Variable Pulmonary Responses from Exposure to Concentrated Ambient Air Particles in a Rat Model of Bronchitis

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Chronic bronchitis may be considered a risk factor in particularative matter (PM)-induced morbidity. We hypothesized that a rat model of human bronchitis would be more susceptible to the pulmonary effects of concentrated ambient particles (CAPs) from Research Triangle Park, NC. Bronchitis was induced in male Sprague-Dawley rats (90–100 days of age) by exposure to 200 ppm sulfur dioxide (SO2), 6 h/day × 5 days/week × 6 weeks. One day following the last SO2 exposure, both healthy (air-exposed) and bronchitic (SO2-exposed) rats were exposed to filtered air (three healthy; four bronchitic) or CAPs (five healthy; four bronchitic) by whole-body inhalation, 6 h/day × 2 or 3 days. Pulmonary injury was determined either immediately (0 h) or 18 h following final CAPs exposure. The study protocol involving 0 h time point was repeated four times (study #A, November, 1997; #B, February, 1998; #C and #D, May, 1998), whereas the study protocol involving 18 h time point was done only once (#F). In an additional study (#E), rats were exposed to residual oil fly ash (ROFA), ~1 mg/m3 ×6 h/day × 3 days to mimic the CAPs protocol (February, 1998). The rats allowed 18 h recovery following CAPs exposure (#F) did not depict any CAPs-related differences in bronchoalveolar lavage fluid (BALF) injury markers. Of the four CAPs studies conducted (0 h time point), the first (#A) study (~650 μg/m3 CAPs) revealed significant changes in the lungs of CAPs-exposed bronchitic rats compared to the clean air controls. These rats had increased BALF protein, albumin, N-acetyl glutaminidase (NAG) activity and neutrophils. The second (#B) study (~475 μg/m3 CAPs) did not reveal any significant effects of CAPs on BALF parameters. Study protocols #C (~869 μg/m3 CAPs) and #D (~907 μg/m3 CAPs) revealed only moderate increases in the above mentioned BALF parameters in bronchitic rats exposed to CAPs. Pulmonary histologic evaluation of studies #A, #C, #D, and #F revealed marginally higher congestion and perivascular cellularity in CAPs-exposed bronchitic rats. Healthy and bronchitic rats exposed to ROFA (~1 mg/m3) did not show significant pulmonary injury (#E). Analysis of leachable elemental components of CAPs revealed the presence of sulfur, zinc, manganese, and iron. There was an apparent lack of association between pulmonary injury and CAPs concentration, or its leachable sulfate or elemental content. In summary, real-time atmospheric PM may result in pulmonary injury, particularly in susceptible models. However, the variability observed in pulmonary responses to CAPs emphasizes the need to conduct repeated studies, perhaps in relation to the season, as composition of CAPs may vary. Additionally, potential variability in pathology of induced bronchitis or other lung disease may decrease the ability to distinguish toxic injury due to PM.

Key Words: bronchitis; bronchoalveolar lavage fluid (BALF); concentrated ambient particles (CAPs); Sprague-Dawley rats.

Past air pollution episodes from Donora, Pennsylvania, in 1948 and London in 1952 have shown that incidental exposures to high levels of particulate matter (PM) can markedly increase hospital admissions and mortality among those with preexistent bronchitis, chronic obstructive pulmonary disease, and other cardiopulmonary diseases (Martin, 1964; Schrenk et al., 1949). Recent studies have shown association between subtle increases in air pollution and human health impairments, including mortality among those with underlying pulmonary and/or cardiac disease (Dockery et al., 1993; Pope et al., 1995; Schwartz, 1994; Vedal, 1997). Understanding the role of host susceptibility factors that predispose one to increased adverse health effects of air pollution has become necessary in ascertaining the biologic plausibility of the epidemiologic findings. Thus, use of animal models of human cardiopulmonary disease has recently gained significant interest in evaluating the role of these host factors in PM-associated mortality and morbidity (Kodavanti et al., 1998a; Kodavanti and Costa, 1999).

Individuals with preexistent chronic obstructive pulmonary disease, including bronchitis, constitute an important subgroup...
potentially at risk of PM-induced health effects based on the prevalence of the disease. Nearly 14 million cases of bronchitis are reported annually nationwide (Ball, 1995; Iribarren et al., 1995). Bronchitis is often associated with chronic cigarette smoking and is characterized by chronic productive cough due to mucus hypersecretion and proliferation of mucus-secreting cells, recurrent or persistent airway inflammation, airflow obstruction, and often airway hyperresponsiveness (Jeffery, 1991; O’Byrne and Postama, 1999). A number of animal models of bronchitis have been developed to study the pathogenesis of the disease and the impact of pharmacologic manipulation (Drazen et al., 1982, 1995; Levrier et al., 1992; Shore et al., 1987). The most widely used and characterized animal model involves exposure of animals (dogs and rats) to high levels of sulfur dioxide (SO$_2$) for a duration of 4–8 weeks (Chakrin and Saunders, 1975; Drazen et al., 1982; Shore et al., 1995).

SO$_2$-induced bronchitis in the rat is characterized by increased mucus production, goblet cell hyperplasia, and increased airway responsiveness to methacholine, as in the case of human bronchitis. However, the severity and persistence of neutrophilic inflammation and chronic thickening of airways seen in humans are relatively mild or absent in the rat SO$_2$ model (Farone et al., 1995; Shore et al., 1995).

Ambient particles contain a variety of components such as sulfates, nitrates, metals, organics, and biologic materials (National Research Council, 1998). Each of these components theoretically has the potential to elicit airway injury via one or more mechanisms. Airway mucus has been considered a first line of defense against inhaled toxicants. However, in pathologic conditions of bronchitis, which are associated with increased mucus production and epithelial cell injury, inhaled PM may be more hazardous. Bronchitic rats have been shown to retain more particles in their lungs upon inhalation, and have a multifocal deposition pattern that is different from the more uniform pattern in healthy rats (Sweeney et al., 1995). The purpose of this study was to evaluate the pulmonary health effects of real-time concentrated ambient particles (CAPs) from Research Triangle Park, North Carolina, in healthy and bronchitic rats (airways disease induced by SO$_2$ exposure). Because it is likely that PM concentration and composition vary dependent on the atmospheric conditions and the season, and can influence the health outcome, we elected to conduct the study on four occasions at different times of the year to determine the consistency of observed health effects, and how that relates to water-leachable constituents such as sulfate and metals. Sulfate and minerals are considered primary causative constituents of combustion source fine PM (National Research Council, 1998). The study shows that under some conditions, the bronchitic rat model exhibits increased pulmonary injury from CAPs exposure (a response is not readily repeatable at different times), and that leachable sulfate and metal content do not seem to correlate with the observed effects.

**MATERIALS AND METHODS**

**Animals.** Male Sprague Dawley (SD) rats, 90 days of age, were purchased from Charles River Laboratories (Raleigh, NC) in three different shipments and were divided into six studies (Table 1). Rats used in the studies #A and #F (defined below) were acclimatized for 1 week in an AAALAC-approved animal facility (21 ± 1°C, 50 ± 5% relative humidity, 12:12 h light/dark cycle), where they were housed in plastic cages with pine shavings bedding, then relocated to exposure chambers for an additional 2 days of acclimatization in wire mesh cages. Rats used for the remaining studies were housed in exposure chambers with wire mesh cages for the entire acclimation and exposure periods. All animals received standard Purina rat chow (Brentwood, MO) and water *ad libitum* except during exposures.

**Development of bronchitis.** Rats in each shipment were divided in two groups for which experiments began 1 week apart (Fig. 1). This arrangement was made to maintain consistency in bronchitis model development and to utilize the PM concentrator exposure unit at full capacity. This also allowed exposure of reasonable numbers of healthy and bronchitic rats at one time to CAPs for fair statistical evaluation of each study separately.

Rats were housed individually in stainless steel wire mesh cages placed in 422-liter stainless steel Rochester-type inhalation exposure chambers. Clean air (healthy rats) or SO$_2$ exposures (bronchitic rats) for each study began between 8 and 9 A.M. for 6 h, 5 days/week. Food and water were not provided during this exposure period. At all other times, rats were exposed to clean air and provided standard Purina rat chow (Brentwood, MO) and water *ad libitum*.

**TABLE 1**

Experimental Design Showing Study Designation, Exposure, Postexposure Analysis Time and Number of Rats Used per Group

<table>
<thead>
<tr>
<th>Study designation$^a$</th>
<th>Exposure</th>
<th>Analysis time, post exposure</th>
<th>Air + Air</th>
<th>Air + CAPs</th>
<th>SO$_2$ + Air</th>
<th>SO$_2$ + CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CAPs</td>
<td>0 h</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>F</td>
<td>CAPs</td>
<td>18 h</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>E</td>
<td>ROFA</td>
<td>0 h</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>CAPs</td>
<td>0 h</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
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<td>4</td>
</tr>
<tr>
<td>D</td>
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<td>0 h</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

$^a$ Study designations listed in the first column are in the order that the studies were performed.

$^b$ At 18 h post CAPs exposure, no CAPs-related changes were apparent in BALF of rats, and therefore this time point was not repeated and the toxicity data for this group of rats are not given. Rats were ordered in three batches (batch 1 for A and F studies; batch 2 for E and B studies, and batch 3 for C and D studies). All rats in each batch were divided in two groups to begin SO$_2$ exposures 1 week apart for each study.
Anhydrous grade SO₂ (Air Products, Allentown, PA) was metered by a Tylan mass flow controller (FC-260, Tylan General, Torrence, CA) and mixed with clean air stream (scrubbed through water-charcoal and HEPA filter) prior to the metering orifice. Chamber flow allowed 14 ± 1 air changes/h. Chamber temperature, relative humidity, and static pressure ranges were 72 ± 3°F, 41 ± 10, 0.5 ± 0.2 inches H₂O, respectively, for all exposures. Chamber concentrations of SO₂ were measured continuously using a long-path dispersive infrared spectrophotometer (Mirax 1A, Foxoro Company, East Bridgewater, MA) and calibrated using a closed-loop calibration method. The chamber concentration was maintained at 200 ppm for each day and the standard deviation never exceeded 10% of the target concentration. The SO₂ exposures began in the middle of the week (Wednesdays) and were conducted 6 h/day, 5 days/week for about 6 weeks. During the week 6, SO₂ exposures were continued over the weekend (Fig. 1), as CAPs exposure depended on ambient weather and they were set to begin 1 day post SO₂. During SO₂ exposure, rats were weighed twice per week to determine overall health in the chambers. Many incidences of sneezing were noted in rats exposed to SO₂ but not in control rats exposed in identical chambers to clean air.

**Concentrated ambient particle (CAPs) exposure.** One day following the last air (healthy) or SO₂ (bronchitic) exposure, rats of each category were randomized into two groups by first sorting them from low weight to high weight. Then starting with the lowest weights, animals equal to the number of groups were selected. These animals were randomly (based on computer random number generator) placed in a group. This process was repeated with the next lowest body weight animals until all animals were used and all groups were filled. One group was exposed to clean air and the other was exposed to CAPs using the concentrator exposure unit (Table 1). The concentrator exposure unit utilizes virtual impactor technology in which massive airflow (~4000 l/min) through a series of narrow slits allows minor flow containing ambient particles of ~0.1 to 2.5 μm to be concentrated (Sioutas et al., 1995). The virtual impacters were set up in a bleed flow manner where only 20% of the total flow exits the impacter at each stage, resulting in a concentration factor including slit losses of about 3× at each stage. A series of 4 virtual impacters produced ambient PM2.5 concentration enhancement of about 40 times in the chamber. Air containing real-time CAPs was directed through a 80-liter stainless steel and glass exposure chamber with 7.5 air changes/h. This chamber accommodated a maximum of nine rats in individual wire mesh cages (one layer) to be exposed to CAPs. Chamber temperature and relative humidity were 80 ± 2°F and 68 ± 2%, respectively. Each study included exposure of five healthy and four bronchitic rats to CAPs for 6 h/day. Three healthy and four bronchitic rats were simultaneously exposed to filtered nonconcentrated ambient air in a similar manner. All animals were weighed before and after the CAPs or filtered air exposure to determine health status. The ambient concentrator exposure unit could not be operated during unfavorable weather conditions (i.e., rain) and therefore, although the exposures were planned for 6/day for 3 consecutive days, in some cases, the exposures were conducted only for two consecutive days.

**PM mass and elemental analysis.** PM samples were collected on pre-weighed Teflon filters (Gelman, Teflon 47mm diameter, 2 μm; Gelman Sciences, Ann Arbor, MI) for the duration of each exposure. At the end of exposure, filters were weighed using a microbalance (Cahn-C33; Ryan Research, Beverly, ME). Concentrations were determined by sample mass/sample flow volume (μg/m³). Aerosol size distribution in the CAPs system inlet was determined for each exposure by an eight-stage MOUDI (MSP, Minneapolis, MN) cascade impactor. Chamber temperature, relative humidity, airflow, and pressure were monitored continuously.

Each filter was individually extracted in 8.0 ml distilled water for 1 h by continuous agitation. The filtrates were centrifuged at 17000 × g for 30 min and filtered through a teflon syringe filter, then acidified to a pH of 2.0 using concentrated HCl to keep soluble metal salts in soluble form (0.1 μM). The acidified filtrates were analyzed for presence of sulfur, zinc, iron, vanadium, nickel, manganese, and copper using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES), as described in Kodavanti et al. (1998b).

For calibration of the instrument, metals calibration standard (100 ppm multi element standard; p/n QC-21) and sulfate calibration standard (1000 ppm standard; p/n AS-SO₄9-2X/2Y) from SPEX Certiprep (Metuchen, NJ) were used. Quality control (QC) standards used to check calibration standards were from VHG Labs, Manchester, VT. For metals, the calibration standard is 10 ppm multielement standard; p/n LCAL6020–100, and for sulfate, calibration standard is 3000 ppm standard; p/n ASW-100. The minimum detection limits for filter extract solutions were sulfate = 0.7 μg/ml, zinc = 3 ng/ml, manganese = 1 ng/ml, iron = 10 ng/ml, and copper = 8 ng/ml. In some filter extracts, the levels or iron and copper were below the detection limit, whereas sulfate, zinc, and manganese were clearly detectible in all samples.

**Nose-only residual oily fly ash (ROFA) inhalation exposure.** In one study (#E) healthy or bronchitic rats were exposed to a dry aerosol of ROFA by nose-only inhalation (6 h/day × 3 days) at a concentration of 1 mg/m³ (Ledbetter et al., 1997). Briefly, the generator, designed on the principle of a carpenter’s chalk line, used a continuously moving string to carry adherent particles upward from a reservoir containing ROFA. Aerosolization was accomplished using compressed air to pulse-dislodge particles from the string into the dilution airstream. The ROFA-laden air was directed through a 2-mCi ⁸⁵Kr charge neutralizer/mixing tube (Thermal Systems, Inc., St. Paul, MN), then into a four-row, 24-port, nose-only exposure chamber. Each exposed animal was restrained in a conical, plastic restrainer. All rats were acclimatized to this setup 3 days) at a concentration of 1 mg/m³ (Ledbetter et al., 1997). Briefly, the generator, designed on the principle of a carpenter’s chalk line, used a continuously moving string to carry adherent particles upward from a reservoir containing ROFA. Aerosolization was accomplished using compressed air to pulse-dislodge particles from the string into the dilution airstream. The ROFA-laden air was directed through a 2-mCi ⁸⁵Kr charge neutralizer/mixing tube (Thermal Systems, Inc., St. Paul, MN), then into a four-row, 24-port, nose-only exposure chamber. Each exposed animal was restrained in a conical, plastic restrainer. All rats were acclimatized to this setup.

Either within 3 h post CAPs (studies #A–#D) or ROFA (study #E), or 18 h post CAPs (study #F), rats were anesthetized with sodium pentobarbital (Nembutal, Abbott Lab., Chicago; 50 –100 mg/kg body weight, ip) and exsanguinated via the abdominal aorta. The tracheas were cannulated, and the left lungs were ligated. The right lung was lavaged using phosphate-buffered saline (pH 7.4) at a volume of 28 ml/kg body weight (approximately 75% total lung capacity). Three in-and-out washes were performed using the same fluid. Following the lavage, the right lung was ligated and the left lung bronchus was opened. The left lung was then fixed through tracheal infusion of 4% buffered paraformaldehyde at a volume based on 28 ml/kg total lung capacity and the left lung being 40% of the total lung mass. The trachea was tied and the lung was submerged in a jar containing 4% paraformaldehyde for histologic evaluation. One aliquot of lavage fluid was used for determining total cells using...
a Coulter Counter (Coulter, Inc., Miami FL), and a second aliquot was centrifuged using a Shandon 3 Cytospin (Shandon) for preparing cell differential slides. The slides were dried at room temperature and stained with LeukoStat (Fisher Scientific Co., Pittsburgh, PA). Macrophages, neutrophils, eosinophils, and lymphocytes were quantitated using light microscopy (200 cells/slide).

The remaining BALF was centrifuged at 1500 \( \times g \) to remove cells, and the supernatant fluid was analyzed for protein, albumin, N-acetyl glucosaminidase (NAG) activity, and lactate dehydrogenase (LDH) activity. Assays for protein, albumin, NAG, and LDH activity were modified and adapted for use on a Hoffmann-La Roche Cobas Fara II clinical analyzer (Roche Diagnostics, Branchburg, NJ). Total protein content was determined using a Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. BALF samples were analyzed for albumin content using a commercially available kit and controls from ICN Star Corporation (Stillwater, MN). NAG activity was determined using a kit and controls from Boehringer Mannheim Corporation Products (Indianapolis, IN).

**Histopathology.** After fixation, the left lung tissues were embedded in paraffin and 4-\( \mu \)m thick transverse sections were mounted and stained with hematoxylin and eosin (Experimental Pathology Laboratory, Research Triangle Park, NC). Pathology evaluations were made in a nonblinded fashion by Dr. Peter Mann (Experimental Pathology Laboratory, Research Triangle Park, NC) in studies #A, #C, #D, and #F.

**Statistics.** The data were analyzed using a two-way analysis of variance (ANOVA) model. The independent variables were model (healthy or bronchitic) and exposures (air or CAPs). Pairwise comparisons were performed as subtests of the overall model. Effects and comparisons were declared significant if the \( p \)-value was \(< 0.05 \). Adjustment in the significance level, for multiple comparisons, was made using a modified Bonferroni correction. The \( p \)-value of \( \leq 0.05 \) was indicated by an asterisk (*) for comparisons between healthy:air and healthy:CAPs or bronchitic:air and bronchitic:CAPs groups; and as † for comparisons between healthy:air and bronchitic:air or healthy:CAPs and bronchitic:CAPs groups.

**RESULTS**

**Development of Bronchitis-like Disease in Rats Exposed to \( \text{SO}_2 \)**

Exposure of rats to 200 ppm \( \text{SO}_2 \), 6 h/day, 5 days per week (bronchitic:air and bronchitic:CAPs) resulted in mucus hypersecretion, and goblet cell hyperplasia and hypertrophy (Fig. 2). BALF contained a visible semitranslucent mucus layer that was apparent upon centrifugation of BALF. Later in the exposures to \( \text{SO}_2 \), especially toward the weekends, audible clicking could be heard upon opening the chamber doors, suggesting a cough or sneezelike response involving excess mucus. Histologic examination of the lung tissue sections with PAS positive stain revealed hyperplastic goblet cells containing purple-stained mucus in some but not all of the major airways as well as bronchioles. Occasionally, an airway with sloughed ciliated cells and congestion was also present. Chronic airway inflammation and thickening, associated with human bronchitis, appeared to be minimal in these rats after 6 weeks of \( \text{SO}_2 \) exposure followed by 2–3 days of clean air exposure. The alterations seen in \( \text{SO}_2 \)-exposed rats were minimal in filtered air-exposed control rats.

**CAPs-Induced Lung Injury in Healthy and Bronchitic Rats**

This study included six experiments that were conducted over a period of 6 months, where any given two were done at one particular time (Fig. 1, Table 2). This manipulation was made to accomplish maximum usage of available space in the concentrator exposure chamber. In four of the studies, rats were euthanized immediately following 2- or 3-day exposure to CAPs (Table 1; studies #A–#D). In one study, rats were...
TABLE 2

<table>
<thead>
<tr>
<th>Study</th>
<th>CAPs exposure days</th>
<th>CAPs exposure dates</th>
<th>CAPs concentration, µg/m³</th>
<th>Leachable sulfate, ng/µg CAPs</th>
<th>Leachable zinc, ng/µg CAPs</th>
<th>Leachable manganese, ng/µg CAPs</th>
<th>Leachable iron, ng/µg CAPs</th>
<th>Leachable copper, ng/µg CAPs</th>
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<td>11/11/97</td>
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<td>15.7</td>
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<td>531</td>
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Note. Each study is listed in the order that it was done. During CAPs exposure, samples were collected on Teflon filters, weighed and separated in distilled water by agitation (see Materials and Methods). Elemental and sulfate analysis of water-leachable components of CAPs was done using ICP-AES. In some filter extracts, the levels of iron and copper were below the minimum detection limit before multiplication by the dilution factor. 

† In this study rats were sacrificed 1 day following last CAPs exposure while in all other studies rats were sacrificed immediately after the last CAPs exposure and therefore, this group is not included in figures. The BALF injury data of this study show no CAPs related changes.

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exposed to CAPs for 3 days and allowed 18 h recovery (study #F). In the sixth study (#E), rats were exposed nose-only to ROFA particles at 1 µg/m³, 6 h/day for 3 consecutive days and euthanized immediately to ascertain whether ROFA and CAPs produce a similar pulmonary injury response.

A number of indices were analyzed in BALF to determine the extent of pulmonary injury. Statistically, no increases in BALF markers of lung injury and inflammation were noted in healthy or bronchitic rats that were allowed 18 h recovery following air/CAPs exposure. Therefore, the data are not shown for this group (study #F), and this time point analysis was not repeated in the remainder of the CAPs studies. However, based on the variable response seen in all CAPs studies, ROFA at 1 µg/m³ caused only a modest change. The data for the ROFA group are given for comparison. BALF biochemical and inflammation indices were compared among studies #A–#D to determine repetitiveness of observed effects.

BALF protein levels in bronchitic rats subsequently exposed to clean air were not higher than in healthy rats exposed to clean air in any of the studies. Exposure to CAPs or ROFA did not result in increased BALF protein in healthy rats. However, in one study (#A), exposure to CAPs was associated with a significant increase in BALF protein in bronchitic rats when compared to air-exposed bronchitic rats; and there was a modest increase in another study (#D) (Fig. 3). BALF albumin followed the same pattern of changes as protein in all studies (Fig. 4). BALF LDH activity did not reveal any significant increase associated with either CAPs or ROFA exposure in any study (Fig. 5), except that there appeared to be an increase (nonsignificant) in study #A (Fig. 5) following CAPs exposure in both healthy and bronchitic rats. BALF NAG activity, measured as one of the indicators of pulmonary injury, was not increased in healthy rats exposed to CAPs but was increased significantly in three of the four CAPs exposures in bronchitic rats (CAPs exposed healthy rats vs CAPs exposed bronchitic rats; Fig. 6). ROFA exposure did not increase NAG activity in healthy or bronchitic rats (Fig. 6, study #E).

BALF total cells were counted and cell differentials were performed to evaluate inflammatory response due to bronchitis or due to CAPs exposure. Total numbers of cells recovered in the BALF were not significantly increased following CAPs or ROFA exposure in either healthy or bronchitic rats (Fig. 7). In two of the studies, bronchitic rats exposed to air appeared to have higher number of neutrophils compared to healthy rats exposed to air (Fig. 8; study #A, p = 0.15 and #B, p = 0.19). CAPs exposure of bronchitic rats was associated with increase in BALF neutrophils in two of four studies when compared to healthy rats exposed to CAPs (#A and #D). However, in #B, neutrophil counts in bronchitic:air appeared to be higher than bronchitic:CAPs (p = 0.18). ROFA exposure was also associ-
ated with a mild neutrophilic inflammatory response in bronchitic rats; however, because of the limited number of observations in each study and within group variability, the level of significance was not reached.

Histologic lesions were evaluated in the lung following CAPs exposure of healthy and bronchitic rats (studies #A, #C, #D, and #F). As described earlier, exposure of rats to SO\textsubscript{2} (bronchitic:air and bronchitic:CAPs groups) was associated with mucus hypersecretion, bronchial goblet cell hyperplasia, and a slightly increased edema. However, alveolar, peribronchial, and perivascular inflammatory cell infiltration did not appear to be different from that of healthy controls (air:air and air:CAPs groups) (Tables 3 and 4). Exposure of healthy and bronchitic rats to CAPs seemed to cause a slight increase in edema, goblet cell hyperplasia, and perivascular, focal mononuclear cell infiltration (Tables 3 and 4). These effects of CAPs appeared to be slightly greater in bronchitic rats when compared to healthy rats; however, statistical evaluation was not done with histology analysis.

Elemental Analysis of CAPs Collected on Filters

To quantitate and determine the leachable elemental composition of CAPs, CAPs samples were collected on filters. Either one or two filters were collected on each day of exposure. The data in Table 2 represent the mean value from combined filters of the same day. Depending on the time of the year and the weather condition, the concentrations of CAPs varied between each day and each study (Table 2). ICP-AES analysis of these CAPs showed that there were significant amounts of sulfate present in these filters, ranging from 148 µg/mg to 458 µg/mg CAPs. Previously characterized ROFA used in one of the studies had only slightly higher sulfate levels (531 µg/mg) than maximum levels present in CAPs, suggesting that ambient fine PM mass is associated with high levels of sulfate and may originate from combustion sources emitting sulfate in the air. Of all the metals analyzed, only a few were detectable using ICP-AES (Table 2). The amount of water

FIG. 3. Repetitive CAPs exposure studies showing changes in BALF protein as an index of lung injury in healthy and bronchitic rats. In each study, rats were euthanized immediately following the last particle exposure and BALF was analyzed for lung injury markers. Values represent mean ± SE of 3–5 rats. Asterisk indicates significant difference (p ≤ 0.05) between healthy:air and healthy:CAPs or bronchitic:air and bronchitic CAPs. Cross indicates significant difference (p ≤ 0.05) between healthy:air and bronchitic:air or healthy:CAPs and bronchitic:CAPs.

FIG. 4. Repetitive studies showing BALF albumin in healthy and bronchitic rats exposed to CAPs or ROFA for 2–3 days. In each study, rats were euthanized immediately following the last particle exposure and BALF was analyzed for lung injury markers. Values represent mean ± SE of 3–5 rats. Asterisk indicates significant difference (p ≤ 0.05) between healthy:air and healthy:CAPs or bronchitic:air and bronchitic CAPs. Cross indicates significant difference (p ≤ 0.05) between healthy:air and bronchitic:air or healthy:CAPs and bronchitic:CAPs.
leachable zinc present on CAPs was similar to what was present in water extracts of ROFA (Table 2). However, zinc was only a minor component of ROFA, as vanadium, nickel, and iron constitute the predominant metal mass of ROFA. Zinc levels of CAPs filters ranged from 0.87 to 1.857. Although the levels were low, consistency in its detection shows that zinc may represent one of the critical metal constituents of ambient PM in Research Triangle Park, NC. Manganese, iron, and copper were also detectable in water extracts of filters; however, these metals varied significantly between samples.

**DISCUSSION**

This study examined pulmonary injury from real-time CAPs in a rat model of SO₂-induced bronchitis, consistency of responsiveness in repetitive exposure studies, and the leachable sulfate and elemental composition of CAPs in each study. CAPs effects as determined by histologic examination of the lung and BALF markers of pulmonary protein leakage/cell injury and inflammation were noted in one study, with a suggestion of effects in second study, and only a modest effect in the other two studies. Elemental analysis of leachable components of CAPs indicated presence of sulfate and zinc. Other metals of potential anthropogenic origin such as vanadium, iron, manganese, nickel, and copper were variable and present in smaller quantities. There appears to have been no association between the levels of CAPs or its leachable sulfate/elemental composition and the responsiveness of bronchitic rats.

SO₂-induced bronchitis in the rat has been considered a model of chronic human bronchitis because mucus hypersecretion and chronic mucus cell metaplasia are persistent and progressively increased during the course of SO₂ exposure (Farone et al., 1995; Knauss et al., 1976). The concentrations of SO₂ used for developing bronchitis range from 200 to 600 ppm, depending on animal species and strain. Increased mucus production and goblet cell hyperplasia have been noted with this concentration range when the duration of exposure is 4–8 weeks (5 days/week). These characteristic features of bronchitis were readily apparent in the present study. However, the results of this study show that chronic airways inflammation and

**FIG. 5.** Lactate dehydrogenase (LDH) activity in BALF of healthy and bronchitic rats exposed to CAPs (four studies) or ROFA (one study) for 2–3 days. In each study, rats were euthanized immediately following the last particle exposure and BALF was analyzed for lung injury markers. Values represent mean ± SE of 3–5 rats.

**FIG. 6.** Repetitive studies showing N-acetyl glutaminidase (NAG) activity in BALF of healthy and bronchitic rats exposed to CAPs or ROFA for 2–3 days. In each study, rats were euthanized immediately following the last particle exposure and BALF was analyzed for lung injury markers. Values represent mean ± SE of 3–5 rats. Asterisk indicates significant difference ($p \leq 0.05$) between healthy:air and healthy:CAPs or bronchitic:air and bronchitic CAPs. Cross indicates significant difference ($p \leq 0.05$) between healthy:air and bronchitic:air or healthy:CAPs and bronchitic:CAPs.
fibrosis, which are critical pathophysiologic features of human bronchitis (Jeffery, 1991; Thurlbeck, 1990), were not readily apparent or persistent in rats following 200 ppm (used in this study) or 250 ppm SO\(_2\) exposure (data not shown). It is likely that, as in human bronchitis (Fietta et al., 1988; Jansen et al., 1995), host defense mechanisms involved in the clearance of particles and pathogens are impaired in the SO\(_2\)-induced model; however these responses are not well characterized. Thus, the rat model of SO\(_2\)-induced bronchitis could be considered a model of acute airways mucus hypersecretion and goblet cell metaplasia. We chose to expose rats to 200 ppm SO\(_2\) for a longer duration because this may allow relatively milder but likely more consistent and persistent mucus production. A similar protocol and rat strain have previously been employed in PM deposition (Sweeney et al., 1995) and PM susceptibility studies (Clarke et al., 1999).

This report demonstrates that CAPs exposure can result in pulmonary injury in an animal model of preexistent disease. The congestion and cellularity appeared to be increased in histologic evaluation. BALF markers of lung injury (protein, albumin, NAG activity) and neutrophilic inflammation seemed to be slightly increased only in bronchitic rats following CAPs exposure. It is likely that airway epithelial cells, stimulated via SO\(_2\) exposure to produce excess amounts of inflammatory mediator cytokines, elicit greater inflammatory and pulmonary injury response upon exposure to ambient particles that likely contain sulfate, metals, organics, and biologic materials. It has been presumed that in vitro macrophage activation occurs more readily when metals of ambient particles interact with biologic materials in a synergistic manner (Imrich et al., 1999). Previous reports have shown that PM deposition patterns in rats with SO\(_2\)-induced bronchitis differ from those of healthy rats such that focal areas of heavy deposition are evident, presumably due to airway mucus and altered deposition mechanics (Sweeney et al., 1995). Recently Clarke et al. (1999) have shown that bronchitic rats are susceptible to pulmonary injury caused by ambient particles in a relatively more industrialized and populated area in Boston, Massachusetts. The presence of detectable pulmonary injury from CAPs exposure could have been due to increased focal deposition of CAPs within the lung. Alternatively, clearance of

FIG. 7. Total lavageable cells in BALF of healthy and bronchitic rats exposed to CAPs (four studies) or ROFA (one study) for 2–3 days. In each study, rats were euthanized immediately following the last particle exposure and BALF cells were counted using a Coulter Counter. Values represent mean ± SE of 3–5 rats.

FIG. 8. Repetitive studies showing number of neutrophils in BALF of healthy and bronchitic rats exposed to CAPs or ROFA for 2–3 days. In each study, rats were euthanized immediately following the last particle exposure and BALF neutrophils were counted from a stained Cytospin slide. Values represent mean ± SE of 3–5 rats. Cross indicates significant difference (p ≤ 0.05) between healthy:air and bronchitic:air or healthy:CAPs and bronchitic:CAPs.
particles via ciliary transport may also have been impaired in the rats with bronchitis, as SO$_2$ exposure has been shown to damage ciliary cells lining the airways (Asmundsson et al., 1973; Reid, 1970). How extra mucus would affect the dissolution and transport of ambient particles to pulmonary epithelium, leading to increased vascular leakage and inflammation, is not known.

Although the epidemiologic evidence of PM and associated human health effects is consistent regardless of location (suggesting varied composition of PM) and the time of the year, variability in responsiveness is often encountered in humans and animal models studies (Bertranpetit and Calafell, 1996; Kodavanti et al., 1999). To determine variability of pulmonary response to inhaled CAPs in bronchitic rats, the study was repeated four times during the fall/winter and the spring of 1997. CAPs exposure was associated with significant pulmonary vascular leakage and inflammation in one of the four studies, with a trend (not significant) in the other two studies that was not consistent with the maximum levels of CAPs achieved during the exposure. A borderline effect was noted in some of the BALF parameters in the third study, indicating considerable variability in the response to CAPs at different times during the fall/winter and the spring. The following speculations can be made regarding variability of the pulmonary response: a) the composition of CAPs collected at different times was likely different; b) the bronchitic response in rats obtained at different times could quantitatively vary in such a way that the degree of mucus hypersecretion and lesions present at the beginning of CAPs exposure was sufficiently different at different seasons to cause variation in the host responsiveness to CAPs. It is noteworthy that healthy animals did not show detectible CAPs effects.

The presence of significant amounts of leachable sulfate and zinc (20–50% of total mass) suggests that the fine fraction of ambient PM may originate from anthropogenic combustion sources. Burning of sulfur-containing fossil fuel has been shown to contribute to the fine particulate fraction of many locations in the eastern United States (Lippmann and Thurston, 1996). High sulfate concentrations in the ambient air, including the London fog episode, has been frequently associated with

### TABLE 3
Severity Incidence Table Showing Number of Rats with Changes in the Pulmonary Pathology

<table>
<thead>
<tr>
<th>(Total number of lungs examined)</th>
<th>Air + Air (12)</th>
<th>Air + CAPs (20)</th>
<th>SO$_2$ + Air (16)</th>
<th>SO$_2$ + CAPs (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar macrophage accumulation, focal</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Alveolitis, focal</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Congestion</td>
<td>3</td>
<td>8</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Goblet cell hyperplasia, bronchus</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Peribronchial infiltration, mononuclear cell</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Perivascular infiltration, polymorphonuclear cell, focal</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Perivascular infiltration, mononuclear cell, focal</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Subpleural infiltration, mononuclear cell, focal</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Note. Hematoxylin and eosin stained lung tissue sections of studies A, E, C, and D were evaluated by a pathologist (Experimental Pathology Labs, Research Triangle Park, NC) and the data in the table shows combined number of animals used and affected. Among all rats in the group, the number of rats showing pathology were identified by a pathologist. The values in the table for specific indices reflect the number affected per total number of rat lungs within the group.

### TABLE 4
Histopathology Incidence Showing Severity Score Normalized per Rat

<table>
<thead>
<tr>
<th>(Total number of lungs examined)</th>
<th>Air + Air</th>
<th>Air + CAPs</th>
<th>SO$_2$ + Air</th>
<th>SO$_2$ + CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar macrophage accumulation, focal</td>
<td>0.33</td>
<td>0.15</td>
<td>0.38</td>
<td>0.25</td>
</tr>
<tr>
<td>Alveolitis, focal</td>
<td>0.17</td>
<td>0.20</td>
<td>0.19</td>
<td>0.06</td>
</tr>
<tr>
<td>Congestion</td>
<td>0.50</td>
<td>0.55</td>
<td>0.75</td>
<td>1.13</td>
</tr>
<tr>
<td>Goblet cell hyperplasia, bronchus</td>
<td>0.00</td>
<td>0.10</td>
<td>0.50</td>
<td>1.06</td>
</tr>
<tr>
<td>Peribronchial infiltration, mononuclear cell</td>
<td>0.17</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Perivascular infiltration, polymorphonuclear cell, focal</td>
<td>0.00</td>
<td>0.00</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>Perivascular infiltration, mononuclear cell, focal</td>
<td>0.50</td>
<td>0.35</td>
<td>0.31</td>
<td>0.63</td>
</tr>
<tr>
<td>Subpleural infiltration, mononuclear cell, focal</td>
<td>0.17</td>
<td>0.15</td>
<td>0.19</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Note. Hematoxylin and eosin stained lung tissue sections of studies A, E, C, and D were evaluated by a pathologist (Experimental Pathology Labs, Research Triangle Park, NC) and the data in the table show an average severity score given to a rat from a total number of animals used. Histopathology severity scoring was given to all individual rats where 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, and 5 = severe. Mean individual severity within a group was calculated by adding severity scores of all animals within a group and dividing that by total number of animals.
the incidence of bronchitis and also the exacerbation of bronchitic symptoms in humans (Abbey et al., 1998; Schremk et al., 1949). This study utilized a rat model of bronchitis to support the epidemiologic association of increased morbidity with ambient PM containing sulfur. It is likely that sulfur exists largely as sulfate (Stevens, 1986) associated in part with the bioavailable metals, because metals such as zinc and manganese were detectible in extracts of CAPs samples. Ammonium ion is the most common ligand for sulfates, but this was not measured. No apparent relationship could be established between pulmonary injury and the concentration of CAPs achieved during the exposure or its leachable metal or sulfate content. Because ambient particles contain many other nonleachable and leachable components (e.g., organics, biologicals), it is possible that those components play a role in biologic responsiveness. Many studies need to be done in order to determine specificity and interactions of causative constituents of real ambient PM from different origins in causing pulmonary response.

Our previous studies using ROFA combustion PM have shown that the metallic constituents of the material were responsible for lung injury in healthy rats (Kodavanti et al., 1997, 1998b). ROFA contains significant quantities of leachable sulfur, nickel, vanadium, and iron; biologic contamination has been shown to be negligible (Dreher et al., 1997). In order to determine similarities and differences in pulmonary response from CAPs and ROFA, in an additional study, healthy and bronchitic rats were exposed nose-only to ROFA at 1 mg/m³ 6 h/day for 3 consecutive days. The lack of response in the bronchitic rats following ROFA exposure and a positive response with CAPs at a similar concentration suggest that there may be components present in CAPs that are more hazardous than anticipated based on bioavailable metal composition. It is also likely that biologic organic constituents synergistically interact with CAPs components to elicit pulmonary response (Imrich et al., 1999). Additionally, host responsiveness of bronchitic rats, depending on the degree or state of the impairment, may modulate pulmonary injury or response to different PM constituents. Constituents such as organics and biologicals were not determined in the present study because of the lack of sufficient sample. Thus, what caused the pulmonary responses in bronchitic rats remains to be investigated.

In summary, real-time CAPs exposure is associated with detectable pulmonary injury in a rat model of relatively mild bronchitis. However, the response is variable if the study is conducted at different times of the year. There seems to be no apparent association between CAPs concentrations achieved, or its leachable sulfate or metal, and the extent of pulmonary injury.

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