# UC Irvine
## UC Irvine Previously Published Works

**Title**
Effect of Nitric Acid Vapor on the Response to Inhaled Ozone

**Permalink**
https://escholarship.org/uc/item/4rq9x0n9

**Journal**
Inhalation Toxicology, 4(4)

**ISSN**
0895-8378

**Authors**
Nadziejko, CE
Nansen, L
Mannix, RC
et al.

**Publication Date**
1992

**DOI**
10.3109/08958379209145313

Peer reviewed

# EFFECT OF NITRIC ACID VAPOR ON THE RESPONSE TO INHALED OZONE

C. E. Nadziejko, L. Nansen

Department of Pathology, University of California, Irvine, California

R. C. Mannix, M. T. Kleinman, R. F. Phalen

Air Pollution Health Effects Laboratory, Department of Community and Environmental Medicine, University of California, Irvine, California

*Studies concerned with the adverse health effects of acidic atmospheres have focused primarily on sulfuric acid aerosols and other acid sulfates. Little information is available on the health effects of nitric acid ($HNO_3$), an important atmospheric pollutant in southern California. The purpose of this study was to investigate the effects of exposure to nitric acid vapor, alone and in combination with ozone ($O_3$), on the rat lung. Groups of rats were exposed once for 4 h to purified air, 0.6 ppm $O_3$, 1.0 mg/m$^3$ $HNO_3$, or 0.6 ppm $O_3$ plus 1.0 mg/m$^3$ $HNO_3$. Other rats were exposed for 4 days, 4 h/day to purified air, 0.15 ppm $O_3$, 0.25 mg/m$^3$ $HNO_3$, or 0.15 ppm $O_3$ plus 0.25 mg/m$^3$ $HNO_3$. The animals were lavaged 18 h after exposure, and cells and fluid were assayed for changes in lavage cell population and lavage fluid protein content, pulmonary macrophage respiratory burst activity and leukotriene production, and elastase inhibitory capacity of lavage fluid. A separate series of rats was lavaged 2 h after exposure to high concentration atmospheres for measurement of lavage fluid pH and $PCO_2$ and macrophage intracellular pH. Exposure to 0.25 mg/m$^3$ $HNO_3$ alone decreased spontaneous and PMA-stimulated respiratory burst activity in freshly isolated macrophages, while the high concentration mixed atmosphere was also found to cause a significant decrease in respiratory burst activity in freshly isolated macrophages, while the high concentration mixed atmosphere was also found to cause a significant decrease in respiratory burst activity of macrophages maintained overnight in culture. $HNO_3$ at both concentrations resulted in a modest but significant increase in elastase inhibitory capacity of lung lavage fluid. Exposure to 0.6 ppm $O_3$ resulted in an increased number of neutrophils in the lavage cell population as well as in increased lavage fluid protein content and elastase inhibitory capacity. These changes were also observed following exposure to 1.0 mg/m$^3$ $HNO_3$ plus 0.6 ppm $O_3$, although not to the same extent as following $O_3$ alone. Linear regression analysis indicated that an antagonistic interaction occurs between $HNO_3$ and $O_3$ for changes in lavage cell population, lavage fluid protein content, and elastase inhibitory capacity.*

## INTRODUCTION

Data from epidemiological studies have suggested a link between exposure to acidic atmospheres and adverse health effects (Speizer, 1989). However, regulators cannot easily establish air contamination criteria for acids because their effects are difficult to distinguish from those produced by other copollutants. In addition, exposures to pollutant mixtures containing acids can produce complex toxicological interactions due to modifications of pollutant chemistry, changes in dose deposition in the respiratory tract, and interactions between toxic effects of compounds in tissues.

The majority of studies concerned with the effects of acids in combination with other agents focus on interactions between ozone ($O_3$) and sulfuric acid ($H_2SO_4$) aerosols. Studies by Last and co-workers demonstrated a synergistic interaction between $O_3$ and acidic sulfates. $H_2SO_4$ and ammonium sulfate were found to potentiate ozone-induced increases in lavage fluid protein, total lung protein, soluble proline, collagen synthesis rate, and lung lesion size (Last et al., 1986; Warren et al., 1986; Warren and Last, 1987). The extent of the interaction between $O_3$ and $H_2SO_4$ appeared to be dependent on acid droplet size: $H_2SO_4$ droplets having a mass median aerodynamic diameter (MMAD) of 0.02 $\mu$m had no synergistic effect on $O_3$, while 0.5-$\mu$m $H_2SO_4$ droplets potentiated ozone-induced lung injury (Last et al., 1986).

Setting acid exposure standards in southern California is complicated by the fact that nitric acid ($HNO_3$) is the dominant form of acid present in that region (Munger et al., 1990). Considerably less is known about the possible adverse health effects of $HNO_3$, either alone or in combination with $O_3$, than about those of $H_2SO_4$. Aris et al. (1991) conducted pulmonary function measurements in exercising humans following sequential exposure to $HNO_3$ fog and $O_3$. They found that preexposure to $HNO_3$ tended to attenuate $O_3$-induced alterations in pulmonary function, suggesting that an antagonistic interaction may occur between the two pollutants. More information is needed on the potential health effects of $HNO_3$ alone as well as on its interactions with $O_3$.

We conducted a series of single, 4-h acute inhalation exposures of rats to $HNO_3$, $O_3$, or $O_3$ plus $HNO_3$ in order to better understand the potential adverse effects of $HNO_3$ vapor on the lung. An additional series of 4-h exposures, repeated over 4 consecutive days at one-fourth the acute exposure concentration, was also performed to examine the importance of exposure concentration versus total integrated exposure dose. In order to study the interactive effects of $O_3$ and $HNO_3$ on the lung, we measured seven biochemical and cellular parameters in lung lavage fluid that have been shown to be affected by inhalation of either $O_3$ or $H_2SO_4$. Lavage fluid protein content, elastase inhibitory capacity, and lavage cell number and type are end points that are sensitive to $O_3$

(Bhalla and Young 1992; Guth et al., 1986), while short-term exposure to $H_2SO_4$ has been shown to affect macrophage intracellular pH (Chen et al., 1992), macrophage respiratory burst activity (Zelikoff et al., 1992), and pulmonary arachidonic acid metabolism (Schlesinger et al., 1990).

## METHODS

### Animal Exposures

Certified viral-antibody-free Fischer 344 male rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing approximately 250 g were used in this study. The rats were delivered in filtered shipping boxes and were housed in a particle-filtering and gas-scrubbing laminar-flow caging system for about 1 wk prior to the start of the experimental protocol in order to minimize prior exposure of the animals to pollutants and pathogens. Serologic testing and histopathologic examinations were performed by our laboratory on sentinel rats from each batch of animals received, to confirm that the rats were virus-free.

Groups of rats were exposed to either purified air or pollutant atmospheres using 1-m$^3$ stainless-steel University of Rochester-type chambers modified for nose-only exposure (Prasad et al., 1988). Rats from each supply batch were randomly assigned to exposure groups and were exposed nose-only following identical experimental protocols. The chambers were supplied with air passed through a coarse particulate filter, a gas/vapor scrubber, a humidifier, and a HEPA filter. When appropriate, $O_3$ and/or $HNO_3$ was injected into the airstream just prior to the exposure chamber. Air temperature and relative humidity were maintained at 23 °C and 60%, respectively. Because only small numbers of rats could be handled at any one time in the biological assessment protocols, the 1-day exposures were performed over 12 days and the 4-day exposures were performed over 10 days. $HNO_3$ was sampled twice (2 h each) during each 4-h exposure, and the results were averaged to provide a daily measurement. The daily measurements were then averaged and exposure group means and standard deviations were calculated (see Table 1). Relative humidity, temperature, and $O_3$ were monitored continuously and recorded every 30 min during the exposures (see Table 1).

$O_3$ was generated by passing medical-grade oxygen gas through corona-discharge ozonizers (Sander type III, Osterberg, Germany). The concentration of the $O_3$ was monitored by sampling through fluorocarbon tubing from the rat breathing zone with ultraviolet light-absorption continuous monitors (Dasibi Environmental Corp., Glendale, Calif.). The monitors were calibrated prior to the start of the study, as well as at weekly intervals throughout the duration of the study, using a factory-calibrated in-house ozone transfer standard.

Vapor-phase $HNO_3$ was generated by atomizing a dilute, aqueous $HNO_3$ solution using a Collison nebulizer. The droplets were then evapo-

**TABLE 1.** Summary of Atmosphere Characteristics

| Exposure atmosphere | Parameter/ pollutant | Target | Measured (mean ± SD) |
|---|---|---|---|
| | 4 h/day × 1 day | | |
| Purified air | Relative humidity | 60% | 60.7 ± 1.0 |
| | Temperature | 22–24 °C | 23.7 ± 0.8 |
| 0.6 ppm $O_3$ | Relative humidity | 60% | 60.7 ± 1.0 |
| | Temperature | 22–24 °C | 23.7 ± 0.8 |
| | Ozone | 0.6 ppm | 0.61 ± 0.02 |
| 1 mg/m$^3$ $HNO_3$ | Relative humidity | 60% | 60.9 ± 0.9 |
| | Temperature | 22–24 °C | 23.3 ± 0.5 |
| | Nitric acid | 1.0 mg/m$^3$ | 1.08 ± 0.13 |
| 0.6 ppm $O_3$ + 1 mg/m$^3$ $HNO_3$ | Relative humidity | 60% | 60.5 ± 1.0 |
| | Temperature | 22–24 °C | 24.1 ± 0.8 |
| | Ozone | 0.6 ppm | 0.60 ± 0.01 |
| | Nitric acid | 1.0 mg/m$^3$ | 1.01 ± 0.12 |
| | 4 h/day × 4 day | | |
| Purified air | Relative humidity | 60% | 60.2 ± 0.9 |
| | Temperature | 22–24 °C | 22.5 ± 0.8 |
| 0.15 ppm $O_3$ | Relative humidity | 60% | 60.2 ± 0.9 |
| | Temperature | 22–24 °C | 22.5 ± 0.8 |
| | Ozone | 0.15 ppm | 0.15 ± 0.01 |
| 0.25 mg/m$^3$ $HNO_3$ | Relative humidity | 60% | 60.4 ± 0.9 |
| | Temperature | 22–24 °C | 22.3 ± 0.6 |
| | Nitric acid | 0.25 mg/m$^3$ | 0.27 ± 0.08 |
| 0.15 ppm $O_3$ + 0.25 mg/m$^3$ $HNO_3$ | Relative humidity | 60% | 60.0 ± 0.8 |
| | Temperature | 22–24 °C | 22.8 ± 0.8 |
| | Ozone | 0.15 ppm | 0.15 ± 0.01 |
| | Nitric acid | 0.25 mg/m$^3$ | 0.26 ± 0.09 |

*Note.* No aerosol-phase $HNO_3$ was observed; all of the $HNO_3$ was in the vapor phase.

rated by dilution with dry, purified air, and the resulting vapor was injected into the chamber airstream. Sampling was performed from the rat breathing zone using two filters in tandem: a Teflon-coated glass fiber prefilter, which was used to check for the presence of any aerosol-phase $HNO_3$, and a nylon backup filter, which was used to collect $HNO_3$ vapor. Filter samples were extracted in aqueous media, and aliquots of each extract were analyzed by ion chromatography to determine the nitrate concentration. Data obtained in this manner were then used to determine the $HNO_3$ concentration in the rat breathing zone.

The groups of animals were exposed to high and low concentrations of $O_3$, $HNO_3$, or $O_3$ plus $HNO_3$, and the durations of the exposures were adjusted so that the integrated exposure dose (concentration × time) remained constant for all groups. High concentration atmospheres (purified air, 0.6 ppm $O_3$, 1.0 mg/m$^3$ $HNO_3$, or 0.6 ppm $O_3$ plus 1.0 mg/m$^3$ $HNO_3$) were given in a single 4-h exposure, whereas low concentration

exposures were done for 4 days, 4 h/day (purified air, 0.15 ppm $O_3$, 0.25 mg/m$^3$ $HNO_3$, or 0.15 ppm $O_3$ plus 0.25 mg/m$^3$ $HNO_3$). Summaries of the atmosphere characteristics for all exposures are given in Table 1.

Animals were lavaged 18 h after exposure except for one series of animals, exposed to the high concentration atmospheres, that was lavaged within 2 h of exposure to measure lavage fluid pH and macrophage intracellular pH.

### Lung Lavage

Rats were deeply anesthetized by intraperitoneal administration of sodium pentobarbital (65 mg/kg). Invasive procedures were initiated only after the loss of deep-tendon reflexes was observed. The aorta was then severed, the trachea exposed and cannulated with a thin-walled plastic catheter, and the diaphragm exposed and cut away from the anterior rib cage. To minimize variability in the lavage procedure, a multichannel peristaltic pump was employed to lavage five rats at a time. The lungs were lavaged with calcium- and magnesium-free Hanks balanced salt solution (HBSS). A volume of 7 ml was used for the first lavage, which was centrifuged to obtain acellular supernatant fluid for the lavage fluid protein and elastase inhibitory capacity assays described below. Four additional lavages of 10 ml each were then done for a total instilled lavage volume of 47 ml. The cells from all 5 lavages were pooled and counted with a bright line hemacytometer, then resuspended in HBSS at a concentration of approximately $1 \times 10^6$ cell/ml. Differential cell counts were performed on a small aliquot of cells from each rat, pelleted onto a glass microscope slide using a cytospin (Shandon, Inc., Pittsburgh, Pa.), and stained with Diff-Quick (Baxter Healthcare, McGaw Park, Ill.). The cellular content of the lavage fluid from air-exposed and pollutant-exposed rats was greater than 90% macrophages so no further enrichment of macrophage content was attempted. We have used the term "pulmonary macrophages" to refer to the total cell population obtained by lavage, even though a small number of other cell types were present. This term is more suitable than "alveolar macrophages" because it is unclear how many of the macrophages in the lavage fluid were from the airways and how many were alveolar macrophages.

### Lavage Fluid pH and PCO$_2$ and Macrophage Intracellular pH

Rats were exposed to the high concentration atmospheres and lavaged within 2 h. A 1-ml sample of supernatant fluid from the first lavage was stored in an air-tight syringe at 4°C and analyzed for pH and PCO$_2$ with a blood gas analyzer (Radiometer America Inc, Westlake, Ohio). To measure macrophage intracellular pH, pulmonary macrophages were suspended in an aliquot of lung lavage fluid from the same rat as the macrophages and allowed to adhere to round glass coverslips in an atmosphere containing 5% $CO_2$ (95% air). The cells were incubated for 45

min at 37°C with lung lavage fluid containing 7 $\mu$g/ml 2',7'-bis-(carboxyethyl)-5 (and -6) carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Molecular Probes, Eugene, Ore.), a pH-sensitive fluorescent dye. The coverslips were then placed into square fluorimeter cuvettes containing fresh lavage fluid and positioned with hairpins to hold them at a 45° angle to the incident light. Fluorescence intensity at an emission wavelength of 526 nm was measured following excitation at both 506 (pH-sensitive) and 430 (isosbestic) nm, and the ratio of the 2 readings was calculated. A standard curve was then used to obtain a measurement of intracellular pH from each ratio. This curve was prepared by removing the lavage fluid from the sample cuvette and replacing it with buffered solutions of known pH values containing 130 m$M$ KCl, 10 m$M$ NaCl, 1 m$M$ CaCl, 1 m$M$ MgSO$_4$, 2 m$M$ NaH$_2$PO$_4$, 5 m$M$ glucose, and 6 m$M$ HEPES. Nigericin ionophore (10 $\mu$M) was then added to equilibrate intracellular pH with that of the medium, and equilibrated pH values were plotted against excitation ratios (Lubman et al., 1989).

## Respiratory Burst Activity

Respiratory burst activity of pulmonary macrophages maintained in culture overnight was measured by lucigenin chemiluminescence (Williams and Cole, 1981). An aliquot containing 0.5 × 10$^6$ pulmonary macrophages was pipetted into sterile polystyrene cuvettes and incubated in RPMI 1640 medium (Gibco, Grand Island, N.Y.) at 37°C for 18–20 h. After the culture medium was removed, the cells were preincubated for the chemiluminescence assay in 0.4 ml Dulbecco's phosphate-buffered saline containing 5 m$M$ glucose for 30 min at 37°C. Respiratory burst activity was stimulated by adding opsonized zymosan (50 $\mu$l of 2.5 mg/ml), following which 50 $\mu$l of 2 m$M$ lucigenin (Sigma, St. Louis, Mo.) was added and chemiluminescence was measured using an LKB 1251 luminometer (Pharmacia-LKB Nuclear Division, Gaithersburg, Md.). Measurements continued until the peak emission rate was reached and light emission began to decline. Data were expressed as peak height (in millivolts) versus milligrams adherent cell protein.

A cytochrome c reduction assay was used to measure the respiratory burst activity of freshly isolated pulmonary macrophages from low concentration exposure groups, according to the method of Kemmerich et al. (1987). An aliquot containing 0.2 × 10$^6$ pulmonary macrophages was added to microtiter plate wells containing 100 m$M$ cytochrome c as well as buffer, 0.125 mg/ml opsonized zymosan, or 50 ng/ml phorbol myristate acetate (PMA). Cytochrome c reduction [(OD at 500 nm) − (OD at 540 nm)] was then measured after incubation at 37°C for 30 min. Superoxide dismutase (SOD) (60 units) was added to duplicate samples. Data were expressed as SOD-inhibitable cytochrome c reduction in nmol/30 min/mg cell protein.

## Leukotriene Production

To measure in vitro production of leukotrienes $B_4$ ($LTB_4$) and $C_4$ ($LTC_4$) by pulmonary macrophages, cells obtained from the lavage fluid of each rat were suspended in RPMI 1640 medium at a concentration of $1 \times 10^6$ cells/ml and incubated in 24-well culture dishes at 37°C. After 45 min, the culture medium was removed and rapidly centrifuged to remove non-adherent cells, and the cell-free supernatant fluid was returned to the wells. The macrophages were then incubated for an additional 3¼ h, after which the medium was again removed and centrifuged and the cell-free supernatant fluid was stored at −70°C. $LTB_4$ and $LTC_4$ levels in the supernatant fluid were subsequently measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, Mich.) according to the manufacturer's protocol.

## Elastase Inhibitory Capacity

Pancreatic elastase in 0.1 M Tris-HCl buffer (0.25–1.0 $\mu$g, 130 U/mg, pH 8.8) was added to a 300-$\mu$l aliquot of lung lavage fluid and maintained at room temperature for 5 min, followed by 2 mg fluorescein-labeled elastin (Pickrell et al., 1987). The assay mixtures were incubated with continuous mixing at 37°C for 30 min, after which they were centrifuged to remove all nonsolubilized elastin. Residual elastase activity was determined by the fluorescence of the supernatant fluid at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. Elastase inhibitory capacity was then calculated using a standard curve obtained by measuring fluorescence of mixtures containing pancreatic elastase and elastin in the absence of lavage fluid.

## Statistical Analysis

A batch correction factor described by Lee et al. (1990) was applied to all data to eliminate artifactual findings caused by batch-to-batch variations. The correction factor used was $C_g/C_b$, where $C_g$ is the mean value for all controls and $C_b$ is the mean control value for a given sacrifice batch of rats. All of the end-point data except lavage cell type were corrected in this manner before means and standard deviations were calculated. Due to the limited numbers of lavage cells that can be recovered from the rat, only one measurement was made per assay from each animal. Differences between all group means were evaluated nonparametrically using both the Kruskal-Wallis method and the pair-wise Mann-Whitney $U$-test. Group differences were considered to be statistically significant when a test value of $p < .05$ was obtained.

A linear regression model with dummy variables (Lee et al., 1990) was also used to test for a possible interaction between the effects of $O_3$ and $HNO_3$. Percentage data are not normally distributed, and an arcsine transformation was therefore applied prior to regression analysis (Snedecor and Cohran, 1969).

## RESULTS

### Lavage Fluid pH and PCO$_2$ and Macrophage Intracellular pH

No significant differences in lavage fluid pH, lavage fluid PCO$_2$, or intracellular pH were found between any of the groups exposed to high concentration atmospheres. Macrophage intracellular pH was 7.08 ± 0.16 and lavage fluid pH was 6.87 ± 0.06 (mean ± SD, $n$ — 40). It is possible that changes in lavage fluid pH were masked by the buffering action of the HBSS that was used for the lavages (which contains H$_2$CO$_3$ at approximately 4 mEq/ml). However, the average pH of the lavage fluid was found to be 6.8–6.9, which is in the same range as has been previously reported for the pH of the alveolar lining fluid in vivo (Nielson et al., 1981).

### Lavage Cell Number and Type

Table 2 summarizes the changes in the lavage cell population observed 18 h after exposure. Exposure to 0.6 ppm O$_3$ resulted in a statistically significant increase in the percentage of neutrophils, accompanied by a corresponding decrease in the percentage of macrophages that was also statistically significant. Exposure to 1 mg/m$^3$ HNO$_3$ caused no changes in cell population. The combination of HNO$_3$ plus O$_3$ also caused a statistically significant increase (relative to controls) in the number of neutrophils in the lavage fluid, with the accompanying decrease in

**TABLE 2.**  Effect of Exposure of Rats to HNO$_3$ and O$_3$, Alone and in Combination, on Lavage Cell Number and Type

| Parameter[a] | 4 h/day × 1 day | | | |
|---|---|---|---|---|
| | Purified air | 1 mg/m$^3$ HNO$_3$ | 0.6 ppm O$_3$ | 0.6 ppm O$_3$ + 1 mg/m$^3$ HNO$_3$ |
| Total cell number[a] (× 10$^6$) | 3.5 ± 0.5 | 3.5 ± 0.8 | 2.8 ± 0.7 | 3.0 ± 0.7 |
| Percent macrophages[a] | 98.6 ± 0.7 | 98.7 ± 1.0 | 95.1 ± 1.8[b] | 96.5 ± 2.2[b] |
| Percent neutrophils[a] | 0.6 ± 0.4 | 0.6 ± 0.7 | 3.7 ± 1.7[b] | 2.5 ± 2.0[b] |

| Parameter[a] | 4 h/day × 4 day | | | |
|---|---|---|---|---|
| | Purified air | 0.25 mg/m$^3$ HNO$_3$ | 0.15 ppm O$_3$ | 0.15 ppm O$_3$ + 0.25 mg/m$^3$ HNO$_3$ |
| Total cell number[a] (× 10$^6$) | 3.0 ± 0.4 | 2.8 ± 0.9 | 2.6 ± 0.6 | 3.2 ± 0.7 |
| Percent macrophages[a] | 97.4 ± 2.5 | 97.8 ± 2.8 | 98.3 ± 1.2 | 97.5 ± 2.3 |
| Percent neutrophils[a] | 1.0 ± 1.1 | 0.9 ± 1.7 | 0.9 ± 1.1 | 1.3 ± 1.4 |

[a]The data represent means and standard deviations of measurements made on 20 rats per group.
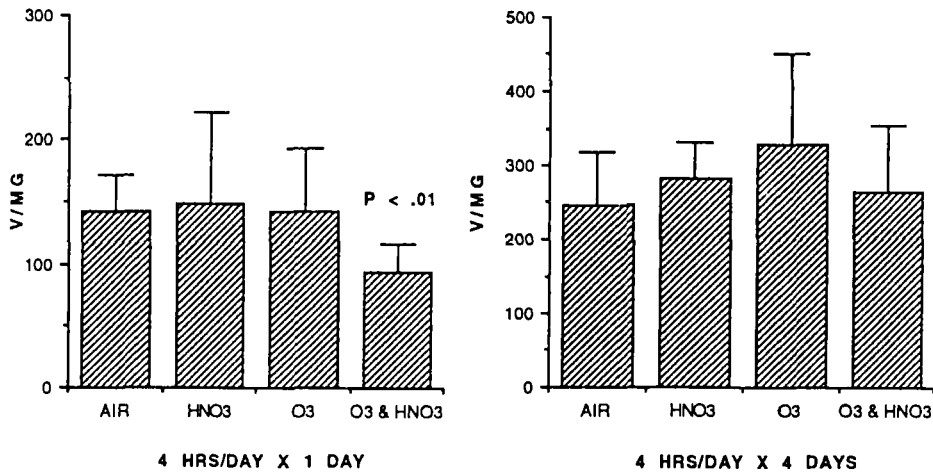[b]Significant difference relative to the purified air group ($p$ < .05).

FIGURE 1. Effect of inhalation of $HNO_3$ vapor and $O_3$, alone and in combination, on respiratory burst activity of lung macrophages. Pulmonary macrophages were obtained 18 h after exposure of rats to $HNO_3$ and $O_3$. Cells were maintained in culture for 20 h, and opsonized zymosan-stimulated respiratory burst activity was measured by lucigenin chemiluminescence. Results are mean ± SD of 10 rats/group. The $p$ values indicate statistical significance relative to the air-exposed group. Left panel: effects of a single 4-h exposure to purified air, 0.6 ppm $O_3$, 1.0 mg/m$^3$ $HNO_3$, or 0.6 ppm $O_3$ plus 1.0 mg/m$^3$ $HNO_3$. Right panel: effects of 4 consecutive days of exposure (4 h/day) to purified air, 0.15 ppm $O_3$, 0.25 mg/m$^3$ $HNO_3$, or 0.15 ppm $O_3$ plus 0.25 mg/m$^3$ $HNO_3$.

macrophages. However, the changes resulting from exposure to the mixture were significantly less than changes caused by exposure to $O_3$ alone. There were no significant differences observed in the numbers of monocytes, lymphocytes, and eosinophils. The number of red blood cells per high-power microscopic field was also unchanged following any of these exposures. Total lavage cell number was the same in all groups.

There were no significant differences in lavage cell number or type between groups after any of the exposures to low concentration atmospheres.

## Respiratory Burst Measurements

Zymosan-stimulated respiratory burst activity was measured by lucigenin chemiluminescence in pulmonary macrophages that were maintained in culture overnight to eliminate spontaneous activity. Single 4-h exposures of rats to 1 mg/m$^3$ $HNO_3$ plus 0.6 ppm $O_3$ resulted in a significant depression of zymosan-stimulated respiratory burst activity (Fig. 1, left panel). No effects were observed from exposure to any of the low concentration atmospheres (Fig. 1, right panel).

Freshly isolated pulmonary macrophages from rats exposed to 0.25 mg/m$^3$ $HNO_3$ demonstrated significantly less spontaneous and PMA-stimulated respiratory burst activity when measured by the cytochrome $c$

reduction assay. No effects were seen following exposure to the combination of 0.25 mg/m$^3$ HNO$_3$ and 0.15 ppm O$_3$ or to O$_3$ alone (Fig. 2).

### Leukotriene Production

In the groups exposed to the high concentration atmospheres, pulmonary macrophages from rats exposed to 1 mg/m$^3$ HNO$_3$ released slightly more LTB$_4$ than controls (2.3 ± 2.2 vs. 0.6 ± 0.2 $\mu$g/mg, $p$ < .10). Macrophages from animals exposed to 0.6 ppm O$_3$ released significantly less LTC$_4$ than macrophages from air-exposed animals (11.2 ± 12.1 vs. 21.6 ± 10 ng/mg, $p$ < .05). No changes in LTB$_4$ or LTC$_4$ production were measured following exposure to the combination of HNO$_3$ and O$_3$.

None of the low concentration exposures had any effect on pulmonary macrophage production of LTB$_4$ or LTC$_4$.

### Lavage Fluid Protein Content

Lavage fluid protein content was markedly increased ($p$ < .01) after exposure to 0.6 ppm O$_3$ (Fig. 3A). Exposure to 0.6 ppm O$_3$ plus 1 mg/m$^3$ HNO$_3$ also resulted in increased lavage fluid protein ($p$ < .01), but the
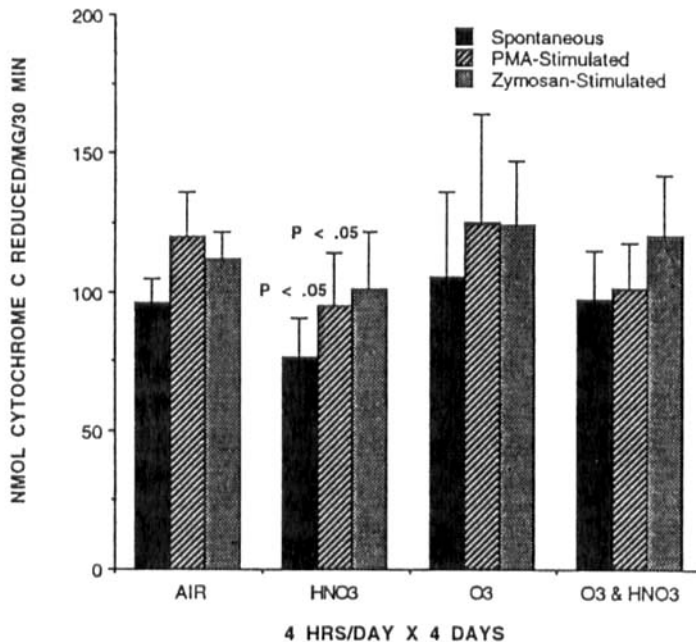


FIGURE 2. Effect of in vivo exposure on respiratory burst activity of freshly isolated pulmonary macrophages following 4 consecutive days (4 h/day) to purified air, 0.15 ppm O$_3$, 0.25 mg/m$^3$ HNO$_3$, or 0.15 ppm O$_3$ plus 0.25 mg/m$^3$ HNO$_3$. Spontaneous and stimulated respiratory burst activity was measured by the reduction of cytochrome c. Results are mean ± SD of 10 rats/group. The $p$ values indicate statistical significance relative to the air-exposed group.
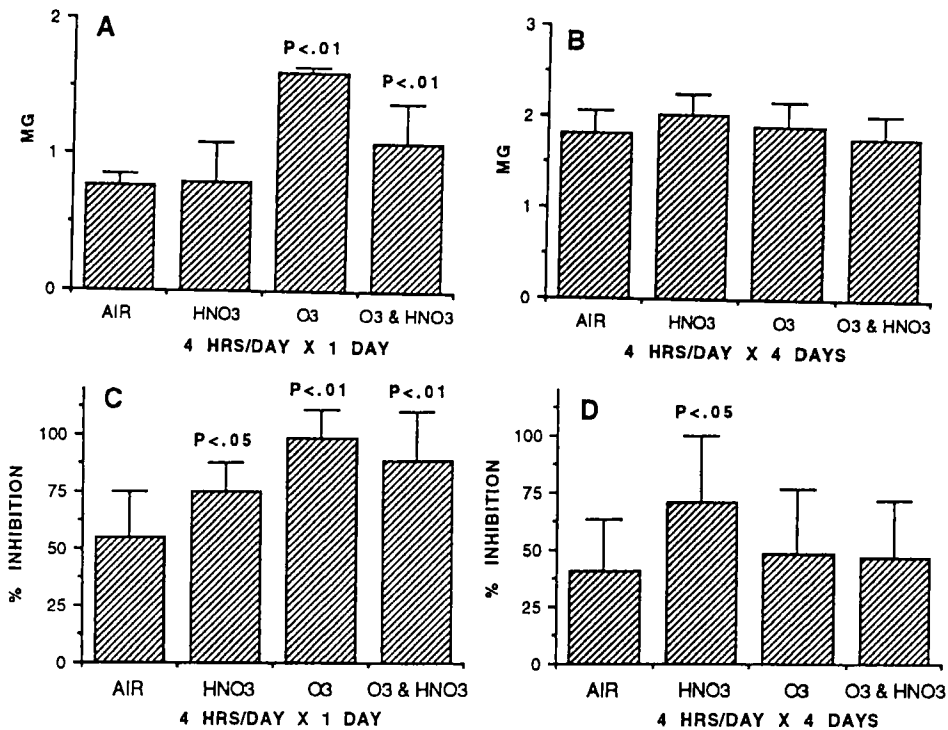
FIGURE 3. Effects of exposure to $HNO_3$ vapor and $O_3$ on lavage fluid protein content (top panels) and elastase inhibitory capacity (bottom panels) of lung lavage fluid obtained 18 h after exposure. Results are mean ± SD of 10 rats/group. The $p$ values indicate statistical significance relative to the air-exposed group. (A) and (C): Effects of a single 4-h exposure to purified air, 0.6 ppm $O_3$, 1.0 mg/$m^3$ $HNO_3$, or 0.6 ppm $O_3$ plus 1.0 mg/$m^3$ $HNO_3$. (B) and (D): Effects of 4 consecutive days of exposure (4 h/day) to purified air, 0.15 ppm $O_3$, 0.25 mg/$m^3$ $HNO_3$, or 0.15 ppm $O_3$ plus 0.25 mg/$m^3$ $HNO_3$.

effect of the mixture was less than the effect of $O_3$ alone. Lavage fluid protein was unchanged by exposure to 1 mg/$m^3$ $HNO_3$ alone, or to any of the low concentration atmospheres (Fig. 3B).

## Lavage Fluid Elastase Inhibitory Capacity

The effects of the exposures on lung lavage fluid elastase inhibitory activity are shown in Figs. 3, C and D. In the high concentration exposure groups, elastase inhibitory capacity was markedly increased in the lung lavage fluid of rats after exposure to 0.6 ppm $O_3$ ($p < .01$). Exposure to 1 mg/$m^3$ $HNO_3$ alone and to $O_3$ plus $HNO_3$ also significantly increased lavage fluid elastase inhibitory capacity but to a lesser extent than exposure to $O_3$ alone. In the low concentration exposure groups, only $HNO_3$ alone caused an increase in elastase inhibitory capacity.

### Interactive Effects of Combined Exposures to HNO₃ and O₃

Table 3 summarizes the lung lavage cell and lavage fluid parameters that were significantly affected by exposure to $O_3$, $HNO_3$, or $O_3$ plus $HNO_3$, as determined by nonparametric one-way analysis of variance and pairwise contrasts of group means. A number of parameters were affected by exposure to the high concentration $O_3$ or $O_3$ plus $HNO_3$ atmospheres; however, relatively few of the end points were affected by low concentration exposures, even though the total integrated exposure dose was the same for both concentrations. Exposure to both high and low concentrations of $HNO_3$ alone resulted in a modest but significant increase in lavage fluid elastase inhibitory capacity. Respiratory burst activity of freshly isolated lung macrophages was also inhibited by exposure to the low concentration of $HNO_3$; this assay was not done for the high concentration exposure.

Linear regression analysis showed that the effects of exposure to mixtures of $O_3$ and $HNO_3$ on lavage fluid protein content, elastase inhibitory capacity, and percentage of neutrophils in the lavage cell population were significantly less than the sum of changes caused by exposures to either $HNO_3$ or $O_3$ alone. The effects of the combined exposure were additive for pulmonary macrophage respiratory burst activity, production of $LTC_4$, and percentage of macrophages, indicating a lack of detectable interaction between the two pollutants for these endpoints.

### DISCUSSION

One purpose of this study was to examine the effects of short-term in vivo exposure to $HNO_3$ vapor on a variety of lung lavage parameters. This study has shown that exposure to $HNO_3$ results in a decrease in lung macrophage respiratory burst activity and an increase in the elastase inhibitory capacity of lung lavage fluid.

The term "respiratory burst" refers to the ability of phagocytes to generate a burst of superoxide in response to a variety of stimuli, including infectious agents. Single 4-h in vivo exposures to 1 mg/m³ $HNO_3$ plus 0.6 ppm $O_3$ significantly decreased the respiratory burst activity (measured by lucigenin chemiluminescence) of lung lavage cells recovered 18 h after exposure. Respiratory burst activity was unchanged (relative to controls) in macrophages from animals exposed to $HNO_3$ alone or to $O_3$ alone. It is important to note that, in this case, respiratory burst activity was measured after the lung lavage cells had been maintained in cell culture overnight to eliminate spontaneous respiratory burst activity. Macrophages in culture spontaneously release a wide variety of factors, such as arachidonic acid metabolites, interferons, and other cytokines, which are known to moderate respiratory burst activity (Kemmerich et al., 1987; Warren et al., 1988). Thus, the inhibitory effect of $O_3$ and $HNO_3$ in combination on respiratory burst activity could have been due to a

**TABLE 3.** Summary of the Effects of Exposure of Rats to $HNO_3$ and $O_3$, Alone and in Combination, on Lung Lavage Cells and Lavage Fluid Parameters

| Parameter | $HNO_3$ | $O_3$ | $O_3$ + $HNO_3$ | $O_3/HNO_3$ interaction[c] |
|---|---|---|---|---|
| *Lavage cell parameters* | | | | |
| Percent macrophages | | | | |
| 1 day [a] | ns | ↓ | ↓ | ns |
| 4 days[b] | ns | ns | ns | ns |
| Percent neutrophils | | | | |
| 1 day | ns | ↑ | ↑ | < Additive |
| 4 days | ns | ns | ns | ns |
| $LTC_4$ production | | | | |
| 1 day | ns | ↓ | ns | ns |
| 4 days | ns | ns | ns | ns |
| Respiratory burst activity | | | | |
| 1 day (20 h in vitro) | ns | ns | ↓ | ns |
| 4 days (20 h in vitro) | ns | ns | ns | ns |
| 4 days (0 h in vitro) | ↓ | ns | ns | ns |
| *Lavage fluid parameters* | | | | |
| Protein | | | | |
| 1 day | ns | ↑ | ↑ | < Additive |
| 4 days | ns | ns | ns | ns |
| Elastase inhibitory capacity | | | | |
| 1 day | ↑ | ↑ | ↑ | < Additive |
| 4 days | ↑ | ns | ns | ns |

*Note.* ↓, Significantly decreased ($p < .05$) relative to purified air group. ↑, Significantly increased ($p < .05$) relative to purified air group. ns, Not statistically significant ($p > .05$).

[a]For 1-day exposure: Purified air, 1 mg/m³ $HNO_3$, 0.6 ppm $O_3$, or 1 mg/m³ $HNO_3$ + 0.6 ppm $O_3$ for 4 h.

[b]For 4-day exposure: purified air, 0.25 mg/m³ $HNO_3$, 0.15 ppm $O_3$, or 0.25 mg/m³ $HNO_3$, 0.15 ppm $O_3$, 4 h/day for 4 days.

[c]Determined by linear regression.

direct effect on the respiratory burst mechanism or an indirect effect on respiratory burst modulatory factors. To clarify this issue, in the second series of exposures to the low concentration atmospheres, respiratory burst activity of freshly isolated lung macrophages was measured as well as the activity after the cells were cultured overnight. Exposure to 0.25 mg/m³ $HNO_3$, 0.15 ppm $O_3$, or 0.25 mg/m³ $HNO_3$ plus 0.15 ppm $O_3$ had no effect on respiratory burst activity measured after 18 h in culture. However, when respiratory burst activity was measured immediately after cell isolation, lung lavage cells from animals exposed to 0.25 mg/m³ $HNO_3$ had significantly decreased respiratory burst activity. This suggests that short-term culture of lung macrophages tended to reverse the inhibitory effect of $HNO_3$ exposure on respiratory burst activity.

Inhibition of pulmonary macrophage respiratory burst activity has also been observed in our laboratory after subchronic exposure of rats to

$HNO_3$ at concentrations of 0.15 and 0.45 $mg/m^3$ for 4 h/day, 3 days/wk, for 4 wk (unpublished data). Exposure of rabbits to $H_2SO_4$ for 2 h at concentrations ranging from 0.075 to 0.5 $mg/m^3$ has also been reported to decrease the respiratory burst activity of pulmonary macrophages by 45% (Zelikoff et al., 1992). It is not known if the decreased macrophage respiratory burst activity observed by us and others would result in increased susceptibility to respiratory tract infection in individuals exposed to acidic atmospheres. However, the Harvard Six-Cities Study (Speizer, 1989), which showed that the prevalence of bronchitis in children was directly related to the mean annual acid aerosol levels for each of four cities, suggests that pulmonary immune defenses are compromised by acid air pollution.

When we measured elastase inhibitory capacity of lung lavage fluid we found that it was increased in rats exposed to both high and low concentrations of $HNO_3$. However, we do not know whether this increase was caused by enhanced production of an elastase inhibitor within the lung or whether it was due to increased permeability and leakage of elastase inhibitors from the plasma into the lung lining layer. The lack of an accompanying significant change in lavage fluid protein content argues against $HNO_3$-induced changes in pulmonary permeability. However, more sensitive indices of serum influx into the lung, such as lavage fluid albumen content, would need to be examined before one can rule this out as the cause of the increased elastase inhibitory capacity that we observed. A more complete investigation of the effects of $HNO_3$ on elastase inhibitory capacity is needed to understand the implications of our finding in terms of human health effects.

The elastase inhibitory capacity of lung lavage fluid was also markedly increased (and was accompanied by increases of lavage fluid protein content and the percentage of neutrophils in the lavage cell population) after exposure to 0.6 ppm $O_3$. In this case, the results are consistent with numerous studies that have shown that acute $O_3$ exposure causes increased lung permeability and an influx of neutrophils (Bhalla and Young, 1992; Guth et al., 1986).

Our work is the first to examine the effects of exposure to $HNO_3$ vapor (as opposed to fog) and the interaction of this vapor with $O_3$. Under most conditions, atmospheric $HNO_3$ in southern California exists in vapor phase rather than as a fog (Larson, 1989). It is of interest, therefore, that the effects of $HNO_3$ vapor in combination with $O_3$ on elastase inhibitory capacity (high concentration exposure only), lavage fluid protein content, and percent neutrophils in the lavage cell population were found to be significantly less than the effects of $O_3$ alone (Table 3). Because the changes caused by the combination exposures were less than the sum of the effects caused by $HNO_3$ or $O_3$ alone, these findings indicate that an antagonistic interaction occurs between $HNO_3$ and $O_3$ under some circumstances. Equally of interest was our observation that this

antagonistic interaction was only observed for end points that are affected by $O_3$-only exposure. For parameters that are changed by exposure to $HNO_3$ alone, such as respiratory burst activity, an additive effect of combined exposure was observed instead.

Other research has demonstrated an antagonistic interaction between acids and $O_3$. Aris et al. (1991) recently reported that prior exposure to either $HNO_3$ fog or $H_2O$ fog did not potentiate $O_3$-induced changes in pulmonary function; in fact $HNO_3$ fog and $H_2O$ fog tended to antagonize the effects of $O_3$, but differences were not statistically significant. Grose et al. (1980) have shown that exposure of hamsters to $H_2SO_4$ followed by exposure to $O_3$ decreased ciliary activity but to a lesser extent than $H_2SO_4$ alone. Exposure to $H_2SO_4$ and $O_3$ in combination was found to have no effect on ciliary beat frequency (Grose et al., 1982). In contrast, $H_2SO_4$ and $O_3$ were found to act in an addictive fashion on host susceptibility to infection when mice were exposed to a mixture of $H_2SO_4$ and $O_3$ (Grose et al., 1982) or to a sequential exposure to $H_2SO_4$ followed by $O_3$ (Gardner et al., 1977). A statistically significant antagonistic effect of $H_2SO_4$ on $O_3$-induced changes in macrophage respiratory burst activity and phagocytosis has been reported following a 3-h exposure of rabbits to 0.075 and 0.15 $mg/m^3$ $H_2SO_4$ alone and in combination with various concentrations of $O_3$ (Schlesinger et al., 1992). Antagonistic effects of $H_2SO_4$ on $O_3$-induced changes in in vitro bronchial contractility at 0.05 $mg/m^3$ $H_2SO_4$ and a synergistic effect at 0.125 $mg/m^3$ were also reported.

While it is encouraging that a number of studies do not indicate an additive interaction between $O_3$ and $HNO_3$ or $H_2SO_4$ in animals or humans, short-term acute studies on healthy subjects do not necessarily predict the effects of long-term repeated exposure of a diverse population. There is a need for additional chronic studies focusing on the potential effects of repeated inhalation of nitric acid, both alone and in combination with ozone, at more environmentally realistic levels. Such studies might examine changes in pulmonary immune defenses, because macrophage respiratory burst activity was found to be altered following short-term exposure to $HNO_3$. Because $HNO_3$ is highly water-soluble and is expected to interact with the upper airway epithelium, changes in parameters such as mucus production and bronchial inflammation also warrant investigation.

## REFERENCES

Aris, R., Christian, D., Sheppard, D., and Balmes, J. R. 1991. The effects of sequential exposure to acidic fog and ozone on pulmonary function in exercising subjects. *Am. Rev. Respir. Dis.* 143:85–91.

Bhalla, D. K., and Young, C. 1992. Effects of acute exposure to $O_3$ on rats: sequence of epithelial and inflammatory changes in distal airways. *Inhal. Toxicol.* 4:17–31.

Chen, L. C., Fine, J. M., Qu, S. Q., Amdur, M. O., and Gordon, T. 1992. Effects of fine and ultrafine

sulfuric acid aerosols in guinea pigs: Alterations in alveolar macrophage function and intracellular pH. *Toxicol. Appl. Pharmacol.*, 113:109–117.

Gardner, D. E., Miller, F. J., Illing, J. W., and Kirtz, J. M. 1977. Increased infectivity with exposure to ozone and sulfuric acid. *Toxicol. Lett.* 1:59–64.

Grose, E. C., Gardner, D. E., and Miller, F. J. 1980. Response of ciliated epithelium to ozone and sulfuric acid. *Environ. Res.* 22:377–385.

Grose, E. C., Richards, J. H., Illing, J. W., Miller, F. J., Davies, D. W., Graham, J. A., and Gardner, D. E. 1982. Pulmonary host defense responses to inhalation of sulfuric acid and ozone. *J. Toxicol. Environ. Health* 10:351–362.

Guth, D. J., Warren, D. L., and Last, J. A. 1986. Comparative sensitivity of measurements of lung damage made by bronchoalveolar lavage after short-term exposure of rats to ozone. *Toxicology* 40:131–143.

Kemmerich, B., Rossing, T. H., and Pennington, J. E. 1987. Comparative oxidative microbicidal activity of human blood monocytes and alveolar macrophages and activation by recombinant gamma interferon. *Am. Rev. Respir. Dis.* 136:266–270.

Larson, T. V. 1989. The influence of chemical and physical forms of ambient air acids. *Environ. Health Perspect.* 79:7–13.

Last, J. A., Hyde, D. M., Guth, D. J., and Warren, D. L. 1986. Synergistic interaction of ozone and respirable aerosols on rat lungs. I. Importance of aerosol acidity. *Toxicology* 39:247–257.

Lee, J. S., Mustafa, M. G., and Afifi, A. A. 1990. Effects of short-term, single and combined exposure to low-level $NO_2$ and $O_3$ on lung tissue enzyme activities in rats. *J. Toxicol. Environ. Health* 29:293–305.

Lubman, R. L., Danto, S. I., and Crandall, E. D. 1989. Evidence for active $H^+$ secretion by rat alveolar epithelial cells. *Am. J. Physiol.* 257(*Lung Cell. Mol. Physiol.* 1):L438–L445.

Munger, J. W., Collett, J., Jr., Daube, B., Jr., and Hoffmann, M. R. 1990. Fogwater chemistry at Riverside, California. *Atmos. Environ.* 24B:185–205.

Pickrell, J. A., Gregory, R. E., Cole, D. J., Hahn, F. F., and Henderson, R. F. 1987. Effect of acute ozone exposure on the proteinase-antiproteinase balance in the rat lung. *Exp. Mol. Pathol.* 46:168–179.

Prasad, S. B., Rao, V. S., Mannix, R. C., and Phalen, R. F. 1988. Effects of pollutant atmospheres on surface receptors of pulmonary macrophages. *J. Toxicol. Environ. Health* 24:385–402.

Nielson, D. W., Goerke, J., and Clements, J. A. 1981. Alveolar subphase pH in the lungs of anesthetized rabbits. *Proc. Natl. Acad. Sci. USA* 78:7119–7123.

Schlesinger, R. B., Gunnison, A. F., and Zelikoff, J. T. 1990. Modulation of pulmonary eicosanoid metabolism following exposure to sulfuric acid. *Fundam. Appl. Toxicol.* 15:151–162.

Schlesinger, R. B., Zelikoff, J. T., El-Fawal, H., Kinney, P., and Chen, L. C. 1992. Pulmonary responses to sulfuric acid and ozone—Assessing toxicologic interactions. *Toxicologist* 12:230.

Snedecor, G. W., and Cohran, W. G. 1969. *Statistical Methods.* Ames: Iowa State University Press.

Speizer, F. E. 1989. Studies of acid aerosols in six cities and in a new multi-city investigation: Design issues. *Environ. Health Perspect.* 79:61–67.

Warren, D. L., and Last, J. A. 1987. Synergistic interaction of ozone and respirable aerosols on rat lungs. III. Ozone and sulfuric acid aerosol. *Toxicol. Appl. Pharmacol.* 88:203–216.

Warren, D. L., Guth, D. J., and Last, J. A. 1986. Synergistic interaction of ozone and respirable aerosols on rat lungs. II. Synergy between ammonium sulfate aerosol and various concentrations of ozone. *Toxicol. Appl. Pharmacol.* 84:470–479.

Warren, J. S., Kunkel, S. L., Cunningham, T. W., Johnson, K. J., and Ward, P. A. 1988. Macrophage-derived cytokines amplify immune complex-triggered $O_2^-$ responses by rat alveolar macrophages. *Am. J. Pathol.* 130:489–495.

Williams, A. J., and Cole, P. J. 1981. Investigation of alveolar macrophage function using lucigenin-dependent chemiluminescence. *Thorax* 36:866–869.

Zelikoff, J. T., Parsons, E., Sisco, M., Thomas, D., Yang, Z. J., and Schlesinger, R. B. 1992. Inhaled air pollutants disrupt macrophage-mediated immunity in the lung. *Toxicologist* 12:46.