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# Toxicological Interactions in the Respiratory System after Inhalation of Ozone and Sulfuric Acid Aerosol Mixtures

## Michael T. Kleinman and Robert F. Phalen

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**A factorial design study was performed to examine the acute effects of inhaled acid particles alone and in mixtures with ozone to test the hypothesis that acid particles and ozone would act synergistically. Sprague-Dawley rats were exposed nose-only for a single 4-h period to all 9 possible combinations of purified air and 2 concentrations each of $O_3$ (0.3 and 0.6 ppm) and submicrometer (0.3 $\mu$m mass median diameter [MMD]) sulfuric acid aerosols $H_2SO_4$ (0.5 and 1.0 mg/m$^3$). Respiratory-tract injury and impairment of alveolar macrophage functions were evaluated. Two-way analyses of variance were used to test for significance of main effects and statistical interactions, and Tukey multiple comparison tests were used to test the significance of differences between group mean values. Addition of $H_2SO_4$ to $O_3$-containing atmospheres resulted in significant $H_2SO_4$ concentration-dependent reductions in $O_3$-induced inflammatory responses, and $H_2SO_4$, alone and in combination with $O_3$, depressed some functions of innate immunity. DNA synthesis in nasal, tracheal, and lung tissue following pollutant exposure, which is an index of injury or killing of epithelial cells, was significantly increased by $O_3$ but not by $H_2SO_4$ when administered alone, compared to purified air. When administered with $O_3$, $H_2SO_4$ did not reduce the effects of $O_3$ on DNA synthesis in the trachea or the lung, but did reduce the DNA synthesis response to $O_3$ in the nose. No significant changes in antibody-directed Fc receptor (FcR) binding of sheep red blood cells by alveolar macrophages were observed, but macrophage phagocytic activity was significantly reduced by the pollutant exposures. In summary, the results of this study indicate significant interactions between $O_3$ and $H_2SO_4$ in concurrent exposures; however, the findings do not support the hypothesis that $O_3$ and $H_2SO_4$ act synergistically in rats after single 4-h exposures.**

Interactions between components of air pollutant mixtures can lead to synergism (in which the action of two agents combined is greater than the sum of each acting alone), additivity, or antagonism. Whether or not interactions are observed may depend on the endpoint that is examined. Although some strategies exist for addressing interactions of contaminants in occupational exposures to multiple chemicals, such strategies have not in general been applied to the protection of public health from exposures to pollutants in ambient air, primarily because sufficient data are not yet available.

Human population-based studies have associated air pollution exposure with adverse health effects and, in some instances, have suggested increased effects when individuals were exposed to both oxidant gases (predominantly $O_3$) and acidic particles (predominantly acidic sulfur oxides) (Lippmann & Schlesinger, 1984; Lippmann, 1989). Some short- and intermediate-term animal toxicological studies have demonstrated significant synergistic interactions with respect to increased susceptibility to infection (Gardner et al., 1977; Grose et al., 1982), respiratory-tract cell damage (Juhos et al., 1978; Bhalla et al., 1987; Kleinman et al., 1989; Chen et al., 1991), bronchoalveolar clearance and inflammatory responses (Wolff, 1986; Schlesinger, 1989; Schlesinger et al., 1992a), and changes in lung permeability and collagen composition (Last et al., 1986; Warren & Last, 1987; Last, 1991). However, some human studies (Horvath et al., 1987; Aris et al., 1991) and some studies using laboratory animal models (Cavender et al., 1977, 1978; Silbaugh &

Mauderly, 1986) have shown a lack of synergism, or even antagonistic effects (Schlesinger et al., 1992b; el-Fawal et al., 1995; Kimmel et al., 1997). Moore and Schwartz (1981) reported that long-term (180-day) exposures of rats to $H_2SO_4$ might protect against the inflammatory effects of $O_3$. Kleinman et al. (1999) showed, however, that repeated exposures of rats to ozone/acid mixtures blunted or blocked the attenuation of inflammatory responses induced by repeated exposures to $O_3$, alone.

The objective of this present study was to systematically examine the effects of submicrometer acid particles, alone and in mixtures with $O_3$, and to test the hypothesis that synergistic interactions between $O_3$ and $H_2SO_4$ would be seen after single acute exposures. A factorial design study was performed in which Sprague-Dawley rats were exposed nose-only for a single 4-h period to all 9 possible combinations of purified air and 2 concentrations each of $O_3$ (0.3 and 0.6 ppm) and $H_2SO_4$ aerosols (0.5 and 1.0 mg/m$^3$). Endpoints were evaluated that were indicative of acute respiratory system inflammation and injury repair responses. These endpoints included: (1) area occupied by focal lesions in lung parenchyma; (2) DNA synthesis in nasal, tracheal, and lung epithelia, secondary to injury or killing of epithelial cells; and (3) alveolar macrophage innate immunological functions.

## MATERIALS AND METHODS

### Exposures

In total, 190 barrier-reared male Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA), 6 wk old, weighing approximately 200 g, were delivered to the laboratory in filter-equipped shipping containers. The rats were housed in a laminar-flow air-barrier caging system equipped with gas and particle scrubbers. Ten rats were randomly selected for quality control analyses. Their lungs were excised, examined grossly for the presence of cysts or nodules, and then formalin fixed and sectioned. The left lobe was cut longitudinally along the main bronchus, and paraffin sections were made from the cut face. The sections were evaluated for the presence of histopathologic markers of infectious disease. The quality-control animals showed no evidence of active respiratory disease. Other internal organs (liver, heart) were examined grossly but not microscopically. The rats were certified by the supplier to be free of the commonly encountered murine pathogens reovirus type 3, pneumonia virus of mice, encephalomyelitis, Sendai virus, mouse adenovirus, mouse hepatitis, Toolan-H1, Kilham rat virus, lymphocytic choriomeningitis, and sialodacryoadenitis virus.

The remaining 180 rats were randomly assigned to groups. These groups were exposed nose-only, at rest, for 4 h to (a) purified air, (b) 0.3 ppm $O_3$, (c) 0.6 ppm $O_3$, (d) 0.5 mg/m$^3$ $H_2SO_4$, (e) 1.0 mg/m$^3$ $H_2SO_4$, (f) 0.3 ppm $O_3$ + 0.5 mg/m$^3$ $H_2SO_4$, (g) 0.3 ppm $O_3$ + 1.0 mg/m$^3$ $H_2SO_4$, (h) 0.6 ppm $O_3$ + 0.5 mg/m$^3$ $H_2SO_4$, or (i) 0.6 ppm $O_3$ + 1.0 mg/m$^3$ $H_2SO_4$. The exposure temperature was $22 \pm 3°C$, and the relative humidity was $83 \pm 1\%$ (mean $\pm$ SD). Nose-only exposures were

performed because they eliminated fur and dander as potential atmospheric contaminants and prevented acid neutralization by excreta-generated ammonia. The nose-only system was designed to minimize the rat's discomfort by using a carefully engineered facial interface and by allowing for adequate thermal regulation of the rat during exposure (Phalen, 1997).

It was not possible to perform all of the exposures at a single time. In total, 15 histology rats and 5-macrophage function rats were exposed to each of the 9 atmospheres. The exposures were conducted in four sessions; rats were exposed to two of the pollutant containing atmospheres in each session. The control rat group was subdivided and three to four control rats were exposed to purified air during each of the four sessions.

### Atmosphere Generation and Characterization

Sulfuric acid particles (0.3 $\mu$m mass median diameter [MMD]) were generated by nebulizing dilute $H_2SO_4$ solutions into highly purified dilution air (1:10 dilution) using a Collison nebulizer (May, 1973; Ho et al., 1978). Aerosols were passed through a $^{85}$Kr discharger to reduce static charges to the Boltzmann equilibrium level. A proportional flow splitter directed appropriate fractions of the diluted aerosol to each of two exposure chambers, and the aerosol was further diluted with purified air to obtain the final $H_2SO_4$ concentrations (0.5 mg/m$^3$ and 1.0 mg/m$^3$). Ozone was generated from medical grade oxygen using a commercial ozonizer (Sander ozonizer, Type III; Osterberg, Germany). The ozone concentration was adjusted by changing the voltage applied to the ozonizers using variable transformers.

Ozone was measured using an ultraviolet absorption monitor (Dasibi model 1003-AH; Glendale, CA) which was calibrated against a transfer standard certified by the California Air Resources Board. An optical particle detector (GCA RAM-1; Bedford, MA) was used to measure relative particle concentrations, real time, during each exposure. An eight-stage cascade impactor (model 210K, Anderson Samplers; Atlanta, GA) was used to collect size-classified samples for gravimetric and chemical analyses. Aerosol samples were also collected on precleaned fluorocarbon-coated glass-fiber filters for gravimetric determinations and for chemical analyses of atmospheric components. Ion-exchange chromatography was used to analyze for sulfate ion in filter extracts. The hydrogen ion content of filter extracts was determined potentiometrically to ascertain the degree, if any, of acid neutralization prior to inhalation by the rats.

### Histopathological Measurements

Eighteen hours after the end of each pollutant or purified air exposure, the histology animals were injected intraperitoneally with tritiated thymidine ($^3$H-TdR; 2 $\mu$Ci/g of body mass). Twenty-four hours later (42 h postexposure) the rats were deeply anesthetized with sodium pentobarbital (80 mg/kg body weight ip) and exsanguinated via the dorsal aorta or femoral arteries. The left main bronchus was ligated at the hilum and the left lobe was cut free distal to the ligation. The lobe was

placed in ice-cold Liebowitz medium (a balanced salt solution buffered with amino acids) and reserved for subsequent DNA analyses. The remaining lung lobes were excised, inflated to 30 cm $H_2O$ pressure, and fixed by airway perfusion with 10% buffered formalin, pH 7.0, for 72 h (McClure et al., 1982). The trachea and larynx of each rat were dissected out and immersed in formalin. The heads were removed and the portions of the heads containing the intact nasal cavity were fixed in formalin and decalcified with neutral Tris-buffered 6% ethylenediamine tetraacetic acid (EDTA) for 3 wk. The trachea was split longitudinally, embedded in glycol methacrylate, and sectioned at 2 $\mu$m.

Specimens of the nasal cavity, 2 to 3 mm thick, were cut vertically through the hard palate and nasal septa at level II (Young, 1981), embedded in glycol methacrylate, and sectioned at 2 $\mu$m. DNA synthesis in tracheal and nasal sections, which can be used as an index of cell replication to replace pollutant-injured or killed epithelial cells, was evaluated using autoradiography (Evans & Bils, 1969; Kleinman et al., 1989). Slides were dipped in Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY), and exposed for 30 days in a light-tight refrigerated box. After exposure, the slides were developed with Kodak D-19, stained with toluidine blue, and examined by light microscopy. In the trachea, epithelial cells were quantitated by aligning a microscope eyepiece grid with the basement membrane and counting all cells, differentiating by cell type, within each of 10 grid spaces at a magnification of $\sim$800$\times$. The number of cells labeled with ^{3}H-TdR per unit grid space was expressed as the percent of labeled to total cells, by cell type. At minimum, 400 cells were scored for each rat. In the nasal sections, assay was performed on respiratory epithelium. Previous studies (Evans & Bils, 1969; Kleinman et al., 1989) had demonstrated that squamous and olfactory epithelial cells were relatively unaffected by $O_3$ exposures.

The volume of the fixed right middle lung lobe was measured gravimetrically by water displacement. Longitudinal sections of this lobe, after paraffin embedding, were cut at 6 $\mu$m thickness and stained with hematoxylin and eosin. In preliminary studies, we compared rat lung lobes for the distribution and volume fraction of inflammatory foci in the alveolar zone. There were no significant differences among lobes; hence, the middle lobe of the right lung was used routinely. Histologic sections were analyzed quantitatively by light microscopy using a modification of the morphometric process described by Elias and Hyde (1980). Each lung lobe section was scanned systematically at low microscopic magnification to record the number of times the scanning point overlaid predominantly alveolar areas made up of centriacinar units, as opposed to nonalveolar areas that contained structures including bronchi, blood vessels, and connective tissue. This process defined the alveolar and nonalveolar lung volume fractions. Regions with inflammation were differentiated from those of normal appearance; normal tissue exhibited delicate walls of alveolar septa, alveolar ducts, and terminal bronchioles, and neither free cells in the alveolar lumen nor excess cell nuclei in the walls of alveoli. The alveolar volume density was recorded and the systematic scan was repeated at higher magnification to record the frequency with which the scanning point overlaid areas of inflammatory lesion in centriacinar units in an alveolar zone. This frequency, divided by the alveolar volume density, was used to calculate the extent of lung injury, expressed as the volume density of centriacinar units with inflammatory lesions within the total volume of the alveolar zone. This datum was multiplied by 100 and recorded as the percent lesion area. All lesion area measurements were made without knowledge of the exposure. Lesions were characterized as follows:

Type 1: The criterion for type 1 lesion is the presence of pulmonary macrophages or shed epithelial cells in alveolar lumina. This is normally observed at relatively low frequency in control animals. It is the frequency of this "lesion," and hence the area of the tissue section involved, that is related to the pollutant exposure.

Type 2: The type 2 lesion consists of a region of contiguous alveolar septa, varying in extent, in which cells have assumed a cuboidal or ovoid shape, rather than the thinly extended form characteristic of type I alveolar cells and normal alveolar walls. The appearance is of thickened alveolar walls containing closely packed cells. The cell types present, as judged by both light and electron microscopy, include type I and type II pneumocytes, alveolar macrophages, and neutrophils in varying proportions. Pulmonary macrophages are frequently present in the alveolar spaces, as are occasional neutrophils. The lesions differ from those seen in pneumonitis caused by infectious agents in that lymphocytes are rarely seen in association with the lesion.

## DNA Synthesis in the Lung

The left lobe that had been reserved in ice-cold Liebowitz medium was analyzed for DNA. In brief, weighed samples of the fresh tissue were digested overnight at 37°C in 1 $N$ NaOH. The DNA, together with some proteins, was precipitated by adding 6 $N$ HCl to adjust the pH to 2.0, and the precipitate was washed twice with 1 $M$ NaCl, pH 2.0. The DNA in the precipitate was hydrolysed with 1 $N$ $HClO_4$ for 20 min at 70°C. After centrifugation, the clear supernatant was removed, an aliquot was taken for radioactivity determination, and the absorbance was measured at 266 nm. (This is the wavelength of peak absorbance for DNA in perchloric acid solution.) A standard solution of salmon sperm DNA in 1 $N$ $HClO_4$ gave an absorbance of 1.0 at a concentration of 50 $\mu$g DNA/ml. The absorbance of the DNA solution was a linear function of the concentration, at least up to an absorbance of 1.0, and the concentrations of all DNA solutions were adjusted by dilution with 1 $N$ $HClO_4$ to fall below this value. The number of disintegrations per minute of tritium (^{3}H-dpm) was determined using liquid scintillation counting, with appropriate standards, and reported as ^{3}H-dpm/$\mu$g DNA.

## Macrophage Fc Receptor Binding and Phagocytic Activity

Immediately following exposure, rats assigned for macrophage function assay were deeply anesthetized with a lethal dose of sodium pentobarbital (65 mg/kg ip), a slit incision was made in the top of the trachea and a saline-rinsed cannula (2 cm long, 2 mm OD, 1 mm ID) was inserted. The lungs were lavaged twice with 7 ml of $Ca^{2+}$ and $Mg^{2+}$-free pH 7.3 HEPES-buffered Hanks balanced salt solution (HHBS). Following lavage, rats were euthanized by exsanguination. The lavage fluid was transferred to a 15-ml polystyrene centrifuge tube and centrifuged at 2000 rpm (300 × 9g) for 10 min. The supernatant solution was reserved for subsequent analyses, the cell pellet was resuspended in HHBS with $Ca^{2+}$ and $Mg^{2+}$, and the concentration of viable cells, determined by hemocytometry and trypan blue exclusion, was adjusted to $1 \times 10^6$ cells/ml. Phagocytic activity (Prasad et al., 1988; Ziegler et al., 1994; Kleinman et al., 2000) was measured by adding $10^5$ cells to 0.5 ml HHBS in an 8-well Lab Tek chamber (VWR Scientific, Cerritos, CA). The cells were incubated for 90 min at 37°C and the nonadherent cells were removed by washing. Then 0.1 ml of a suspension ($10^9$ particles/ml) of spherical latex microparticles (2.0 $\mu$m diameter; Duke Scientific, Palo Alto, CA) was added to each chamber; the cells were incubated for 60 min and washed with HHBS to remove the free latex particles. The slides were air dried under HEPA-filtered laminar flow air, the cell chambers were dismantled, and the cells were stained with a Wright–Giemsa monochromatic cytological stain (Diff-Quik, Baxter Health Care, McGaw Park, IL). The slides were immersed in xylene for 2 h to dissolve the polystyrene particles; phagocytized spheres were visualized as unstained voids in the cell cytoplasm. The percentage of latex-positive cells (>2 spheres/cell) was determined. The cutoff of at least two latex spheres per cell was set based on the possibly that one sphere might become associated with a macrophage by chance, but the presence of two or more would more likely be the result of active phagocytosis. At least 200 cells were scored in each assay.

A sheep red blood cell (SRBC) rosette assay (Prasad et al., 1988; Ziegler et al., 1994; Kleinman et al., 2000) was used to determine the effect of exposure on Fc receptor binding of an antigen (sheep red blood cells). Chambers with 8 wells, each containing $10^5$ macrophages in 0.5 ml of HHBS, were incubated for 90 min at 37°C. Nonadherent cells were removed by washing with medium, and the adherent cells were incubated with 0.1 ml of rat anti-SRBC antibody for 30 min at 37°C. The macrophages were washed gently to remove excess antibody, 0.1 ml SRBC ($10^7$ cells/ml) was added, and the chambers were incubated for 30 min at room temperature. The unbound SRBCs were removed by gentle washing, and cells with three or more attached SRBC's were counted as positive rosettes (300 cells were counted in each well). Control chambers were prepared in an identical manner, except that no antibody was added. The number of rosettes in control chambers was determined as already described. These control counts, which represent non-specific rosetting, were subtracted from the total number of rosettes observed formed by the antibody-treated cells to compute Fc receptor binding fractions. Macrophages from five rats per atmosphere were tested, and all Fc receptor and phagocytosis assays were performed in duplicate.

## Statistical Analyses

Data were analyzed using analyses of variance (ANOVAs) and post hoc Tukey multiple comparison tests were used to test for significant differences between group mean values as a function of exposure. All tests were two-tailed. Significance was ascribed to a test at the $p = .05$ level. Since not all exposures were performed on the same day, the ANOVA tested for main effects of $O_3$, $H_2SO_4$, and day of exposure, as well as for statistical interactions between $O_3$ and $H_2SO_4$.

## RESULTS

### Atmosphere Concentrations

Exposure concentrations are summarized in Table 1. Each value in the table represents the average of two 2-h integrated samples. The range to mean ratio, expressed as a percentage, of the 2-h samples did not exceed 20%. Particle size distributions were in good agreement for the particle-containing atmospheres, but the MMDs were slightly below the target value of 0.3 $\mu$m. Sulfuric acid concentrations determined from $H^+$ and $SO_4^{2-}$ ion analyses were not significantly different, indicating that no detectable acid neutralization occurred. The $H_2SO_4$ values in Table 1 are based on the $SO_4^{2-}$ ion analyses.

TABLE 1
Mean concentrations and particle size characteristics of exposure atmospheres

| | Concentrations | | H₂SO₄ aerosol Particle size distribution | |
| | $O_3$ (ppm) | $H_2SO_4^a$ (mg/m³) | $MMD^b$ ($\mu$m) | $S_g^c$ |
| Atmosphere | | | | |
|---|---|---|---|---|
| 1 | <0.01 | <0.02 | — | — |
| 2 | 0.30 | — | — | — |
| 3 | 0.61 | — | — | — |
| 4 | <0.01 | 0.48 | 0.23 | 2.3 |
| 5 | <0.01 | 1.00 | 0.28 | 2.1 |
| 6 | 0.31 | 0.41 | 0.23 | 2.3 |
| 7 | 0.31 | 1.04 | 0.28 | 2.1 |
| 8 | 0.60 | 0.52 | 0.23 | 2.3 |
| 9 | 0.60 | 0.86 | 0.28 | 2.1 |

[a] Determined based upon $SO_4^{2-}$ ion chromatographic analyses.
[b] Mass median diameter.
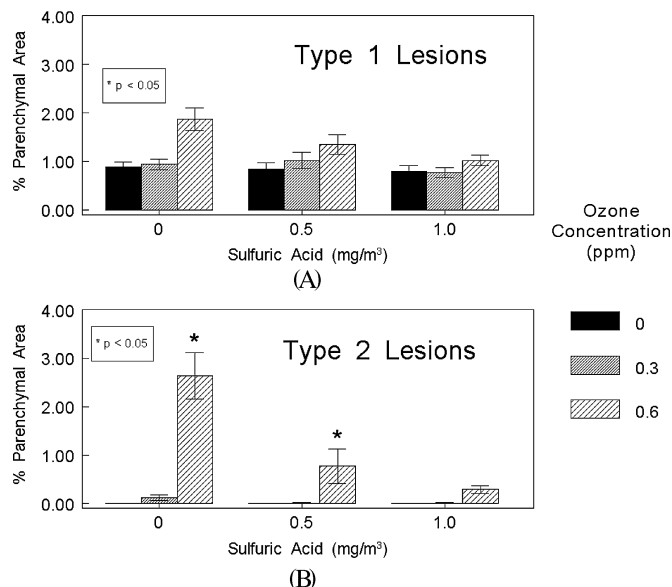[c] Geometric standard deviation.

FIG. 1. Histopathological lesions in lung parenchyma. (A) Percent lung area with free cells in alveolar air spaces (Type 1 lesion). (B) Percent lung area with more advanced (Type 2) pathology, including thickened alveolar walls and evidence of cellular infiltration. Intensities of inflammatory lesions induced by ozone were reduced by coexposures with sulfuric acid in a dose-dependent manner. Error bars are $\pm 1$ SEM.

## Inflammatory Lesions in Lung Parenchyma

The percent areas for Type 1 and 2 lesions are shown in Figure 1. Lesion areas in rats exposed to $H_2SO_4$ alone were not different from those in control rats, for either Type I or Type II lesions. Analysis of variance of Type 1 lesion data showed significant main effects of both $O_3$ ($p < .0001$) and $H_2SO_4$ ($p < .01$); there was no significant effect due to day of exposure. The interaction between $O_3$ and $H_4SO_4$ approached significance ($p = .057$); the magnitude of the $O_3$ lesion tended to be reduced by increasing concentrations of $H_2SO_4$. The percent lesion area in rats exposed to the mixture of 0.6 ppm $O_3$ and 0.5 mg/m$^3$ $H_2SO_4$ was significantly greater than that seen in rats exposed to 0.6 ppm $O_3$, 0.5 mg/m$^3$ $H_2SO_4$, or 1 mg/m$^3$ $H_2SO_4$ alone. $O_3$ at 0.3 ppm did not significantly increase lesion area compared to controls. The ANOVA of Type 2 lesion data showed significant ($p < .0001$) main effects of both $O_3$ and $H_2SO_4$ and a significant interaction between $O_3$ and $H_2SO_4$ ($p < .0001$). Posthoc multicomparison test showed that, for rats exposed to 0.6 ppm $O_3$, the ozone lesion areas were significantly reduced ($p < .01$) in the presence of either 0.5 or 1.0 mg/m$^3$ $H_2SO_4$ as a copollutant. The lesion area results demonstrate a significant antagonistic effect between 0.6 ppm $O_3$ and $H_2SO_4$ at 0.5 and 1.0 mg/m$^3$ concentrations. Similar effects were observed with rats exposed to 0.3 ppm $O_3$ that were coexposed to $H_2SO_4$ aerosols; however, the degree of antagonism did not achieve statistical significance.

## DNA Synthesis in Nasal and Tracheal Tissue

When lung epithelial cells are injured or killed, progenitor cells replicate to provide replacement cells. During the cell replication process, new DNA is formed. When $^3$H-TdR is present, the new DNA incorporates the $^3$H-TdR, which can be detected by autoradiography or scintillation counting. $^3$H-TdR uptake data are presented in Figure 2 as a means to quantify DNA synthesis in nasal, tracheal, and lung tissue. Exposures to $H_2SO_4$ alone did not produce significantly increased labeling of nasal or tracheal tissues with $^3$H-TdR, which would have been consistent with cell replication secondary to cytotoxicity. However, as shown by the data in Figure 2, autoradiographic analysis of these tissues suggested the presence of cytoxic effects after exposure of rats to $O_3$-containing atmospheres. In Figure 2, the ratios of $^3$H-TdR-labeled cells to unlabeled cells in nasal and tracheal epithelia of exposed rats were expressed as percentages. Cell labeling in the
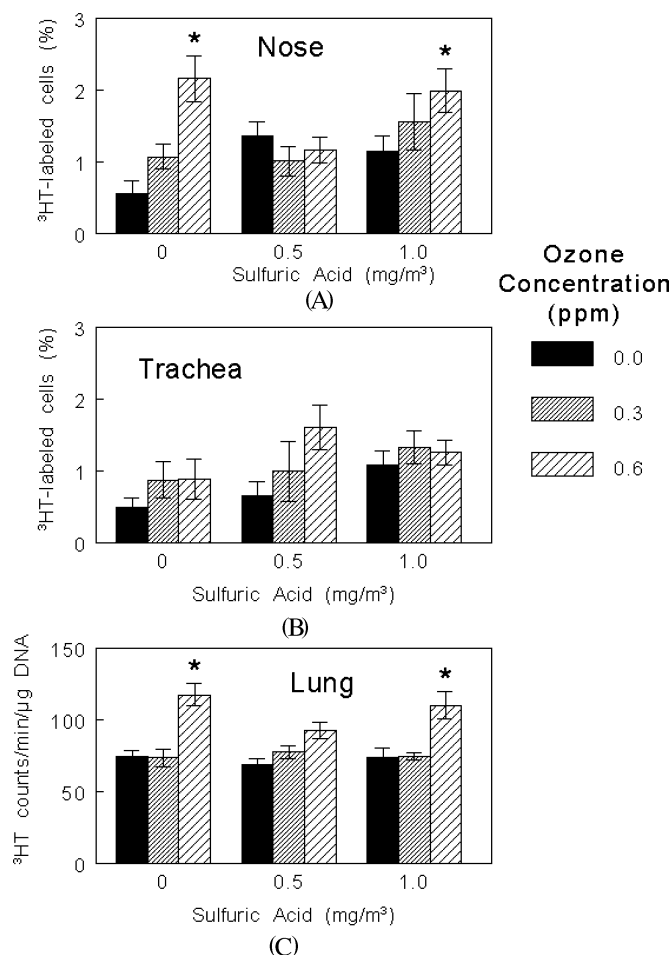


FIG. 2. DNA synthesis in response to cellular injury in (A) nasal, (B) tracheal, and (C) lung epithelia is evidenced as increased uptake of radiolabeled DNA precursor by epithelial cells. Nasal and lung epithelial injuries appeared to be related to ozone exposures and were not reduced by coexposures with sulfuric acid. Error bars are $\pm 1$ SEM.

nose showed a significant main effect of $O_3$ ($p < .001$). The main effect of acid approached, but did not achieve significance ($p = .10$). As shown in the upper panel of Figure 2, labeling increased with increasing $H_2SO_4$ in rats with 0.0 ppm $O_3$ exposure, but group mean values were not significantly different from controls. Labeling was also increased with increasing $O_3$ concentrations, in the absence of $H_2SO_4$, with significance ($<.01$) achieved at 0.6 ppm $O_3$. Acid had no effect on cell labeling index at 0.3 ppm $O_3$ and decreased labeling slightly, but not significantly, in rats exposed to 0.6 ppm $O_3 + 1$ mg/m$^3$ $H_2SO_4$, compared to labeling in rats exposed to 0.6 ppm $O_3$ alone. Although mean values within the $O_3$ groupings were not significantly different, the net differences in these trends gave rise to a significant $O_3 \times$ acid interaction ($p < .001$).

In the trachea (middle panel of Figure 2), $O_3$ exposure increased cell labeling ($p = .04$). Acid exposure increased cell labeling ($p = .01$), but there was no significant $O_3 \times$ acid interaction. The cell labeling data for nasal, tracheal, and lung epithelia showed that $H_2SO_4$ alone did not cause increased cell labeling, that $O_3$ exposure resulted in significantly increased cell labeling in nasal and lung tissues, and that $H_2SO_4$ coexposure did not significantly reduce the intensity of the $O_3$-induced cell injury.

## DNA Synthesis in Lung Parenchymal Tissue

DNA synthesis, as measured by uptake of $^3$H-TdR in lung DNA, was significantly increased in atmospheres containing 0.6 ppm $O_3$ ($p < .01$), compared to either purified air or atmospheres containing 0.3 ppm $O_3$ (lower panel of Figure 2). A two-factor ANOVA demonstrated no direct or interactive effects of $H_2SO_4$; the presence of acid did not reduce the lung cell injury caused by 0.6 ppm $O_3$ exposure.

## Fc Receptor (FcR) Binding and Phagocytosis

The effects of acid alone and $O_3$ plus acid mixture exposures on macrophage Fc receptor binding and phagocytosis are summarized in Figure 3. Data on FcR binding and phagocytosis were not acquired for animals exposed to 0.3 or 0.6 ppm $O_3$ alone. The values shown in Figure 3 are reported as percent of the average of control values. FcR binding in pollutant-exposed groups was not significantly different from that in controls. Phagocytic activity was significantly reduced by pollutant exposures relative to purified air controls. The ANOVA showed a near-significant ozone $\times$ acid interaction ($p = .056$), but the main effects of either ozone or acid were not significant.

## DISCUSSION

The overall goal of this study was to examine the toxicological interactions resulting from single, acute exposures to mixtures of sulfuric acid and ozone. The results show that the nature of the interaction observed depended on the endpoint being measured and that effects at high concentrations were not necessarily greater than those at low concentration. The upper
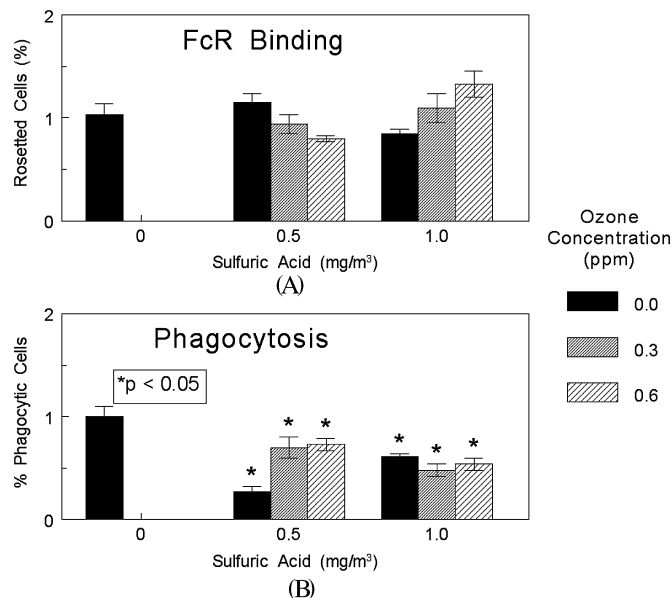


FIG. 3. Changes in (A) Fc receptor (FcR) binding of sheep red blood cells and (B) phagocytosis of latex particles were measured using alveolar macrophages obtained by bronchoalveolar lavage. FcR binding was not affected by exposures, but sulfuric acid alone, and in mixtures with ozone, suppressed phagocytic activity. Error bars are $\pm 1$ SEM.

concentration of $O_3$ and $H_2SO_4$ used in this study (0.6 ppm $O_3$ and 1 mg/m$^3$ $H_2SO_4$) are much higher than current ambient levels. However, the highest concentration reported for ambient $O_3$ in the Los Angeles area was approximately 0.6 ppm and occurred in 1967. Concentrations of $H_2SO_4$ approached 1 mg/m$^3$ during the 1962 London smog episode. Additionally, the concentrations used in this study had been used in previous studies (Chen et al., 1992; Kimmel et al., 1997), and effects had been elicited at these levels, allowing for the possibility of comparing outcomes between studies. Two assays of macrophage function were selected for this study. Fc receptor (FcR) binding is a measure of nonspecific antibody-directed activity, which is important for scavenging and removal of opsonized materials, including pathogens, from the lung. The rosette assay described later was previously shown to correlate with expression of Fc receptors on macrophage plasma membranes measured using confocal microscopy and fluorescently tagged antibody (Kleinman et al., 2000). The phagocytosis assay used examined overall, inate, nonspecific macrophage function and did not differentiate between possible mechanisms of action of the exposures or components of the macrophage activity such as motility, scavenger receptor function, or recruitment, all of which play a role in phagocytosis.

The present study found that inflammatory lesions in the lung were produced by $O_3$ but not by $H_2SO_4$ when administered separately. When $O_3$ and $H_2SO_4$ were administered as mixtures, the magnitude of the $O_3$-induced lesion response was

reduced, suggesting an antagonistic interaction. The degree of antagonism was greater in rats exposed to mixtures containing 0.6 ppm $O_3$ than in rats exposed to mixtures containing 1.0 mg/m$^3$ $H_2SO_4$. Kimmel et al. (1997) exposed rats to $O_3$ alone or to mixtures containing 0.5 ppm $O_3$ and 0.5 mg/m$^3$ $H_2SO_4$ for 4 h per day, on 2 consecutive days. They did not observe a significant difference in lung lesions between rats exposed to $O_3$ and those exposed to the mixture when the $H_2SO_4$ particle size was 0.3 $\mu$m, whereas we noted a reduction in the lesion volume. However, Kimmel et al. exposed rats for 2 days while we exposed rats for only 1 day, and it is possible that this might account for the difference in responses. Kimmel et al. (1997) also reported that particle size influenced interactive responses; ultrafine particles (30 nm diameter) evoked a synergistic interaction whereas fine (0.3 $\mu$m) particles did not. Schlesinger et al. (1992b) reported that exposure to mixtures of $O_3$ and $H_2SO_4$, reduced macrophage TNF activity at acid concentrations of 75 $\mu$g/m$^3$ but enhanced macrophage TNF activity at acid concentrations of 125 $\mu$g/m$^3$ and the same $O_3$ concentrations used in the present study (0.3 and 0.6 ppm).

In order to put these results into perspective, it is useful to examine the nature of the inflammatory response, which is a consequence of oxidant exposure, and the relationship of the endpoints we examined to this inflammatory response. Single 4-h inhalation exposures to $O_3$, even at relatively low-concentrations, cause injury or death of respiratory-tract epithelial cells. In the lung, macrophages and other leukocytes infiltrate the injured tissue to remove dead cells. The inflammatory response is seen in scattered proximal acinar units of the alveolar parenchymal zone of the lung. Each proximal acinar unit consists of the terminal bronchiole and the nearby alveolar ducts to which it leads. Only a fraction of these units are injured by exposure to relatively low-concentration oxidants and develop an infiltrate of inflammatory cells, by which they are identified as lesioned. Since most inflamed acinar units are surrounded by units of normal appearance, the lesions are isolated and are referred to as focal lesions. At exposure concentrations of about 0.6 to 0.8 ppm of $O_3$ alone, focal lesions appear in rats by 18 h postexposure, reach maximum intensity at about 48 h postexposure, and subsequently resolve (Stephens et al., 1974a, 1974b; Evans et al., 1976).

What we have termed Type 1 lesion occurs at low incidence in lungs of control rats; however, the number of sampling points that exhibit the Type 1 state increases markedly 18 to 48 h after acute resting exposures of rats to $O_3$ at concentrations of 0.4 ppm or greater. Type 2 and Type 3 lesions have never been seen in parenchyma of healthy control rats in our laboratory. Since these lesions are identified by the presence of inflammatory cells in the lung parenchyma, it is possible to interpret a reduction in lesion area in one group of pollutant-exposed rats, relative to that in another group exposed to a different pollutant atmosphere, either as a reduction in parenchymal injury, or as a reduction in migration of neutrophils and alveolar macrophages to areas of injury.

When inhaled pollutants kill or injure respiratory-tract cells, the damaged tissue is repaired by the replication of cells adjacent to the injured cells (Mustafa, 1990). Such injury can be identified because DNA precursors are taken up by the replicating cells, and, by introducing tritiated thymidine ($^3$H-TdR), a radiolabeled nucleotide, it is possible to radioactively tag these cells and subsequently map their locations and numbers in the airways by autoradiography (Evans & Bils, 1969; Evans et al., 1971). The locations of increased $^3$H-TdR uptake, then, represent epithelia in which cell death has been followed by a wave of new cell production. The number of cells that take up $^3$H-TdR is an index of the magnitude of the tissue damage, which is probably independent of migration of inflammatory cells, as discussed earlier. The data from this study showed that $H_2SO_4$ caused cell injury leading to increased $^3$H-TdR uptake in nasal and tracheal tissue, but not in lung tissue. This study also showed that although $H_2SO_4$ might have reduced the $^3$H-TdR uptake in the nose due to the effects of $O_3$, $H_2SO_4$ did not appear to reduce $^3$H-TdR uptake in the trachea or the lung. The reduced phagocytic activity of macrophages and the interaction of $O_3$ and $H_2SO_4$ observed in other endpoints do, however, suggest that $H_2SO_4$ deposition in the lung parenchyma did occur. This is in contrast to data reported by Schlesinger et al. (1992b) in rabbits, which suggested that $H_2SO_4$ deposition occurred in tracheobronchial airways but not in the alveolar region. The differences in experimental design and between animal species used might account for this discrepancy.

This study has also shown that the influx of inflammatory cells and other indicators of lung inflammatory processes, which are scored as Type 1 and Type 2 lesions, are significantly reduced from values seen after exposure to $O_3$ alone in rats exposed to $O_3$ plus $H_2SO_4$. The reduction in inflammatory response in the lung, despite the presence of epithelial injury indicated by the $^3$H-TdR uptake data, could suggest impairment of macrophage recruitment mechanisms, or some form of macrophage impairment. Direct examination of macrophage functions demonstrated significant acid-related reductions in phagocytic activity; however, the measurements made in this study did not address the recruitment or migration of inflammatory cells to injured parenchyma.

## CONCLUSIONS

Exposure to $O_3$ and $H_2SO_4$ mixtures resulted in damage to respiratory epithelia, and reduced alveolar macrophage phagocytic activity. The data in this study do not support the contention that $O_3$ and $H_2SO_4$ act synergistically in rats after single 4-h exposures. We did observe a reduction in $O_3$-induced inflammation (or influx of inflammatory cells) in the presence of $H_2SO_4$. Our findings could be interpreted to suggest that the acid concentrations studied might be protective against some inflammatory effects of ozone. On the other hand, the observed lack of influx of inflammatory cells might reflect some disruption of macrophage functions and of the mechanisms related to recruitment of phagocytic cells to sites of lung injury.

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