$-exposed animals demonstrated that particles were deposited in the lung parenchyma and phagocytosed by AM. Figure 1, A and B, demonstrates 2 views of particle ingestion by AM in a mouse exposed for 4 h to 10 mg/m^3 CB and 10 ppm SO_2 at 85% RH and subsequently sacrificed 3 days following exposure. Figure 1C represents an air-control.

Previous studies from other groups reported increased pulmonary inflammation following inhalation of pSO_4^{2-} (Conner et al., 1988; Chen et al., 1991). To determine if $cpSO_4^{2-}$ generated in the present work caused inflammation, inflammatory parameters including total BAL cell counts, differential cell counts, and total BAL fluid protein analysis were assessed. Untreated, age-matched controls were included for comparison. Figure 2 shows total BAL cell counts from animals exposed for 4 h to 10 mg/m^3 CB

TABLE 1. Acid-sulfate levels formed on the surface of carbon black aerosol (10 mg/m^3) cogenerated with or without sulfur dioxide (10 ppm) at low (10%) and high (85%) relative humidity

Carbon black aerosol (mg/m^3)	Sulfur dioxide concentration (ppm)	Relative humidity (%)	Acid sulfate concentration (μg/m^3)
10	0	10	0
10	0	85	8
0	10	10	0[a]
0	10	85	0[a]
10	10	10	41
10	10	85	137
1	1	85	20

Note. All measurements were made following 4 h of particle generation and were sampled from filters obtained by gravimetric analysis (Hemenway et al., 1996).

[a]No particles were generated; therefore, no particle-bound sulfate was measured. However, it is possible that sulfate droplets were formed though they were not assessed in the present work.

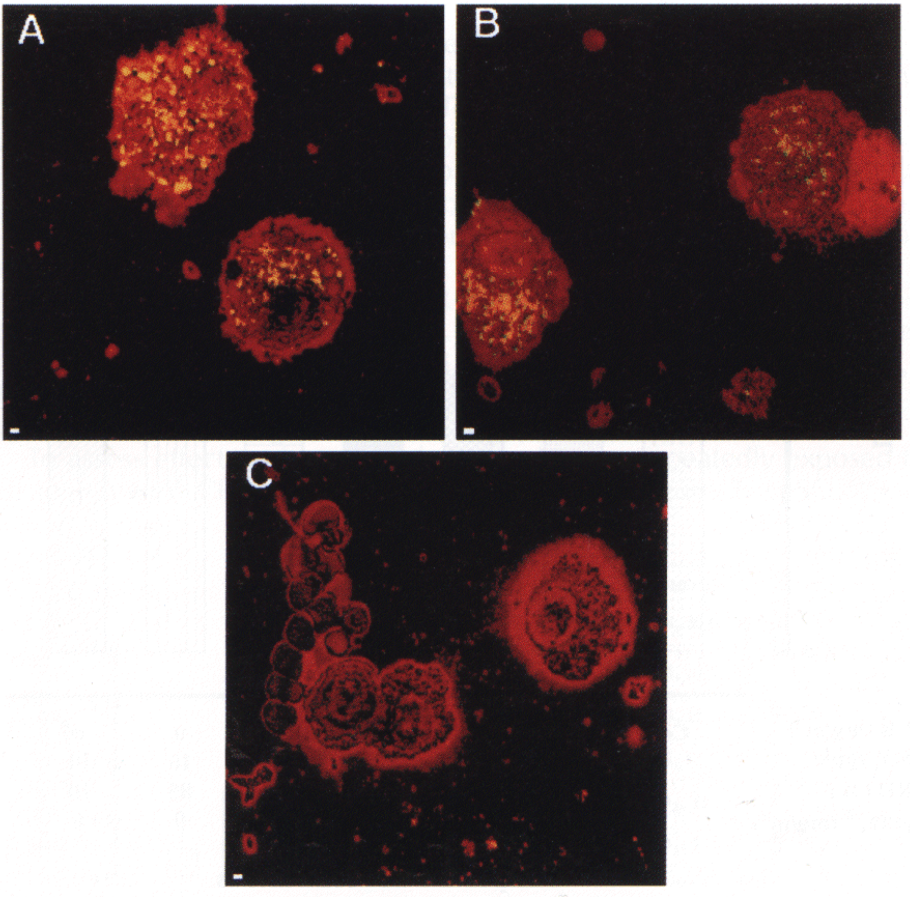

FIGURE 1. Confocal micrograph of alveolar macrophages recovered from mice exposed for 4 h to (A, B) 10 mg/m^3 carbon black (CB) and 10 ppm sulfur dioxide (SO_2) at 85% relative humidity (RH) and (C) room air. Animals were sacrificed at 3 days following exposure. Cells are red; particles are yellow. Bar is 2 μm.

and 10 ppm SO_2 at 85% RH ($cpSO_4^{2-} \approx 140$ μg/m^3) or 10% RH ($cpSO_4^{2-} \approx 40$ μg/m^3), 10 mg/m^3 CB alone at 85% or 10% RH, or 10 ppm SO_2 at 85% or 10% RH. A total of $61 \pm 3.0 \times 10^4$ cells was retrieved from untreated control animals. No significant differences were observed following any of the exposures as compared to controls. Differential cell counts (Figure 3) from untreated control animals exhibited >99.0% AM and <1.0% of both polymorphonuclear leukocytes (PMN) and lymphocytes (LYM). Although both PMN and LYM percentages were slightly elevated in animals exposed to 140 μg/m^3 $cpSO_4^{2-}$, no significant differences were observed. None of the control conditions, 10 mg/m^3 CB at 10% and 85% RH, 10 ppm SO_2 at 10% and 85% RH, or the combination at 10% RH, indicated any significant change in differential profile. Also, no sig-

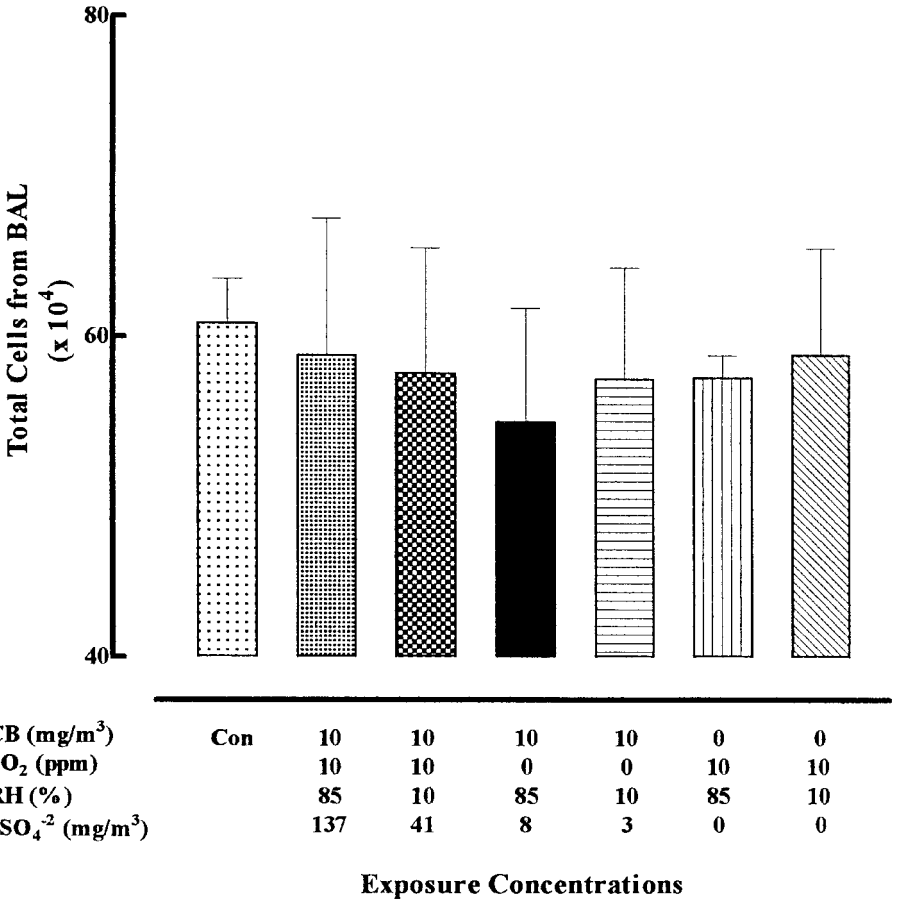

FIGURE 2. Comparison of total cell counts retrieved by BAL at 3 days following exposure to 10 mg/m^3 CB, 10 ppm SO_2, or the combination of the two agents at 85% or 10% RH. Each value represents the mean ± SE of at least 10 determinations. Asterisk indicates significant at $p < .05$ vs. air-exposed control.

nificant differences were measured in BAL total protein following exposure to $cpSO_4^{2-}$ or any of the control conditions (data not shown).

Animals were exposed for 4 h to 10 mg/m^3 CB and 10 ppm SO_2 at 85% RH ($cpSO_4^{2-} \approx 140$ μg/m^3) or 10% RH ($cpSO_4^{2-} \approx 40$ μg/m^3), 10 mg/m^3 CB alone at 85% or 10% RH, or 10 ppm SO_2 at 85% or 10% RH. Three days following exposure, animals were sacrificed and AM phagocytosis was assessed. Significant suppression was only observed following exposure to elevated $cpSO_4^{2-}$ (140 μg/m^3; Hemenway et al., 1996).

To investigate recovery of the phagocytic suppression, animals were exposed for 4 h to 140 μg/m^3 $cpSO_4^{2-}$. Animals were then sacrificed and Fc receptor-mediated phagocytosis was assessed at 1, 3, 7, and 14 days postexposure. Phagocytosis was significantly depressed 1 day following

exposure, reached a maximal low at 3 days, and returned to control levels between 7 and 14 days postexposure (Figure 4).

Suppression of phagocytosis due to inhaled $cpSO_4^{2-}$ suggested immunocompromise in affected animals. To determine potential alterations in immune function in response to a biological agent, IBA to *S. aureus* was assessed. Animals were exposed to 10 mg/m^3 CB, 10 ppm SO_2, or the combination at 85% and 10% RH for 4 h. IBA was assessed at 24 h postexposure. Neither exposure to CB or SO_2 alone at 85% and 10% RH nor exposure to the mixture at 10% RH suppressed staphylococcal killing. In contrast, exposure to the mixture at 85% RH ($cpSO_4^{2-}$ = 140 μg/m^3) significantly suppressed IBA (Figure 5). The kinetics of IBA suppression were also assessed following singular 4-h exposure to 140 μg/m^3 $cpSO_4^{2-}$. Maximal suppression was observed on day 1, and recovery occurred by day 7 postexposure (Figure 6).

To assess effects at lower levels, animals were repeatedly exposed for 4 h to 1 mg/m^3 CB and 1 ppm SO_2 at 85% RH (Figure 7; $cpSO_4^{2-} \approx 20$

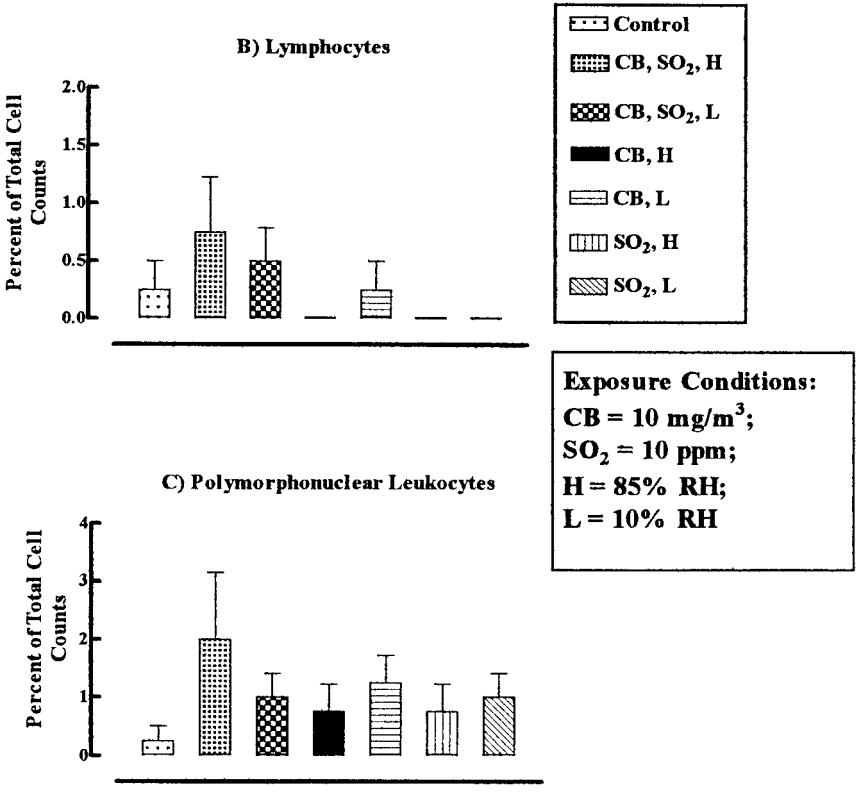

FIGURE 3. Differential cell counts from animals exposed to varying inhalation chamber atmospheres (the same as Figure 2) and lavaged at 3 days postexposure. Each value represents the mean ± SE of at least 10 determinations.

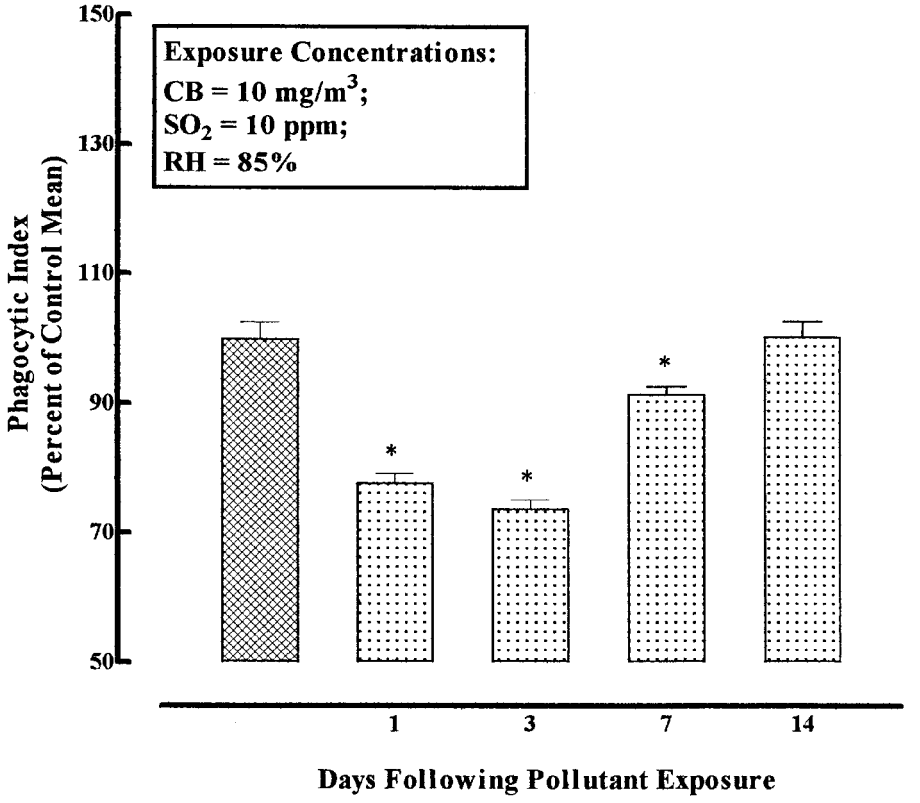

FIGURE 4. Suppression and recovery of alveolar macrophage phagocytosis from mice exposed to 10 mg/m^3 CB and 10 ppm SO_2 at 85% RH. Each value represents the mean ± SE of at least 10 determinations. Asterisk indicates significant at $p < .05$ vs. air-exposed control.

μg/m^3). Exposures were continued for up to 6 days, and IBA was assessed 24 h following the last exposure. Significant suppression of IBA occurred after 5 days of exposure.

DISCUSSION

This study employed the fresh generation of acid sulfate on the surface of an "inert" carbonaceous particle, carbon black, as a surrogate for environmental $cpSO_4^{2-}$. We have previously shown that the combination of CB, SO_2, and high RH led to the formation of $cpSO_4^{2-}$ in the fine, respirable (0.29–0.40 μm) range (Hemenway et al., 1996). The amount of formation of $cpSO_4^{2-}$ was highly dependent on the RH percentage and SO_2 concentration.

Based on previous studies, it was expected that pulmonary inflammation would be observed in the present investigation (Conner et al., 1988; Amdur & Chen, 1989; Chen et al., 1991) upon exposure to $cpSO_4^{2-}$. Increased neu-

trophils and total BAL protein have been reported following exposure to as little as 20 μg H_2SO_4/m^3 layered on the surface of a particle (Chen et al., 1989; Amdur & Chen, 1989). In the present work, $cpSO_4^{2-}$ was observed to reach the lung parenchyma and was ingested by AM (Figure 1). In contrast to previous studies with other types of pSO_4^{2-}, the acid sulfate used in the present study did not induce any significant inflammatory response. It is hypothesized that the carrier particle utilized in the previous studies, zinc oxide (ZnO), may have directly affected the inflammatory response. The concentrations of ZnO used in these previous studies have been shown to induce pulmonary inflammation (Lam et al., 1985; Gordon et al, 1992).

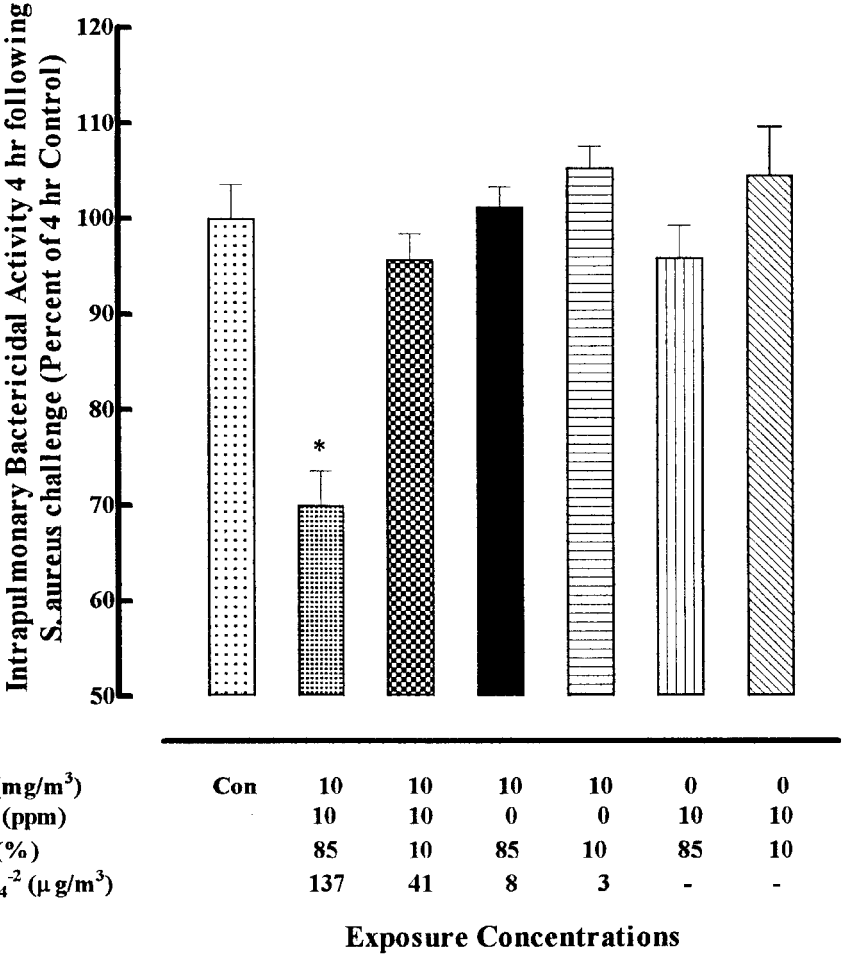

FIGURE 5. Comparison of intrapulmonary bacterial killing at 1 day following exposure of mice to 10 mg/m^3 CB, 10 ppm SO_2, or the combination of the two agents at 85% or 10% RH. The combination at 85% RH led to the formation of 140 $\mu g/m^3$ $cpSO_4^{2-}$. Each value represents the mean ± SE of at least 12 determinations. Asterisk indicates significant at $p < .05$ vs. air-exposed control.

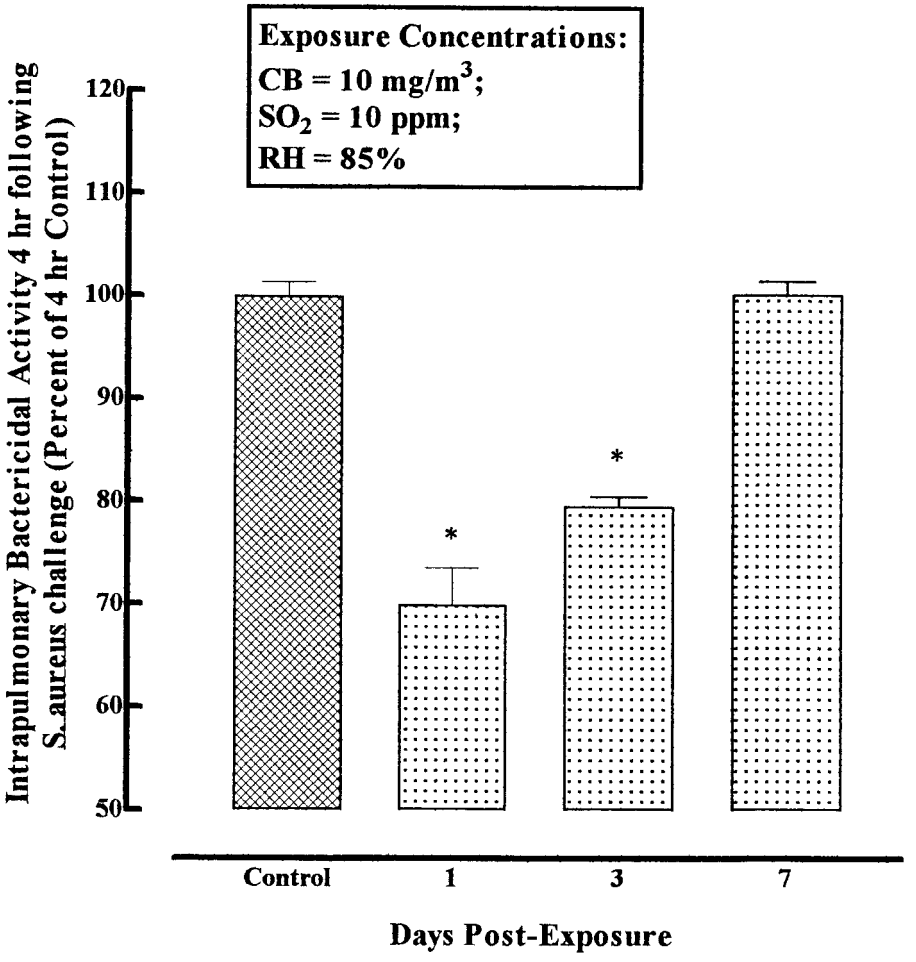

FIGURE 6. Kinetics of intrapulmonary bactericidal activity suppression in mice exposed to 10 mg/m^3 CB and 10 ppm SO_2 at 85% (140 μg/m^3 $cpSO_4^{2-}$). Each value represents the mean ± SE of at least 12 determinations. Asterisk indicates significant at $p < .05$ vs. air-exposed control.

Alveolar macrophage phagocytosis and intrapulmonary bactericidal activity were employed as measures of lung defense function in the distal lung that may be affected by $cpSO_4^{2-}$. These two assays were chosen because they have been previously used as effective indicators of pulmonary toxicity to inhaled toxicants including acid sulfate (Gearhart & Schlesinger, 1986; Schlesinger, 1987; Canning et al., 1991; Chen et al., 1992b; Jakab & Hemenway, 1993, 1994; Zelikoff et al., 1994). These assays represent two approaches for assessment of the lung defense system (Jakab, 1993). Alveolar macrophage Fc receptor-mediated phagocytosis was used as an ex vivo marker of AM toxicity to assess the function of a pulmonary cell specifically involved in the response to inhaled parti-

cles. The second assay, IBA, provides an in vivo marker of AM toxicity. Uptake and killing of *Staphylococcus aureus* in the distal lung is AM mediated (Goldstein et al., 1971a, 1971b, 1978; Kim et al., 1976) and therefore allows for measurement of function in the normal microenvironment of the AM.

We demonstrated previously that AM phagocytosis was shown to be significantly suppressed at 3 days following exposure to 140 μg $cpSO_4^{2-}/m^3$ (Hemenway et al., 1996). However, this was the only time point assessed. It has been observed that acute exposures to other gas/particle combinations can produce long-lasting effects (up to 30 days) on AM phagocytic function

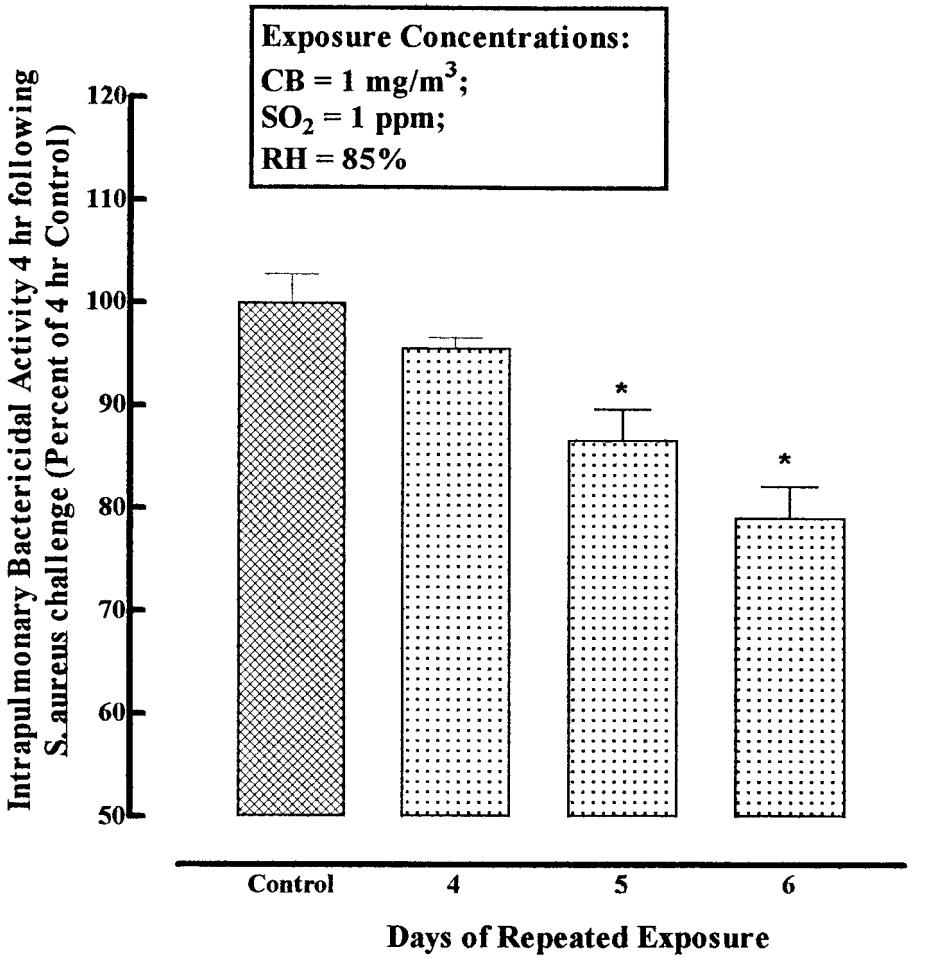

FIGURE 7. Suppression of intrapulmonary bactericidal activity from mice repeatedly exposed for 4 h/day to 1 mg/m^3 CB and 1 ppm SO_2 at 85% (20 $\mu g/m^3$ $cpSO_4^{2-}$) for up to 6 days. Each value represents the mean ± SE of at least 12 determinations. Asterisk indicates significant at $p < .05$ vs. air-exposed control.

(Amdur & Chen, 1989; Jakab, 1992, 1993; Jakab & Hemenway, 1994). In this study, a singular 4-h exposure to 140 μg/m^3 cpSO_4^{2-} caused significant suppression phagocytosis 1 day after exposure that persisted for days afterward (7–14 days in AM phagocytosis). A similar result was observed when IBA was assessed, although recovery occurred more quickly (3–7 days postexposure).

The mechanism of suppression of phagocytosis and IBA due to the cpSO_4^{2-} exposure is not known. However, based on previous studies, it is suggested that internal alterations in the AM are partially responsible. Previous studies have reported decrements in AM intracellular pH (pH_i) following acute exposure to pSO_4^{2-} (Gordon et al., 1992; Qu et al., 1993; Chen et al., 1995b). During phagocytosis of the inhaled particles, the AM phagosome brings in cpSO_4^{2-} along with epithelial lining fluid (ELF) from the epithelial surface (Qu et al., 1993). The cpSO_4^{2-}-associated protons (H^+) subsequently produce decrements in the pH_i. This decrement in pH_i has been associated with alterations in proton (H^+) extrusion mechanisms, including the Na–H^+ exchanger (Gordon et al., 1992). Consequently, decreased pH_i suppresses phagocytosis and IBA either by direct malfunction of these mechanisms or by competitive inhibition of these highly metabolic events as the AM diverts energy to regain homeostasis.

The significant suppression of pulmonary lung defenses at later time points following exposure suggests a "lag" effect in biological responses. Interestingly, epidemiological studies have reported that observed increases in morbidity and mortality due to elevated particle levels occur 1–3 days after the peak exposure (Schwartz, 1991; Dockery et al., 1993). The mechanism for these delayed effects is hypothesized to be related to SO_4^{2-}-induced changes in the milieu of the AM. Suppressed AM pH_i has been shown to linger for at least 24 h (Chen et al., 1992b). The downstream effects of this prolonged internal alteration are expected to include long-term malfunction of normal cellular processes including phagocytosis.

The results from the present study also indicate that a dose threshold following cpSO_4^{2-} exposure exists. Exposure to 140 μg/m^3 cpSO_4^{2-} induced significant pulmonary defense mechanism changes, while exposure to 40 μg/m^3 (10 mg/m^3 CB and 10 ppm SO_2 at 10% RH) caused no significant changes. Previous results have suggested that a threshold appears to exist with acid sulfate inhalation effects (Gearhart & Schlesinger, 1986; Schlesinger, 1987; Schlesinger et al., 1992; Chen et al., 1995a). Chen et al. (1995b) clearly demonstrated that pH_i was significantly altered by exposure to 125 μg/m^3 sulfate droplets while 50 μg/m^3 had no effect. The microenvironment and internal integrity of the AM are expected to be responsible for this difference. It is possible that the ELF buffers the H^+ at lower concentrations in the alveolar epithelium; factors in the AM phagolysosome may also provide sufficient buffering capacity to prevent changes in pH_i. Another possible mechanism involves AM functions designed to maintain pH_i. At a low intracellular burden of H^+, AM proton extrusion

mechanisms are able to keep the H^+ level in a normal range and hence pH_i normal.

The precedent for repeated, lower concentration studies producing similar effects as higher concentration studies has been shown. Repeated exposures at a lower concentration of H_2SO_4 droplets (500 μg H_2SO_4/m^3) induced the same effect as acute exposure at a higher concentration (1000 μg H_2SO_4/m^3; Naumann & Schlesinger, 1986; Schlesinger, 1987). However, neither of these levels was relevant to ambient concentrations of acid sulfate (<50 μg H_2SO_4/m^3).

The use of a repeated-exposure protocol at lower $cpSO_4^{2-}$ concentrations more effectively addressed environmental exposures. The threshold limit values for carbon black and SO_2 are 3.5 mg/m^3 and 2 ppm, respectively. In this study, 1 mg/m^3 CB and 1 ppm SO_2 were cogenerated at 85% RH. Although acute, high-concentration exposures to inhaled hazards such as SO_4^{2-} occur, these events are rare. Inhalation of environmental hazards generally occurs at lower concentrations but involves day after day of exposure. The significant decrement in pulmonary defense mechanisms suggests the importance of investigating chronic exposures. The mechanism for the low-level effects is hypothesized to be due to the achievement of a cumulative H^+ dose that eventually produces decreased pH_i.

In summary, these studies investigated the pulmonary toxicity of an environmental acid aerosol, carbonaceous particles coated with acid SO_4^{2-}. It was demonstrated that these particles reach the alveolar region of the lung and are ingested by AM. These $cpSO_4^{2-}$ particles do not cause any significant inflammation. However, lung defense mechanisms, including Fc receptor-mediated AM phagocytosis and IBA, are depressed by $cpSO_4^{2-}$. Exposures to more environmentally relevant, lower concentrations of $cpSO_4^{2-}$ also depress lung defense mechanisms. The decrements in pulmonary defense mechanisms are theorized to be due to changes in AM induced by H^+ that alter pH_i.

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