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# Nitric Acid Toxicity:

# Potential for Particle and Ozone Interactions

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY
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Research Division

# NITRIC ACID TOXICITY: POTENTIAL FOR PARTICLE AND OZONE INTERACTIONS

Final Report 92-334

Prepared for:

California Air Resources Board
Research Division
2020 L Street
Sacramento, CA 95814

Prepared by:

Michael T. Keinman, Ph.D.
Deepak K. Bhalla, Ph.D.
William J. Mautz, Ph.D.
Katherine Osann, Ph.D.
Carol Basbaum, Ph. D.

Air Pollution Health Effects Laboratory
Department of Community and Environmental Medicine
University of California, Irvine
Irvine, CA 92717-1825

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## ABSTRACT.

The objective of this project was to determine whether or not airborne particles increased the potential for adverse health effects due to exposure to nitric acid ($HNO_3$), under conditions relevant to exposure of people to urban air pollution in California. This project examined air pollutant atmospheres containing $HNO_3$ in combination with ozone ($O_3$) and fine carbon (C) particles. Fischer 344/N rats were exposed, nose-only, to 5 different atmospheres at 60% relative humidity: (1) purified air [PA]; (2) 0.15 ppm $O_3$ [$O_3$]; (3) 50 $\mu g/m^3$ $HNO_3$ + 0.15 ppm $O_3$. [Ac + $O_3$]; (4) 50 $\mu g/m^3$ $HNO_3$ + 50 $\mu g/m^3$ C [Ac + C]; and (5) 50 $\mu g/m^3$ $HNO_3$ + 0.15 ppm $O_3$ + 50 $\mu g/m^3$ C [Ac + $O_3$ + C]. Exposures were for 4 hours/day, 3 consecutive days/week, for up to 4 weeks, with animals evaluated at 6 different time points. The study, in addition to determining whether or not exposure to ambient concentrations of $HNO_3$, $O_3$ and C, alone and in mixtures would cause acute responses, examined whether or not responses would be attenuated (as reported for $O_3$, alone), persist, or intensify during repeated exposures. Rats were killed after 1, 2, 3, 6, 9, and 12 exposure days. The 3, 6, 9 and 12 days of exposure occurred over 1, 2, 3 and 4 weeks, respectively. The regimen represented periodic, elevated concentration human exposures, and the components and concentrations selected for study were based on air monitoring data in a heavily polluted region in South Coast Air Basin of California.

Endpoints were evaluated that were related to human respiratory diseases such as bronchitis, emphysema and respiratory infections. The endpoints included quantification of morphological alterations of respiratory tract tissue compartments, determination of changes in respiratory tract cell populations and mucus secretion, and evaluation of impairment of lung defenses (epithelial permeability and macrophage functions).

The concentrations used were in the range of peak ambient exposures for each of the components. The exposure durations were short, relative to human lifetime ambient exposures. We did not expect, nor did we see, large perturbations in respiratory tissues or pulmonary defenses. The changes we observed were small and most did not achieve statistical significance. There were, however, patterns in the observed responses which suggest potential health consequences of ambient exposures to $HNO_3$, $O_3$ and C.

Alveolar chord length and alveolar wall thickness were increased in the lungs of rats exposed to the $HNO_3$ + C atmosphere. Ozone, alone and in combination with $HNO_3$-containing atmospheres tended to decrease septal wall thickness. The volume of trapped gas in the collapsed lungs from the Ac

+ $O_3$ rats was increased. These trends suggest that the $HNO_3$ + $O_3$ exposures reduced the elasticity of the lungs. People who smoke or who have emphysema display reduced lung elasticity and changes in pulmonary function measures that relate to gas trapping.

Increased mucus secretion and remodeling of small airways are hallmarks of bronchitis. We found increased numbers of secretory cell and increased secretory cell activity in the nasal respiratory epithelium of $HNO_3$-exposed rats. We also found increased (relative to controls) concentrations of mucin glycoprotein in samples from rats exposed to $HNO_3$ + C ($\pm$ $O_3$) for 2 weeks (6 days), but found decreased mucin glycoprotein concentrations in samples from rats exposed for 4 weeks (12 days). Tracheal secretory cell activity was also decreased In the 4 week-exposed rats (not measured in the 2 week-exposed rats).

Macrophage Fc receptor binding and phagocytosis were not significantly altered, but macrophages did tend to exhibit decreased lysosomal enzyme activities, after $HNO_3$ exposures. Respiratory burst activity was cumulatively increased by $O_3$ exposures over the 4 week exposure period; rats exposed to $O_3$ in the presence of $HNO_3$ $\pm$ C did not show this response.

In conclusion, the observed effects were small and few of the responses achieved statistical significance. The directions of changes in morphometric and biochemical endpoints, compared to controls, were consistent with patterns which might be associated with emphysema and bronchitis. There was no evidence of synergy among the atmosphere components, but the data suggest that the potencies of the atmospheres tested could be ranked as Ac+C $\geq$ Ac+C+$O_3$ $\geq$ (Ac+$O_3$ = $O_3$). There were significant differences between responses for short-term ($\leq$ 2 weeks) vs. longer term (4 week) exposures, with respect to macrophage respiratory burst activity and mucus secretion.

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## CONCLUSIONS AND RECOMMENDATIONS

This project examined the potential for airborne particles to modify the adverse health effects due to exposure to nitric acid ($HNO_3$) under conditions relevant to the exposure of people to urban air pollution in California. The atmospheres in communities of the South Coast Air Basin contain complex mixtures, both in terms of size and chemical composition of the ambient aerosol and the concentrations and ratios of gas phase photochemically active compounds such as nitric acid, nitrogen dioxide and ozone. Epidemiological studies of populations in this region have demonstrated possible acute and chronic health effects, indicated by increased rates of loss of pulmonary function and increased reports of respiratory symptoms. Epidemiologically, people living in more polluted urban environments, including several regions in the State of California, demonstrate increased rates of mortality, especially in individuals with pre-existing lung or cardiovascular diseases, increased numbers of hospital visits (especially for asthmatic individuals), and increased reports of respiratory symptoms, days of reduced productivity, and absences from schools than do people living in cities with fewer exceedances of State or Federal air quality standards. The health effects are often significantly associated with either ozone or with the particulate fraction of the urban pollution, but it is not always possible to separate out the effects of the oxidant gases (ozone and nitrogen dioxide) from those of the particulate phase, or from those of acidic copollutants, which are highly correlated with the oxidant gases. Because of the large influence of photochemical processes on air pollution levels in California, $HNO_3$ is a more prevalent source of acidity than is sulfuric acid ($H_2SO_4$), in contrast with other regions of the U.S.

This project examined air pollutant atmospheres containing $HNO_3$ in combination with ozone ($O_3$) and fine carbon (C) particles. Fischer 344/N rats were exposed, nose-only, to 5 different atmospheres at 60% relative humidity: (1) purified air [PA]; (2) 0.15 ppm $O_3$ [$O_3$]; (3) 50 $\mu g/m^3$ $HNO_3$ + 0.15 ppm $O_3$. [Ac + $O_3$]; (4) 50 $\mu g/m^3$ $HNO_3$ + 50 $\mu g/m^3$ C [Ac + C]; and (5) 50 $\mu g/m^3$ $HNO_3$ + 0.15 ppm $O_3$ + 50 $\mu g/m^3$ C [Ac + $O_3$ + C]. More than 95% of the $HNO_3$ in the Ac+$O_3$ mixture was in the vapor phase. However, more than 95% of the $HNO_3$ in the Ac+ C and the Ac + $O_3$ + C atmospheres was particle-associated. Exposures were for 4 hours/day, 3 consecutive days/week, for up to 4 weeks, with animals evaluated at 6 different time points. The study, in addition to determining whether or not exposure to ambient concentrations of $HNO_3$, $O_3$ and C, alone and in mixtures would cause acute responses, examined whether or not responses would be attenuated (as

reported for $O_3$, alone), persist, or intensify during repeated exposures. Rats were killed after 1, 2, 3, 6, 9, and 12 exposure days. The 3, 6, 9, 12 day exposures occurred over 1, 2, 3, and 4 weeks, respectively, and represented periodic, elevated concentration human exposures, and the components and concentrations selected for study were based on air monitoring data in a heavily polluted region in South Coast Air Basin of California.

Endpoints were evaluated that were related to human respiratory diseases such as bronchitis, emphysema and respiratory infections. The endpoints included quantification of morphological alterations of respiratory tract tissue compartments, determination of changes in respiratory tract cell populations and mucus secretion, and evaluation of impairment of lung defenses (epithelial permeability and macrophage functions).

The concentrations used were in the range of peak ambient exposures for each of the components, but were lower than those used in previous toxicological studies of the health effects of inhaled acidic compounds. The exposure durations were short, relative to human lifetime ambient exposures. We did not expect, nor did we see, large perturbations in respiratory tissues or pulmonary defenses. The changes we observed were small and most did not achieve statistical significance. There were, however, patterns in the observed responses which suggest potential health consequences of ambient exposures to $HNO_3$, $O_3$ and C. Responses which were statistically significant were changes in mucin glycoprotein concentrations in bronchoalveolar lavage fluid and a cummulative increase in macrophage respiratory burst activity with increasing $O_3$ exposure time. The implications of these findings, and the observed response trends, are discussed below.

Respiratory tract morphometry was examined in rats exposed for 1 month; 3 hr per day, 3 days per week for 12 total days. None of the exposure atmospheres caused statistically significant changes, compared to the clean air controls. We observed an increase in alveolar mean chord length in rats exposed to $HNO_3$ (50 $\mu g/m^3$) + C (50 $\mu g/m^3$) relative to that in rats exposed to $O_3$. Ozone tended to decrease septal wall thickness in mixtures with $HNO_3 \pm$ C. Both increased chord length and decreased wall thickness could suggest a loss of lung elasticity, but a clear pattern was not seen in this regard On the other hand, some investigators have reported that inhaled nebulized 1% $HNO_3$ significantly increased pulmonary closing volume (CV) and closing capacity (CC), changes which suggest loss of elasticity associated with $HNO_3$. Smokers often exhibit increased CC, compared to non-smokers. Excised lung gas volumes (ELGV), which is a measure of the

volume of gas trapped in a collapsed lung, and is correlated with CC and CV, was significantly increased in rats exposed to 450 $\mu g/m^3$ $HNO_3$ for 1 month and was slightly increased in rats exposed to 50 $\mu g/m^3$ $HNO_3$ + 0.15 ppm $O_3$ for 1 month. All of the pollutant atmospheres in this study caused small, but not significant, increases in ELGV relative to clean air controls. Thus, the observed associations, taken as a whole, are suggestive, but not conclusive evidence, that $HNO_3$ exposures, alone or in combinations with O3 and C, can cause in emphysema-like changes.

The volume fractions of lung tissues were measured and no statistically significant changes were observed. The volume fraction of alveoli was reduced and there was a corresponding increase the volume fractions of alveolar ducts and bronchioles. This so-called "bronchiolization" or remodeling of terminal airways may suggest that inhalation of acids, in conjunction with particles, could play a role in the etiology of bronchitis. A hallmark of bronchitis is changes in mucus secretion. Parameters related to mucus secretion were investigated in the present study. We demonstrated no no significant increase in the numbers of secretory cells in nasal respiratory epithelia, however secretory activity in nasal respiratory epithelia tended to be increased by the exposures, and the largest percent of secratory cells was observed in the nasal epithelia of rats exposed to $HNO_3$+$O_3$+C. On the other hand, secretory activity in tracheal epithelium tended to decrease in rats exposed to acid-containing atmospheres for 1 month and was accompanied by a significant decrease in the mucin glycoprotein content of bronchoalveolar lavage (BAL). In rats exposed for 2 weeks, mucin glycoprotein in BAL was, however, increased after exposure to the acid-containing atmospheres. Thus, there is a tenuous link between $HNO_3$ exposures and respiratory tract changes which might be related to the etiology of bronchitis.

Changes in macrophage functions were studied in rats exposed for 1, 2, 3, 6, 9, and 12 days. The results did not demonstrate any statistically significant changes or coherent patterns of response in macrophage viability, Fc receptor binding or phagocytic activity. Respiratory burst activity in macrophages from rats exposed to $O_3$ increased with increasing days of exposure. The rate of increase was statistically significant for unstimulated macrophages and for macrophages stimulated with PMA. Respiratory burst activity in macrophages from rats exposed to purified air or to $HNO_3$-containing atmospheres with or without $O_3$ did not show an increasing trend. It is of interest to note that macrophage respiratory burst activity demonstrated a cumulative effect of $O_3$ exposure, and that this pattern was not seen after adding $HNO_3$ to the atmospheric mixture.

In summary, all of the biological effects observed in this study were small, which is consistent with the low pollutant concentrations employed. Very few of the biological responses observed achieved statistical significance. The trends observed in morphometric, biochemical and histopathologic endpoints follow patterns which are generally consistent in direction with significant effects of acid-containing atmospheres administered at higher concentrations, and suggest that acid exposures may be associated with lung changes similar to those seen in cigarette smokers who are at risk of developing emphysema and chronic bronchitis. Data suggest that effects seen after short term ($\leq$ 2 week) exposures may be different from those observed after longer exposures. We conclude that the results of this study can not rule out the possibility that exposure to acid vapors and acid-associated particles under ambient conditions can increase the risk of lung diseases.

Recommendations are: (1) that additional studies at somewhat higher concentrations should be undertaken to determine whether or not the tenuous associations between $HNO_3$ exposures and the etiology of emphysema and bronchitis observed in this study are substantiated; (2) that future research include toxicological evaluation of other important constituents of ambient air pollution such as organic aerosols, other acid aerosols, and acid vapors (e.g. nitric, hydrochloric, acetic, and formic acids); (3) that future studies incorporate the use of new animal models, such as models of human respiratory diseases, and the use of "geriatric" animal models to better address the responses of sensitive populations; and (4) that the time course of changes in mucus production and the observed cumulative effects of repeated ozone exposures on macrophage respiratory burst activity and other functions be examined.

# INTRODUCTION

## *A. Objectives.*

The objective of this project was to determine whether or not airborne particles increased the potential for adverse health effects due to exposure to nitric acid ($HNO_3$), under conditions relevant to exposure of people to urban air pollution in California. This project examined air pollutant atmospheres containing $HNO_3$ in combination with ozone ($O_3$) and fine carbon (C) particles. Both $O_3$ and $HNO_3$ in ambient air are formed as products of photochemically-initiated atmospheric reactions of nitrogen oxides (derived primarily from combustion sources). Ozone concentrations in ambient air are, understandably, highly correlated with those of $HNO_3$. Hence, exposure to a combination of $HNO_3$ + $O_3$ (Ac + $O_3$) is relevant to human exposures in California. Elemental carbon, is an important constituent of the fine particle fraction of the urban aerosol and is also associated with emissions from combustion sources. Carbon aerosols are both respirable and capable of adsorbing vapor phase pollutants and of transporting these adsorbates into the lung, thus studies of the potential interaction of C with $HNO_3$ and $O_3$ are also relevant to ambient exposures in California. The questions of primary interest in this study included (1) whether or not the addition of C to $HNO_3$-containing atmospheres, with or without $O_3$ provoked significant biological effects, (2) whether or not the effects of the mixtures differed from those of the $O_3$ component alone, and (3) whether or not acute responses differed from those seen after repeated exposures.

## *B. Background.*

People in Southern California are exposed to complex mixtures of contaminants in ambient air, often including oxidant gases such as $O_3$ and $NO_2$, fine particles which contain elemental and organic carbon, nitrate and sulfate salts, and trace amounts of metals, such as cadmium and lead, coarse particles which contain silicates, metals and compounds derived from resuspended crustal materials, and both vapor phase and particulate phase acidic compounds. There has been a great deal of interest in the potential health risks due to acid air pollutant exposures. This interest has been largely driven by epidemiological associations between acid exposures and human morbidity and mortality (Thurston et al., 1989; Speizer, 1989; Ostro et al., 1991) and toxicological studies demonstrating interactions between acidic pollutants and $O_3$. At the national level most of the interest has related to sulfuric acid ($H_2SO_4$), which is formed in the atmosphere, for the most part, by the oxidation of sulfur dioxide ($SO_2$). The health effects of nitric acid ($HNO_3$), which is an important component of the vapor phase acids in Southern California's ambient air, have been the subject of relatively few studies. Nitric acid in

ambient air is produced by the oxidation of nitric oxide (NO) and nitrogen dioxide ($NO_2$). The process during the day is driven by photochemical processes, but atmospheric chemical processes can also contribute to $HNO_3$ formation at night and possibly indoors. $HNO_3$ contributes about 30% of the total atmospheric hydrogen ion content in Southern California (Solomon et al., 1988a), which averages approximately 0.8 micromoles/$m^3$, over a 24 hr period (Taylor, personal communication). Intensive monitoring studies of diurnal and seasonal changes in the composition of ambient air pollution in the South Coast Air Basin have been conducted and have demonstrated the complex nature of the potential exposure of residents to a number of compounds which have been associated with causing health effects. Perhaps, foremost among these in the relationship to acute health effects is $O_3$, however there have been several investigations which have demonstrated that the effects of $O_3$ can be modified by simultaneous exposure to other pollutants including nitrogen dioxide ($NO_2$) (Mautz et al., 1988; Kleinman et al., 1989), sulfuric acid particles (Schlesinger et al., 1992; Kleinman et al., 1989; Last and Cross, 1978; Warren and Last, 1987); and combinations of C aerosols and sulfuric acid aerosols (Kleinman et al., 1992). It should be noted that mixtures of $O_3$ and $NO_2$ also contain $HNO_3$, hence studies demonstrating interactions between $O_3$ and $NO_2$ may reflect some influence of $HNO_3$. In the atmosphere, variations in $HNO_3$ concentrations are strongly correlated with those of $O_3$ and in the absence of further studies of atmospheric mixtures it may be difficult to accurately assess the risks associated with exposures to ambient acidic pollutants.

Most studies of $HNO_3$ health effects have been performed using concentrations that are high compared to ambient levels, or have used routes of administration other than inhalation, for example intratracheal instillation of dilute $HNO_3$. Transorotracheal instillation of 0.5% $HNO_3$ in saline in Syrian golden hamsters showed, during a 60-day study period, morphometric changes including decreased lung volumes and decreased internal surface areas. Biochemical changes showed increases in lung weight and in total collagen and elastin (Coalson and Collins, 1985). Bronchial injury and lung mechanics (total lung capacity, vital capacity, resistance and compliance) changes were induced in beagle dogs by exposure to nebulized 1% nitric acid ($HNO_3$) on alternate days for 4 wk (Peters and Hyatt, 1986). The changes in these dogs persisted throughout a 5 month post-exposure period, and in addition, hyperresponsiveness to histamine developed after 3 months post-exposure (Fujita et al., 1988). Hamsters exposed by intratracheal instillation of 0.5 ml of 0.08 N nitric, hydrochloric, or sulfuric acid showed statistically significant increases in secretory cell metaplasia and all three acids caused similar interstitial fibrosis, bronchiolectasis, and bronchiolization of alveoli (Christiansen et al., 1988). Human clinical studies of inhaled $HNO_3$ vapor demonstrated small changes in pulmonary function (Koenig et al., 1989). Sequential acute exposures of healthy adult human volunteers to $O_3$ or to $HNO_3$ fog droplets of about 6 mm aerodynamic diameter demonstrated significant pulmonary function decrements with $O_3$, but less effect when $HNO_3$ was added to the $O_3$ atmosphere (Aris et al.,

1991). A subsequent study, in which healthy human volunteers received single 4 hr exposures to 0.2 ppm $O_3$, 500 $\mu g/m^3$ $HNO_3$ vapor, or a mixture of $O_3$ + $HNO_3$, was performed by the same group (Aris et al., 1993). They found no significant differences in pulmonary function tests, or in cellular or biochemical constituents of bronchoalveolar lavage between the acid-exposed and clean air control groups. Nor did they observe significant atmosphere-related differences in bronchial biopsy specimens obtained 18 hr post-exposure. On the other hand, recent ARB-funded studies with both rats and rabbits reported significant effects of $HNO_3$ inhalation on lung morphometry and cellular or biochemical constituents of BAL after repeated (1 month) inhalation exposures at concentrations lower than those used in the studies described above (Mautz et al., 1995). No previous studies examined the question of whether or not particulate components of inhaled atmospheres would potentiate the effects of $HNO_3$. Since $HNO_3$ in ambient air is invariably accompanied by particulate copollutants, the interactions between $HNO_3$ and particles represented an important area for investigation.

The selection of an atmosphere for toxicological evaluation, designed to simulate ambient air to which people are exposed, is never a simple process. For this study we evaluated the available published literature, and consulted with ARB staff and several other scientists. Anlauf et al. (1991) measured diurnal variations in $HNO_3$ concentrations in Glendora, CA during a one week period in the summer of 1986. Peak 4 hr $HNO_3$ concentrations averaged about 50 $\mu g/m^3$, 12 hr daytime (8 AM to 8 PM) concentrations averaged 30 $mg/m^3$, and 24 hr average concentrations were 18 $\mu g/m^3$. $HNO_3$ is however only one of several vapor phase acids present in ambient Southern California atmospheres. Formic and acetic acid vapor 24 hr concentrations in Claremont, CA averaged about 200 nanoequivalents per cubic meter ($neq/m^3$), each (Grosjean, 1990). Hydrochloric acid (HCl) 24 hr vapor concentrations averaged about 100 $neq/m^3$ (Appel et al., 1991) and $HNO_3$ 24 hr average concentration measured about 400 $neq/m^3$ in Claremont, CA (Solomon et al., 1988b; 1992). Thus, one can compute that vapor phase 24 hr average total acidity in the South Coast Air Basin is about 900 $neq/m^3$ which, if it were treated as being just $HNO_3$ would be approximately equivalent to 50 $\mu g/m^3$. C containing aerosols, along with nitrate and sulfate particles make up a major proportion of ambient fine particles (less than 2.5 mm aerodynamic diameter) in the South Coast Air Basin. This study selected, as a reasonable scenario for evaluating $HNO_3$-particle interactions, a case in which elemental C particles were used as a surrogate for PM10. C aerosols have large surface areas should adsorb vapor phase acids, improving their ability to penetrate to the deep lung. Elemental C averages about 13 ± 7 % (mean ± s.d.), by mass, of the fine particle (<2.5 mm) fraction of PM10. Total carbon, organic + elemental, represents about 40% of the fine particle fraction (Cass et al., 1993). The 24 hr average

ambient PM10 concentration often exceeds the State standard in Southern California, and the proposed study is designed to simulate peak 4 hr exposures, which are higher than the 24 hr average. Hence, we have selected a C aerosol concentration that most likely exceeds expected ambient C levels, but not by an unrealistic amount, and were equal to the State PM10 standard (50 $\mu g/m^3$), albeit our exposures are administered for 4 hr per day, whereas the State standard is a 24 hr average.

### *C. Exposure Plan.*

This study was a sub-chronic exposure (4 hours/day, 3 consecutive days/week, for up to 4 weeks), with animals sacrificed at 6 different time points during the study. The goal was to determine whether or not exposure to ambient concentrations of $HNO_3$, $O_3$ and C, alone and in mixtures would cause acute responses and whether or not these responses would be attenuated (as reported for $O_3$, alone), persist, or intensify during the series of repeated exposures. Accordingly, rats were killed after 1, 2, 3, 6 (2 weeks), 9 (3 weeks) and 12 (4 weeks) exposure days. The exposures represented periodic, elevated concentration human exposures. Exposure subjects were Fischer 344/N rats, barrier reared and maintained and specific pathogen free. All exposures were nose-only. Not all the endpoints were evaluated at each sacrifice time.

### *D. Biological Endpoints.*

1. Lung Morphometry and Histopathology. Respiratory tract injury from exposure to a variety of air pollutant compounds alters the fine structure of the lung tissues in a manner that can be quantified by morphometric analysis (Barr et al., 1988, 1990; Tyler et al., 1987; Mauderly et al., 1987; Hyde et al., 1992; Chang et al., 1991, 1992; Pino et al., 1992; Jakab and Bassett, 1990; Gehr et al., 1993). Morphometric analyses for this study included measurement of volume fractions of lung parenchyma tissue compartments (Gehr et al., 1993), and measures of fixed lung volume, average chord length of alveoli, and average septal wall intercept. These measures are sensitive to pulmonary injury and inflammation associated with foci of interstitial infiltration and to changes in inflation of peripheral lung tissues associated with changes in tissue compliance. Nasal epithelial tissues are the front line site for exposure and deposition of inhaled irritants and particles at high concentrations. Sites of particular high sensitivity or high deposition include respiratory and transition epithelia (Buckley et al., 1984; Mautz et al., 1988; St. George et al., 1993). Long-term exposure may alter the distribution

of cell types in these epithelia, and morphometric analyses of nasal epithelial tissues for this study quantified relative numbers of the dominant cell type categories.

In addition to changing representation of cell types, epithelial content of secretory substances may be altered (St. George et al. 1993). We therefore performed ELISA for secreted mucin, and histochemical staining for mucosubstances in nasal and tracheal epithelia and quantified staining density by image analysis to determine if exposures altered secretory substances in these epithelia.

Histochemical staining was also used to quantify acid phosphatase in pulmonary macrophages. Acid phosphatase is a marker for lysosomes in mammalian cells (Duray et al., 1984; Henderson et al., 1979). Changes in population or activation of pulmonary macrophages in response to exposure was quantified as shifts in the distribution of cells differing in density of this lysosomal marker.

2. Epithelial Permeability. A number of studies have shown pulmonary changes following inhalation exposure to air pollutants. An increase in airway permeability appears to be a sensitive indicator of pulmonary effects following exposure to airborne pollutants. In guinea pigs, Hu et al. (1982) found an increase in permeability as indicated by significantly elevated protein levels in the BAL at 10-15 hr after a 3 hr exposure to 0.51 ppm $O_3$. Bassett et al. (1988) found an increase in albumin concentration at 1 and 3 days postexposure. Miller et al. (1986) detected an elevation in HRP transport from airways to blood at 8 hr. after a 1-hr exposure of guinea pigs to 1 ppm $O_3$. Koren et al. (1989) and Devlin et al. (1991) found increased protein levels in BAL collected at 18 hr following an exposure of exercising human subjects to 0.4 ppm $O_3$ for 6.6 hr. Studies from our laboratory have shown time related changes in airway permeability, as detected by increased transport of labeled tracers from the airway to blood and elevated protein and albumin contents of the BAL recovered from rats exposed to $O_3$ or particle containing atmospheres (Bhalla et al., 1986; Bhalla and Young, 1992; Young and Bhalla, 1992; Bhalla et al., 1993; Kleinman et al., 1993).

3. Pulmonary Macrophage Function and Lavage Fluid Analysis. Pulmonary macrophages were evaluated to determine if this crucial element of pulmonary defense is compromised. Epidemiological findings indicate that pollutant exposure can result in increased rates of respiratory infections; this is borne out by studies with laboratory animals which demonstrate increased susceptibility to infection

after exposure to acids and oxidant pollutants (Rose et al., 1988). Changes in macrophage function resulting from pollutant exposure are indicative of these adverse effects of the pollutants.

Macrophages possess receptors on their surfaces for the Fc portion of immunoglobulin (IgG) which help in the recognition of foreign antigens and pathogens (Gaafar et al., 1971). These receptors also mediate phagocytosis and lysis by the macrophages (Boltz-Nitalescu et al., 1981). The binding of these receptors with immune complexes facilitates the phagocytosis of IgG-coated particles (such as bacteria), stimulates both the secretion of the reactive oxygen intermediates and the release of lysosomal hydrolases, and also mediates antibody dependent cellular cytotoxicity (Johnston et al., 1985). Damage to the macrophage cell membrane can also result in leakage of lysosomal enzymes, proteases and $Ca^{+2}$ ion. Modification of the cells $Ca^{+2}$ balance can result in abnormal releases of oxygen free radicals. The phagocytosis of pathogens by macrophages triggers a respiratory burst, which is characterized by the production and release of reactive oxygen species, notably the superoxide radical. This respiratory burst can be modified by inhaled pollutants (Kleinman et al., 1995) and measurement of this function can be a sensitive indicator of potential health effects. These factors may be related to the development of chronic lung disease in that when they are released in abnormal quantities they may adversely affect surrounding cells and tissues.

The ability of macrophages to engulf foreign particles (polystyrene latex microspheres) by phagocytosis was measured in macrophages recovered from lungs of exposed and control rats (Prasad et al., 1988). The function of the macrophage Fc receptor in antigen-antibody response was assessed by measuring the capacity for binding sheep red blood cells in a rosette assay (Rao et al., 1980; Prasad et al., 1988; Kleinman et al., 1993). Several analyses were performed on bronchoalveolar lavage fluid. Cytological smears of the lavage fluid were made and numbers of monocytes (including mature and immature macrophages), PMN's and, when present, inflammatory cells (eosinophils and basophils) and lymphocytes were counted. Total protein concentration in cell free lavage fluid was measured to provide estimates of permeability changes.

# METHODS

### *A. Exposure Atmosphere Generation and Characterization.*

Vapor phase nitric acid for the $HNO_3 + O_3$ mixture was generated by vaporization from a 6N $HNO_3$ solution in a thermostated generator at 30°C. Nitric acid plus C atmospheres were generated by nebulization of a suspension of nitric acid-washed carbon (Monarch, Inc.) in dilute nitric acid. The target $HNO_3$ concentration was 50 $\mu g/m^3$ summed over the particulate and vapor phases. The resulting aerosol was dried by dilution with dry purified air, passed through a $^{85}Kr$ static discharge unit to reduce particle charges to Boltzmann equilibrium levels, and equilibrated to 60% relative humidity with humidified purified air. This technique of generation was selected to maximize the intimate contact of C with $HNO_3$. The dissociation between particulate and vapor phases of $HNO_3$ was determined using an annular denuder-sampling method (John et al., 1988; Appel et al., 1988). Briefly, air was sampled from the rats' breathing zone at 10 L $min.^{-1}$ using a multistage denuder system (URG, Inc., Carrboro, NC). The first stage was an annular denuder coated with $Na_2CO_3$ to quantitatively strip vapor phase $HNO_3$ from the airstream. The second stage was a fluorocarbon-coated filter which to quantitatively collect particles. The third stage was a nylon filter to collect $HNO_3$ stripped from the deposited particles. The denuder and nylon filter stages were extracted in carbonate-bicarbonate buffer and analyzed by ion chromatography. The fluorocarbon filter was extracted in distilled water and analyzed for nitrate and ammonium concentrations by ion chromatography. The vapor phase $HNO_3$ concentration was determined from stage 1. The particulate phase $HNO_3$ concentration was determined as the sum of stages 2 and 3, after correction for ammonium nitrate, if any. A fluorocarbon-coated aluminum nose-only exposure system was used to minimize dissociation of the C-adsorbed $HNO_3$ to form $HNO_3$ vapor. The metal shell of the chamber was grounded to reduce static charge attraction of the aerosol particles to the walls of the ducts and chambers.

Carbon was collected on quartz fiber filters. Sized samples were collected using an 8-stage cascade impactor (Andersen, Inc., Atlanta, GA). Collected samples were analyzed by combustion in oxygen to produce carbon dioxide. The carbon dioxide was quantitatively measured using an infra-red absorption detector (Dasibi Model 3003, Glendale CA). The detector was calibrated using aqueous oxalic acid solutions of known concentration and vs. aliquots of carbon suspension of known concentration.

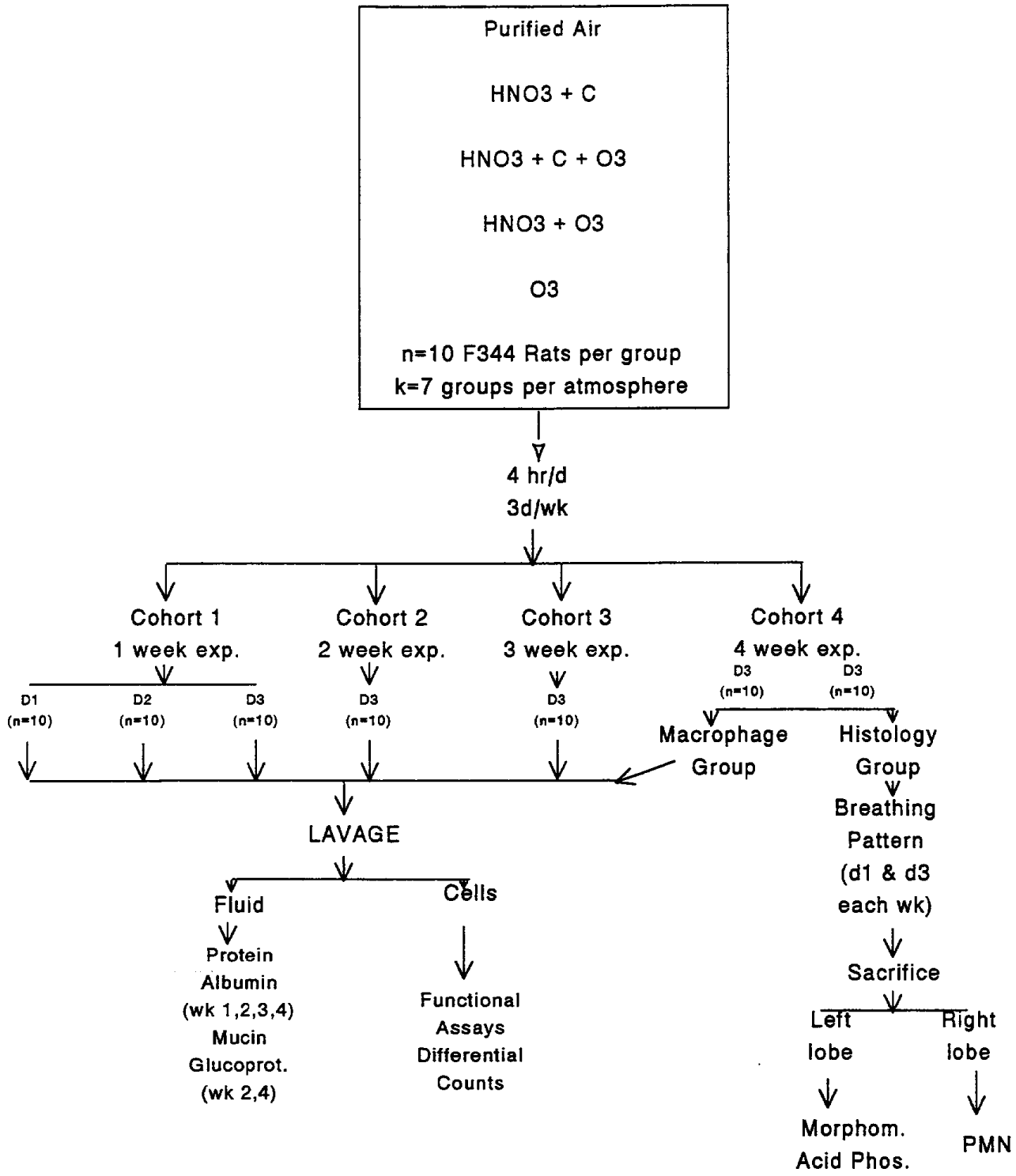
Ozone was generated by passing medical-grade oxygen through an electrical $O_3$ generator (Sander Ozonizer, Type III, Osterberg, Germany). The $O_3$ concentration was monitored continuously using a calibrated ultraviolet monitor (Dasibi Model 1003-AH, Glendale, CA).

### *B. Animal Housing, Quality Control, and Exposure.*

Exposure subjects were male Fischer 344/N rats (Simonsen Laboratories, Inc., Gilroy, CA), barrier reared and maintained in laminar flow isolation units supplied with filtered air. Rats were permanently marked for identification with tattoos. Housing was randomized for rat exposure groups. On arrival from the supplier, 10 animals were sacrificed for quality control analysis. Lungs were examined grossly, and in histological section, for presence of any signs of respiratory infection. Serum samples from 3 animals were collected and tested in a viral and mycoplasma exposure panel by the University of Southern California Animal Diagnostic and Disease Surveillance Laboratory. During the period that the rats were held in the laboratory for exposures, sentinel animals were maintained. Samples of 3 sentinel animals were sacrificed for quality control analyses every 2 weeks. Exposures of rats were nose-only to a continuous stream of the atmospheres. Nose-only exposures prevented artifacts due to airborne dander, ammonia, and dried excreta. Exposures were 4 hours per day on 3 consecutive days per week. Between exposures, rats were housed in a purified air-barrier environment and they were given clean water and dry laboratory chow *ad lib*. Animals were handled by personnel wearing clean lab coats, surgical masks, head covers, shoe covers, and gloves in order to prevent the spread of infections to the rats. Exposure tubes were thoroughly machine-washed in hot soapy water and disinfected with bleach daily. Animal quarters were cleaned twice weekly.

The experimental design is summarized in Figure 1. This figure shows the exposure groups, sacrifice times and endpoints evaluated at each sacrifice time.

Figure 1. Experimental Plan



### *C. Respiratory Tract Morphometry and Histopathology.*

Rats were anesthetized with sodium pentobarbital and killed by exsanguination via the abdominal aorta. The thoracic cavity was opened, and the trachea and lungs exposed. A small opening was made in the trachea just craniad of the bifurcation and a cannula inserted and tied in place. The cranial portion of the trachea was removed and fixed in 10% buffered formalin. The entire lung, with attached cannula, was then fixed by inflation with 10% buffered formalin at a pressure of 30 cm of water for 72 hr, in preparation for morphometric studies (McClure et al., 1982). After fixation, fixed lung volume was determined by the method of Scherle (1970). The left lobe provided all sections used for morphometry analysis. Prior to embedding in paraffin, the lobe was cut longitudinally to expose the left main airway and major intrapulmonary airways. The area of the exposed surface was digitized and stored using a computerized image analysis system (Oncor Model 150, Gaithersberg, MD). After embedding in paraffin, 5 mm sections were cut en face, mounted on glass slides, and again digitized to determine the degree of shrinkage during embedding and sectioning. Separate sections were be stained for morphometry, ARG preparation, acid phosphatase, or carbon particle content of macrophages. The fixed trachea was embedded in paraffin and 5 mm cross-sections cut from the cranial, middle, and distal regions. Sections were either stained with Alcian Blue/Periodic Acid Schiff stain or prepared for ARG.

For analysis of the nasal region, the head was skinned, external tissue and muscle removed, and the nasal section fixed by immersion with vacuum degassing in 10% buffered formalin. Decalcification was performed in 6% EDTA, followed by embedding in paraffin and sectioning at 5 mm for autoradiography and morphometry. Cross-sections of the head were cut in the region designated as Level I by Young (1981), approximately midway between the nares and the eye to provide sections containing squamous, transition, respiratory, and olfactory epithelium.

Morphometric measurements of the lung were made using a computer image analysis system (Summagraphic digitizer interfaced with a PC/AT and proprietary software; Oncor Model 150, Gaithersberg, MD). The subgross volume fractions of parenchyma and nonparenchyma, including large airways, large vessels, and other tissues, were estimated using a lattice system consisting of a 10 x 10 grid based on the methods described by Weibel (1966, 1979). A computer-generated lattice was laid over a video image of the lung section. Intersections (points) falling on structures of interest were scored separately and used to calculate the relative volume fractions of each. The same general

approach was used at higher magnification to estimate the relative fractions of interstitial tissue, cell nuclei, alveolar space, capillaries, macrophages, and alveolar surface. Standard formulae were used to estimate the lung volume fractions (Elias and Hyde, 1983). Data from the subgross level were used to correct data from point counts for parenchymal fractions, so the final data were expressed as volume fractions of the whole lung.

All analyses of histologic preparations were scored blind in that the reader had no knowledge of the exposure groups when slides were scored. In an initial screening, any preparations that showed evidence of problems in processing which could contribute to analysis errors (such as uneven lung inflation or imprecise section plane) were rejected from the analysis.

### *D. Bronchoalveolar Lavage and Airway Epithelial Permeability.*

Procedures for lavage and permeability measurements were similar to those described previously (Bhalla et al., 1986). For measuring permeability, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, IL), 5 mg/100 g body weight. The abdominal aorta was severed and a polyethylene catheter (ID 0.025 cm; OD 0.23 cm) was placed in the trachea and tied in place. The lungs were lavaged via the cannula using 7 ml aliquots of phenol red-free Hank's balanced salt solution, without $Ca^{++}$ or $Mg^{++}$ (HBSS). The HBSS was instilled and withdrawn 3 times and saved. The lavage procedure was repeated with a new aliquot of fluid and the fluids were pooled. The lavage was repeated a third time and the fluid was saved separately. The lavage from each animal was centrifuged at 1000 rpm for 10 min. The supernatant was stored at -70°C for protein analyses at a later time. The cellular fraction was stored on ice for further analyses, as described in Section E. Total protein in the BAL was determined by a bicinchoninic acid (BCA) procedure (Smith et al., 1985). A set of protein standards of concentrations ranging from 0.05 to 0.25 mg/ml was prepared by diluting a stock solution of bovine serum albumin (BSA). Protein standards, unknown samples, and diluent used for blanks (0.10 ml each) were pipetted into appropriately labeled test tubes. Each tube then received 2 ml of BCA working reagent prepared by mixing 50 parts of reagent A with 1 part of reagent B (Pierce BCA Protein Assay Reagent; Pierce Chemical CO, Rockford, IL). All samples were incubated at 60°C for 30 min., then cooled, followed by measurement of absorbance at 562 nm. Absorbance of blanks was subtracted from that for standards and unknown samples. A standard curve was prepared by plotting the net absorbance at 562 nm versus protein concentration, and the protein concentration of unknown samples was determined.

### *E. Macrophage Function Assays.*

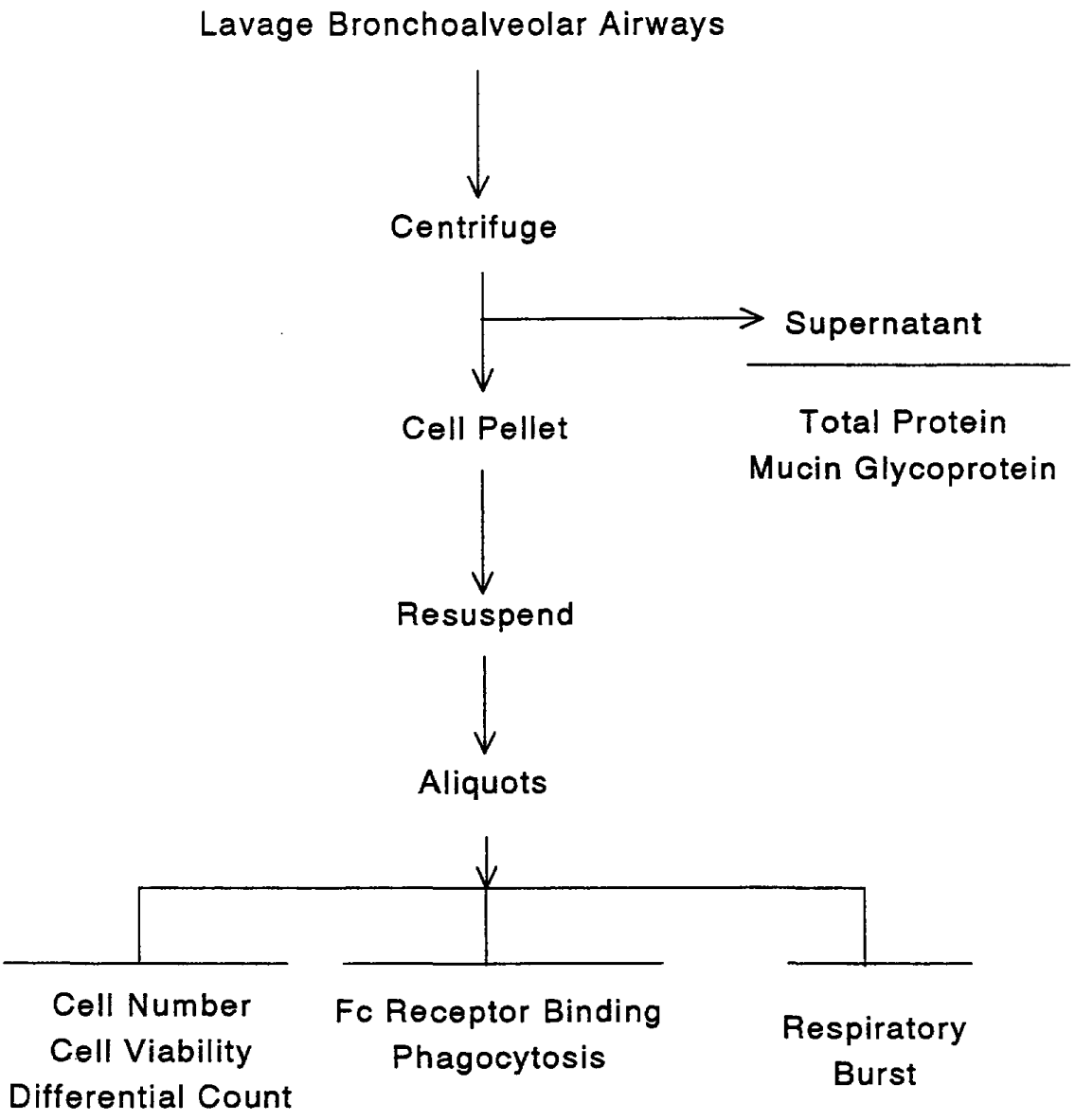
The cells obtained following centrifugation of the BAL were resuspended in HHBS, pooled, and used to prepare slides for differential counts, phagocytosis assays, and Fc receptor assays. The bronchoalveolar lavage procedures and assays are summarized in Figure 2.

Differential cell counts were performed using a bright line hemocytometer. The viable cells were identified by Trypan blue exclusion. The volume of the cell suspension was adjusted to 1 million cells per ml. A 0.1 ml aliquot of cells was pelleted onto a glass microscope slide using a cytocentrifuge. The cells were stained with Wright-Giemsa stain (Dif Quik, Baxter Healthcare Corp., McGaw, IL) and a differential count was made. The remaining cell suspension was aliquoted as described below.

Phagocytic activity of pulmonary macrophages was measured by a suspension assay. In brief, 0.5 ml of each cell suspension was added to a polypropylene tube containing 2.0 ml of culture medium and 5 X $10^8$ polystyrene latex spheres. The tubes were incubated with gentle agitation for 60 min. One ml of the suspension was pelleted onto a slide using a cytocentrifuge. The slides were fixed with methanol and immersed in xylene for 8 hr to remove excess PSL microspheres. The slides were stained with a Wright-Giemsa monochromatic stain and phagocytized spheres were visualized as unstained "ghosts" in the cell cytoplasm. The percentage of PSL positive cells (defined as containing $\geq$ 2 spheres/cell) was determined. A second measure, of cells ingesting $\geq$ 4 PSL spheres, was made as an index of phagocytic capacity. A total of 200 macrophages were counted for these determinations.

A rosette assay was used to determine the effect of exposure on Fc receptors. Lab-Tek chambers, each containing 1 x $10^5$ cells in 0.1 ml of HBBS, were incubated for 1 hr at 37°C and non-adherent cells removed by washing with medium. Fc receptor binding ability of the macrophages was measured by rosette assay (Rao et al., 1980; Prasad et al., 1988; Kleinman et al., 1993). In brief, 0.1 ml of anti-Sheep Red Blood Cells (SRBC) antibody was added to each of the chambers and incubated for 30 minutes at 37°C. After incubation, the macrophages were washed gently to remove excess antibody and 0.1 ml of SRBC (1 x $10^7$) was added to each chamber. The macrophages were then incubated with the SRBC for 30 minutes at 37°C, unbound SRBCs were washed away gently and

Figure 2. Flow Chart of Brochoalveolar Lavage Procedures and Assays



the number of cells (out of a total sample of 300) with three or more SRBC's attached were counted using an inverted microscope.

The antibody was prepared as follows. SRBCs obtained in Alseviers solution were washed three times with PBS and the cell count adjusted to 5 x $10^9$ cells/1.0 ml. Adult rats were injected IP with 5 x $10^8$ SRBC in PBS (0.2 ml). Each rat received four such injections at weekly intervals. Ten days after the last injection the rats were bled and the serum separated. The antiserum was inactivated at 57°C for 30 minutes and the titer of the antibody assayed by its ability to bind to the Fc receptor of macrophages, as determined by the rosette assay.

Macrophage respiratory burst activity was determined spectrophotometrically by measuring the reduction of cytochrome C after a timed incubation with macrophages, with and without stimulation with phorbol myristate acetate (PMA). Replicate samples were incubated with superoxide dismutase (SOD), and respiratory burst activity was reported as SOD-inhibitable activity (Kleinman et al., 1995).

Mucin glycoprotein in BAL was determined by ELISA, under a subcontract with Dr. Basbaum at U.C. San Francisco. Briefly, 50 µl samples containing glycoprotein in 0.05 N sodium bicarbonate and 1 % gluteraldehyde are added to the wells of a polylysine-coated 96 well microtiter plate, and dried overnight at 40°C. The wells were blocked for 1 hr at room temperature with 100 µl PBS containing 0.05% Tween 20 (Sigma Chem, St.Louis, MO). The wells were washed 4 times with PBS containing 0.05% Tween 20 and 1% periodate-treated bovine serum albumin [PBS/Tween/BSA] (Sigma Chem.). Anti-glycoprotein antibody (prepared at UCSF) was diluted 1:100 in PBS/Tween/BSA, 50 µl were added to each well and the plate was incubated 2 hr at room temperature. The wells were emptied and washed 4 times with PBS/Tween/BSA. A galactosidase-conjugated second antibody (Bethesda Research Labs, Bethesda, MD) was diluted in ELISA buffer (0.01 M phosphate, 0.15 M NaCl, 1.5 mM $MgCl_2$, 2 mM mercaptoethanol, 0.05% sodium azide), 50 µl were added to each well, and the plate incubated 2 hr at room temperature. The wells were rinsed 4 times with ELISA buffer. β-galactosidase activity was deterimined spectrophotometrically after incubation with p-nitrophenyl-β-D-galactopyranoside for 30 min.

### *F. Statistical Analysis.*

This study of macrophage functions and permeability followed a nested design, with data collected at 6 different timepoints during an overall 1 month exposure period. Data for each biological endpoint variable were tested for significant effects of exposure atmosphere and exposure duration

using two-way analysis of variance. Significant differences among atmosphere group means were tested with *a posteriori* multiple comparisons using Bonferroni adjustment of critical values. The morphometric and mucin glycoprotein endpoints were only measured in the rats exposed for 1 month. Data for these endpoints were tested for significant effects of exposure atmosphere using a one-way analysis of variance. Again, significant differences among atmosphere group means were tested with *a posteriori* multiple comparisons using Bonferroni adjustment of critical values.

The questions of primary interest included whether the addition of C to $HNO_3$-containing atmospheres, with or without $O_3$ had significant effects and whether the effects of the mixture differed from the effects of the $O_3$ component alone. Thus, 4 comparisons were appropriate: pure air *vs.* $O_3$, , pure air *vs.* mixture with $O_3$, pure air *vs.* mixture without $O_3$, and mixture with $O_3$ *vs.* mixture without $O_3$. Comparisons were two-tailed, and the significance level was set at $p < 0.05$. Results of the exposures show significance tests for levels up to $p < 0.10$ for exposure comparisons to purified air control with Bonferroni correction for multiple comparisons (asterisks). In addition, this study sought to evaluate possible exposure time-dependent changes in biological responses. Significant interaction between exposure time and exposure atmosphere in the two-way analyses of variance would suggest such changes, and if present, orthogonal decomposition would be applied to determine if such interactions represented a linear (monotonic increasing or decreasing) pattern or a quadratic (concave or convex) pattern.

# RESULTS

## *A. Atmosphere Generation and Characterization*

Nitric acid and carbon atmospheres were generated by nebulization of a dilute suspension of carbon black which had been pre-washed with 0.1 N $HNO_3$ to saturate adsorption sites, and then washed with water to remove excess $HNO_3$. On nebulization, sufficient $HNO_3$ was added to achieve a breathing zone total $HNO_3$ concentration of 50 $\mu g/m^3$. Exposures were for 4 hr per day, 3 days per week. Samples were collected continually during each 4 hr exposure period to determine both vapor phase and particle-bound $HNO_3$ levels, and C concentrations. Initially, samples from particle-containing atmospheres were collected using a 3-stage annular denuder collections system. The nitric acid vapor collected on the first stage was less than the detection limit of our procedure (3 $\mu g/m^3$). Because these samplers were cumbersome and the analyses were very time consuming we eliminated the first stage of the collector and continued to use a two-stage system (fluorocarbon-coated prefilter followed by a nylon backup filter). The data presented in Table 1 are the averages for the 23 days on which exposures were performed. Vapor phase $HNO_3$ was at or below the detection limit (3 $\mu g/m^3$), throughout the study in the particle-containing atmospheres. The data reported as $HNO_3$ in Table 1 therefore represent particle-bound $HNO_3$, except for the Ac + O3 atmosphere, in which more than 95% of the acid was in the vapor phase. As shown in Table 1, good agreement with the atmosphere target concentrations was achieved, and the atmospheres were well matched, albeit the C concentrations were slightly below the target value. Particle size for the Ac + C and Ac + C + $O_3$ atmospheres was determined using an 8-stage cascade impactor. The mass median aerodynamic diamters were 0.4 with geometric standard deviationsof 2.3.

**Table 11.** Ozone ($O_3$), Nitric Acid (Ac), and Carbon (C) concentrations in exposure atmospheres averaged over the total exposure period. Data are mean ± SD, n of daily averages. The target concentrations are shown in the last row of the table.

| Atmosphere | Ozone (ppm) | Nitric Acid ($\mu g/m^3$) | Carbon ($\mu g/m^3$) |
|---|---|---|---|
| Purified Air | ≤ 0.03 | ≤ 2 | ≤ 5 |
| $O_3$ | 0.15 ± 0.004 | ≤ 2 | ≤ 5 |
| Ac + $O_3$ | 0.16 ± 0.004 | 54 ± 7.8[A] | ≤ 5 |
| Ac + C | ≤ 0.03 | 50 ± 7.6[B] | 46.9 ± 13 |
| Ac + $O_3$ + C | 0.15 ± 0.006 | 45 ± 7.4[B] | 42.6 ± 11 |
| Target Concentrations | 0.15 | 50 | 50 |

Notes: [A]Vapor-phase $HNO_3$

[B]Particle-bound $HNO_3$

### *B. Airway Epithelial Permeability*

Bronchoalveolar epithelial permeability was measured by determining the total protein concentration in BAL. Measurements were made in rats exposed for 1, 2, 3 (1 week), 6 (2 week), 9 (3 week) or 12 (1 month) days. As shown in Table 2, there was a trend towards lower protein concentrations in rats exposed for longer periods, which was statistically significant ($p \leq 0.001$). There was no significant effect of the atmospheres.

**Table 2.** Epithelial Permeability of rats exposed for 4 hr per day, 3 days per week for 1, 2, 3 and 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Permeability is measured as total protein in bronchoalveolar lavage fluid (BAL) in rats after cumulative exposures of 1, 2, 3, 6, 9, and 12 days. Data are mean ± SE (n).

| | Total Protein in BAL (mg/ml) | | | | |
|---|---|---|---|---|---|
| Days Exposed [A] | Purified Air | $O_3$ | Ac + $O_3$ | Ac + C | Ac + $O_3$ + C |
| 1 | 0.241±0.053 (3) | 0.207±0.016 (3) | 0.234±0.048 (3) | 0.224±0.013 (3) | 0.218±0.037 (3) |
| 2 | 0.221±0.042 (6) | 0.200±0.029 (6) | 0.250±0.025 (6) | 0.265±0.039 (6) | 0.223±0.036 (6) |
| 3 | 0.218±0.014 (6) | 0.216±0.009 (6) | 0.195±0.022 (6) | 0.196±0.013 (6) | 0.235±0.012 (6) |
| 6 | 0.242±0.015 (2) | 0.248±0.023 (2) | 0.205±0.003 (2) | 0.205±0.040 (2) | 0.217±0.024 (2) |
| 9 | 0.178±0.015 (9) | 0.212±0.016 (10) | 0.212±0.018 (10) | 0.225±0.012 (10) | 0.205±0.015 (9) |
| 12 | 0.182±0.021 (10) | 0.177±0.022 (10) | 0.164±0.016 (10) | 0.168±0.018 (9) | 0.178±0.016 (10) |

[A] Significant ($p < 0.001$) trend to decrease concentration over the course of the exposures. Main effect of atmospheres was not significant.

### *C. Respiratory Tract Morphometry and Histopathology*

#### 1. Excised Lung Gas Volume

Excised lung gas volumes (ELGV) are sensitive measures of gas trapping in the lung and an indicator of pulmonary injury and dysfunction. Measurements were made in rats exposed for 1 month (12 total days). As shown in Table 3, the excised lung gas volume was about 0.37 ml, which is consistent with values observed by Mautz et al. (1995) for rats of comparable age exposed for 1 month. The Ac + $O_3$ mixture tended to increase ELGV, however values for other exposure atmospheres were less than controls. There were no significant effects of exposure and none of the group mean values were significantly different from those of the controls..

**Table 3.** Excised lung gas volume (ELGV) in lungs of rats exposed for 4 hr per day, 3 days per week for 4 weeks to $O_3$, and mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Data are mean ± SE, n.

| Group | Atmosphere | Excised Lung Gas Volume (ml) |
|---|---|---|
| 1 | Purified Air | 0.337 ± 0.021, 10 |
| 2 | $O_3$ | 0.366 ± 0.008, 10 |
| 3 | Ac + $O_3$ | 0.392 ± 0.034, 10 |
| 4 | Ac + C | 0.362 ± 0.031, 9 |
| 5 | Ac + $O_3$ + C | 0.364 ± 0.025, 10 |

#### 2. Lung Morphometry.

Lung morphometry measurements were made in rats exposed for 1 month to each of the 5 test atmospheres. Table 4 summarizes the results for fixed lung volume, alveolar mean chord length, and septal wall thickness. Fixed lung volume averaged about 8.2 ml in animals exposed to purified air, and was not significantly affected by any of the exposure atmospheres. Alveolar mean chord length averaged 42.8 μm in the purified air control group; none of the exposure group mean values were significantly different from controls and the main effect of atmosphere in an analysis of variance was not significant. Septal wall thickness averaged 5.79 μm for the

control group. None of the group mean values for the exposure atmospheres were significantly different from controls, and there was no significant effect of atmosphere in the ANOVA. Both alveolar chord length and septal wall thickness were increased relative to controls after exposure to the $HNO_3$ + C (Ac + C) atmosphere; the alveolar chord length for the Ac + C group was significantly increased vs. that for the $O_3$ group. The lack of a significant main effect of atmosphere in the ANOVA for either of these parameters, may be due to a non-uniformity of variances. A nonparametric analogue to the ANOVA, the Kruskal-Wallis test showed a significant effect of atmosphere on alveolar chord length and confirmed that the group mean values for the Ac + C group was significantly greater than that of the $O_3$ group.

**Table 4.** Morphometric measures of lung tissues of rats exposed for 4 hr per day, 3 days per week for 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Data are mean ± se, (n).

| Group | Atmosphere | Fixed Lung Volume (ml) | Alveolar Mean Chord Length (μm) | Septal Wall Thickness (μm) |
|---|---|---|---|---|
| 1 | Purified Air | 8.2 ± 0.15 (10) | 42.8 ± 1.2 (9) | 5.79 ± 0.16 (9) |
| 2 | $O_3$ | 8.6 ± 0.19 (10) | 41.6 ± 1.0 (10) | 5.72 ± 0.25 (10) |
| 3 | Ac + $O_3$ | 8.3 ± 0.19 (8) | 42.8 ± 1.0 (10) | 5.39 ± 0.09 (10) |
| 4 | Ac + C | 8.5 ± 0.26 (8) | 45.4 ± 0.7 (9) | 5.91 ± 0.16 (9) |
| 5 | Ac + $O_3$ + C | 8.2 ± 0.17 (10) | 41.4 ± 1.2 (10) | 5.62 ± 0.19 (10) |
| Significant Differences | | | 2 vs. 4 ($p \leq 0.05$) | 3 vs. 4 ($p \leq 0.10$) |

The volume fractions of lung tissue were measured in the lungs of the same rats used in the morphometric assessments described above. The parameters measured were the fraction of tissue which was parenchymal, and the fractions of tissue represented as alveoli, alveolar ducts, bronchioles, lobar and secondary bronchi, vascular tissue, and bronchus associated lymphoid tissue (BALT). The data are summarized in Table 5. While there were no significant main effects of atmosphere determined by ANOVA for any of these parameters, there was a suggestion that the acid-containing atmospheres caused a small decrease in alveolar volume, with a corresponding increase in the volume fractions of alveolar ducts and bronchioles. None of the increases above control achieved statistical significance. The volume fraction of parenchymal tissue was increased relative to control in groups exposed to the test atmospheres; the increase approached significance ($p \leq 0.10$)

**Table 5.** Volume fraction of lung tissues of rats exposed for 4 hr per day, 3 days per week for 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Data are mean ± SE.

| | Volume Fraction of Lung Tissues | | | | |
|---|---|---|---|---|---|
| | Group = 1 | 2 | 3 | 4 | 5 |
| Tissue | Purified Air (n = 9) | $O_3$ (n = 10) | Ac + $O_3$ (n = 10) | Ac + C (n = 9) | Ac + $O_3$ + C (n = 9) |
| Parenchyma | 0.823±0.012 | 0.848±0.020 | 0.867±0.01[A] | 0.852±0.015 | 0.835±0.028 |
| Alveoli | 0.564±0.016 | 0.546±0.013 | 0.555±0.019 | 0.526±0.015 | 0.532±0.020 |
| Alveolar Ducts | 0.201±0.017 | 0.247±0.016 | 0.247±0.019 | 0.248±0.018 | 0.233±0.015 |
| Bronchioles | 0.051±0.008 | 0.054±0.007 | 0.063±0.011 | 0.079±0.018 | 0.072±0.012 |
| Lobar and Secondary Bronchi | 0.098±0.012 | 0.096±0.021 | 0.071±0.009 | 0.098±0.012 | 0.089±0.017 |
| Vascular Tissue | 0.083±0.015 | 0.054±0.009 | 0.063±0.011 | 0.046±0.009 | 0.071±0.013 |
| BALT | 0.003±0.003 | 0.003±0.002 | 0.000±0.000 | 0.002±0.002 | 0.003±0.003 |

[A] Different from control ($p \leq 0.10$)

**3. Nasal Epithelial Composition.**

The cellular composition of the nasal epithelium of rats at Young's Level I in sections through the rat's head was also measured in the rats exposed as described above. Two types of respiratory epithelium were differentiated; Type R, which is a pseudostratified tissue, rich in secretory cell activity and Type R-1, which is a transitional epithelial region with high columnar structure and less secretory cell activity than the Type R. The fractions of ciliated, secretory and basal cells were determined for both Type R and Type R-1 tissues and the data are summarized in Table 6. There were no significant atmosphere-related differences between group mean fractions for any of the cell types, although there tended to be fewer ciliated cells and more secretory cells in both the Type R and Type R-1 tissue in the noses of rats exposed to the Ac + $O_3$ + C mixture.

**Table 6.** Cellular composition of the nasal epithelium of rats exposed for 4 hr per day, 3 days per week for 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Data are mean ± SE.

| | Cellular Fractions of Nasal Epithelia | | | | |
|---|---|---|---|---|---|
| | Group = 1 | 2 | 3 | 4 | 5 |
| | Purified Air (n = 7) | $O_3$ (n = 8) | Ac + $O_3$ (n = 9) | Ac + C (n = 7) | Ac + $O_3$ + C (n = 9) |
| Type R Epithelium Cells | | | | | |
| Ciliated | 0.357±0.012 | 0.351±0.009 | 0.356±0.009 | 0.365±0.008 | 0.352±0.008 |
| Secretory | 0.374±0.014 | 0.373±0.010 | 0.372±0.008 | 0.370±0.014 | 0.385±0.014 |
| Basal | 0.268±0.015 | 0.276±0.013 | 0.272±0.012 | 0.266±0.013 | 0.263±0.012 |
| Type R-1 Epithelium Cells | | | | | |
| Ciliated | 0.461±0.026 | 0.430±0.005 | 0.444±0.013 | 0.455±0.019 | 0.416±0.016 |
| Secretory | 0.333±0.014 | 0.326±0.012 | 0.324±0.014 | 0.305±0.014 | 0.348±0.017 |
| Basal | 0.230±0.017 | 0.244±0.011 | 0.231±0.020 | 0.240±0.021 | 0.235±0.015 |

**4. Epithelial Secretory Density.**

The secretory activity of these tissue samples was determined by measuring the relative density of alcian blue-periodic acid-Schiff stain over a 0.25 mm region of epithelium. This stain is sensitive to the presence of glycoproteins. In addition, measurements were also made on a sample of tracheal epithelium. As shown in Table 7, all of the atmospheres tended to increase the secretory activity in both types of nasal epithelium, relative to purified air controls. On the other hand, the acid-containing atmospheres tended to reduce secretory cell activity in tracheal tissue. None of the group mean values were, however, statistically different from those of controls.

**Table 7.** Respiratory tract epithelial secretory activity of rats exposed for 4 hr per day, 3 days per week for 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Data are relative density of alcian blue-periodic acid Schiff stain per 0.25 mm of epithelium (mean ± SE).

| | Secretory Activity (Relative Stain Density) | | | | |
|---|---|---|---|---|---|
| Group = | 1 | 2 | 3 | 4 | 5 |
| | Purified Air (n = 8) | $O_3$ (n = 9) | Ac + $O_3$ (n = 9) | Ac + C (n = 8) | Ac + $O_3$ + C (n = 9) |
| *Nasal Epithelium* | | | | | |
| Type R | 1.39±0.12 | 1.47±0.11 | 1.59±0.17 | 1.64±0.24 | 1.40±0.07 |
| Type R-1 | 0.145±0.030 | 0.187±0.043 | 0.209±0.061 | 0.213±0.055 | 0.162±0.026 |
| *Tracheal Epithelium* | | | | | |
| | 0.315±0.078 | 0.318±0.050 | 0.216±0.046 | 0.276±0.043 | 0.219±0.043 |

**5. Acid Phosphatase Stain Density in Pulmonary Macrophages.**

Sections of lung tissue from the 1 month-exposed rats were treated with a histochemical stain for acid phosphatase activity, which is an enzyme expressed by pulmonary macrophages. Activity was quantified by the intensity of staining in macrophages, classified into 3 levels of increasing activity, denoted as I, II or III, respectively. The data are summarized in Table 8.

increasing activity, denoted as I, II or III, respectively. The data are summarized in Table 8. Exposure tended to decrease the fraction of macrophages which were classified in the highest stain intensity classification (III), relative to purified air control. The changes were small and none of the group mean values were significantly lower than that for purified air.

**Table 88.** Acid Phosphatase activity in pulmonary macrophages of rats exposed for 4 hr per day, 3 days per week for 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Activity is quantified by intensity of acid phosphatase stain, and staining intensity in a macrophage is classified into 3 levels. Data are mean ± SE.

| | Fraction of Macrophages in Acid Phosphatase Stain Intensity Classes | | | | |
|---|---|---|---|---|---|
| | Group = 1 | 2 | 3 | 4 | 5 |
| Stain Intensity Class | Purified Air (n = 8) | $O_3$ (n = 9) | Ac + $O_3$ (n = 9) | Ac + C (n = 8) | Ac + $O_3$ + C (n = 9) |
| I | 0.262±0.052 | 0.282±0.046 | 0.307±0.087 | 0.373±0.078 | 0.258±0.078 |
| II | 0.525±0.033 | 0.546±0.045 | 0.597±0.049 | 0.504±0.054 | 0.535±0.032 |
| III | 0.213±0.052 | 0.173±0.042 | 0.171±0.044 | 0.123±0.041 | 0.207±0.029 |

## *D. Bronchopulmonary Lavage Fluid Analysis and Macrophage Function*

### 1. Mucin Glycoprotein

Mucin glycoprotein was measured in BAL from rats exposed for 6 or 12 days (2 weeks or 4 weeks, respectively). The mucin was determined using an ELISA and the results, which are shown in Table 9, are presented as absorbance units. These data show an interesting pattern of responses. At 2 weeks, the average mucin concentration in the control group was lower than that at 4 weeks ($p \leq 0.001$), and the concentration was increased by all of the exposure atmospheres, relative to that in the controls. The concentration of mucin glycoprotein in the BAL from rats exposed to the Ac + $O_3$ + C mixture was significantly higher than that in BAL from rats exposed to purified air ($p \leq 0.05$), in the rats exposed for 2 weeks. In the rats exposed for 4 weeks, however, the mucin glycoprotein concentrations in BAL were reduced, relative to control, by both of the carbon-containing atmospheres, but the differences in group mean values were not significant. The mucin glycoprotein

**Table 9.** Mucin glycoprotein in bronchoalveolar lavage fluid from rats exposed for 4 hr per day, 3 days per week for 2 and 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Mucin glycoprotein was analyzed by ELISA; data are presented as absorbance units, mean ± SE, n = 10.

| | Mucin glycoprotein (Absorbance Units) | | | | |
|---|---|---|---|---|---|
| | Group = 1 | 2 | 3 | 4 | 5 |
| Exposure | Purified Air | $O_3$ | Ac + $O_3$ | Ac + C | Ac + $O_3$ + C |
| 2 Weeks (6 days) | 0.362±0.056 | 0.392±0.055 | 0.441±0.067 | 0.517±0.054 | 0.638±0.04[A] |
| 4 Weeks (12 Days) | 0.500±0.021 | 0.516±0.044 | 0.531±0.066 | 0.349±0.03[B] | 0.345±0.02[B] |

[A] Significantly increased over Group 1 ($p \leq 0.05$).
[B] Significantly less than group 5 at 2 weeks ($p \leq 0.05$).

**2. Pulmonary Macrophage Functions**

The macrophages obtained by BAL represent those found in the airways and alveoli of the lung. Differential cell counts showed that about 95% of the cells recovered in BAL were macrophages or monocytes, and that the remaining cells were lymphocytes (3 to 5%) and polymorphoneuclear leukocytes (PMN) which are present in small numbers (0 to 2%). These are cell counts which are typical for rats with no underlying pulmonary infection or inflammation. There were no significant changes or trends in the values obtained for this study.

Macrophage viability and Fc receptor binding capacity results are summarized in Table 10. Macrophage viability averaged greater than 90%, but tended to be lowest in the samples from rats exposed to Ac + $O_3$ + C. There were no significant main effects of either days of exposure or exposure atmosphere, and none of the observed group mean values were significantly different from controls. Nor were any significant changes seen in FcR binding capacity. There were however small , but not significant, decreases in FcR binding in the $O_3$-exposed rats on days 1 and 2, with a return toward control values in subsequent exposures.

**Table 10.** Pulmonary macrophage functions of rats exposed for 4 hr per day, 3 days per week for 1, 2, 3 and 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Viability was measured by Trypan Blue exclusion and hemocytometry, and Fc receptor binding was measured by a rosette bioassay. Values presented are %s of viable and Fc binding cells, respectively. Data are mean ± SE., n = 10.

| | Macrophage Viability and FcR Binding (%) | | | | |
|---|---|---|---|---|---|
| *Macrophage Viability* | Purified Air | $O_3$ | Ac + $O_3$ | Ac + C | Ac + $O_3$ + C |
| Day = 1 | 90.5±1.6 | 90.5±1.1 | 88.6±2.9 | 88.2±1.6 | 89.7±1.1 |
| 2 | 92.4±1.0 | 91.2±1.0 | 91.8±0.5 | 91.2±0.8 | 89.5±1.3 |
| 3 | 90.8±1.4 | 92.3±1.1 | 91.4±1.6 | 92.5±1.0 | 88.3±2.8 |
| 6 | 91.8±1.6 | 91.8±0.7 | 94.6±0.6 | 92.4±1.0 | 92.7±0.8 |
| 9 | 92.5±1.3 | 91.0±0.9 | 92.6±0.7 | 94.1±0.9 | 91.4±0.7 |
| 12 | 90.8±1.4 | 92.9±1.0 | 88.5±1.9 | 91.5±1.4 | 88.5±2.7 |
| *Fc Receptor Binding* | | | | | |
| Day = 1 | 45.4±3.9 | 44.6±4.3 | 46.0±4.1 | 39.2±3.9 | 48.0±3.4 |
| 2 | 52.2±1.9 | 44.4±3.1 | 47.0±3.2 | 51.2±2.5 | 51.6±3.6 |
| 3 | 53.3±4.1 | 48.4±3.8 | 50.5±3.6 | 50.6±2.9 | 51.0±2.5 |
| 6 | 52.4±1.7 | 52.1±3.0 | 52.8±3.8 | 49.6±3.5 | 53.3±2.2 |
| 9 | 47.1±4.0 | 48.1±4.3 | 46.0±2.8 | 51.1±2.6 | 52.7±2.1 |
| 12 | 49.2±2.1 | 48.0±2.3 | 50.9±2.5 | 55.0±1.2 | 50.2±2.9 |

Phagocytic activity tended to decrease with increasing days of exposure, but none of the group mean values were significantly different from those of controls (Table 11). In 5 out of 6 exposures, $O_3$ tended to decrease phagocytic activity, relative to purified air controls; this finding was not significant when tested using a nonparametric sign test.

**Table 11.** Pulmonary macrophage functions of rats exposed for 4 hr per day, 3 days per week for 1, 2, 3 and 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Values shown are phagocytic activity (macrophages ingesting two or more 2 μm polystyrene latex spheres) and phagocytic capacity (macrophages ingesting ≥ 4 spheres). Data are mean % ± SE, n.

| | Macrophage Phagocytic Activity (%) | | | | |
|---|---|---|---|---|---|
| | Purified Air | $O_3$ | Ac + $O_3$ | Ac + C | Ac + $O_3$ + C |
| *Phagocytic Activity (>2 spheres)* | | | | | |
| Day = 1 | 82.3±4.1, 10 | 74.6±4.7, 10 | 78.7±4.8, 9 | 70.5±4.7, 10 | 73.0±6.0,10 |
| 2 | 75.7±8.3, 6 | 79.0±8.8, 6 | 76.7±7.3, 6 | 85.6±5.7, 6 | 80.7±6.0, 6 |
| 3 | 69.5±4.9, 6 | 63.1±6.5, 6 | 70.4±3.7, 6 | 61.9±2.9, 6 | 60.1±5.8, 6 |
| 6 | 75.0±4.3, 10 | 74.9±4.7, 10 | 81.6±3.8, 10 | 79.9±4.0, 10 | 75.7±5.3, 10 |
| 9 | 64.1±6.2, 6 | 63.8±5.2, 6 | 57.6±2.7, 6 | 71.9±6.1, 6 | 61.1±4.6,6 |
| 12 | 68.8±9.9, 7 | 66.5±7.0, 7 | 65.6±5.3, 7 | 69.5±7.2, 6 | 64.9±5.9, 8 |
| *Phagocytic Capacity (>4 spheres)* | | | | | |
| Day = 1 | 67.9±5.6, 10 | 56.8±5.8, 10 | 61.2±7.0, 10 | 48.8±5.3, 10 | 52.8±6.8, 10 |
| 2 | 50.4±9.5, 6 | 54.5±7.9, 6 | 46.9±8.5, 6 | 62.0±6.6, 6 | 56.2±5.8, 6 |
| 3 | 43.3±6.9, 6 | 37.5±7.1, 6 | 42.3±4.4, 6 | 34.5±4.7, 6 | 30.7±4.4, 6 |
| 6 | 51.5±6.4, 10 | 52.9±5.3, 10 | 58.4±6.3, 10 | 58.6±5.2, 10 | 53.2±6.8, 10 |
| 9 | 34.9±4.4, 6 | 37.1±7.3, 6 | 30.6±5.5, 6 | 47.8±8.1, 6 | 26.7±6.3, 6 |
| 12 | 42.2±10.4, 7 | 37.5±10.4, 7 | 40.0±3.6, 7 | 44.7±11.1, 6 | 36.7±9.4, 8 |

Macrophage respiratory burst activity results are summarized in Table 12. Data shown represent unstimulated respiratory burst activity and PMA-stimulated respiratory burst activity. The values (relative activity) are presented as the reduction in cytochrome C absorbance by macrophage produced free radicals relative to a duplicate sample containing superoxide dismutase

(SOD). ANOVA did not show a consistent atmosphere or exposure day effect for either the stimulated or unstimulated samples. Some interesting patterns are, however, present. Comparing purified air to $O_3$-exposed groups reveals that there is no time trend for either stimulated or unstimulated purified air groups. Regression analysis shows that the slope of the activity vs. time curve is not significant. On the other hand, respiratory burst activity in the unstimulated $O_3$ group increases with increasing exposure time (except for day 6), and that the slope of this line is statistically significant. Furthermore, the addition of PMA increased the daily average respiratory burst activity by about 3 units, but the slope of the activity vs. time curve was the same as that for the unstimulated samples (slope = 0.5 units/day; $r = 0.8$; $p \leq 0.05$). None of other atmosphere groups showed consistent time-dependent activity changes.

**Table 12.** Pulmonary macrophage functions of rats exposed for 4 hr per day, 3 days per week for 1, 2, 3 and 4 weeks to $O_3$, or to mixtures of $O_3$ with $HNO_3$, C or a combination of $HNO_3$ + C. Values shown are respiratory burst activity in unstimulated and PMA-stimulated macrophages relative to duplicate samples containing SOD. Respiratory burst was measured using the cytochrome C reduction method. Data are mean ± SE, n.

| | Macrophage SOD-Inhibitable Respiratory Burst Activity (Relative Activity) | | | | |
|---|---|---|---|---|---|
| *Unstimulated Respiratory Burst Activity* | Purified Air | $O_3$ | Ac + $O_3$ | Ac + C | Ac + $O_3$ + C |
| Day = 1 | 4.9±1.8, 10 | 2.0±0.9, 10 | 1.1±1.7, 10 | 4.0±1.4, 10 | 2.2±1.1, 10 |
| 2 | 0.5±1.9, 6 | 5.0±2.4, 4 | 7.8±2.6, 6 | 1.4±2.7, 5 | 5.7±3.9, 6 |
| 3 | 2.3±3.6, 10 | 6.9±1.7, 9 | 5.3± 2.0, 10 | 10.3±2.9, 10 | 5.1±3.0, 9 |
| 6 | 3.7±4.4, 10 | 2.6±2.0, 10 | 2.9±4.4, 7 | 1.7±3.5, 9 | 0.8±3.3, 9 |
| 9 | 4.7±3.6, 10 | 8.3±2.7, 9 | 1.8±2.1, 10 | 5.1±2.6, 9 | 3.3±2.7, 10 |
| 12 | 4.7±2.4, 10 | 9.8±1.8, 9 | 6.3±1.9, 10 | 4.3±2.3, 9 | 6.2±2.7, 10 |
| *PMA-Stimulated. Respiratory Burst Activity* | | | | | |
| Day = 1 | 7.9±2.6, 10 | 5.1±1.5, 10 | 3.9±2.3, 10 | 7.7±2.4, 10 | 7.7±1.7, 10 |
| 2 | 4.9±2.6, 10 | 7.7±4.0, 10 | 7.9±1.7, 10 | 3.7±2.7, 10 | 6.8±2.5, 10 |
| 3 | 7.3±3.8, 9 | 8.2±2.0, 10 | 8.6±2.3, 10 | 11.0±2.5, 10 | 9.8±3.3, 10 |
| 6 | 6.9±2.0, 10 | 6.5±3.7, 10 | 4.9±3.8, 10 | 8.5±3.1, 10 | 5.2±1.7, 10 |
| 9 | 4.4±2.1, 9 | 8.4±3.4, 9 | 8.8±2.6, 10 | 5.2±2.9, 10 | 6.0±2.6, 10 |
| 12 | 6.6±2.0, 9 | 13.0±3.9, 9 | 8.8±2.6, 10 | 9.1±2.8, 9 | 6.4±1.9, 10 |

## DISCUSSION

This study examined the potential of airborne particles and $O_3$ to modify the biological effects of inhaled $HNO_3$. The design complimented an ongoing investigation of the effects of inhaled $HNO_3$ and $O_3$ on the respiratory system and the endpoints selected for examination were relevant to pulmonary diseases, including bronchitis, upper and lower respiratory infection, and pulmonary fibrosis or emphysema (Mautz et al., 1995). This study had two important aspects. First, it examined the direct effects of two co-pollutants ($O_3$ and C) on the biological effects of inhaled $HNO_3$. Second, it examined the time-dependent characteristics of the toxicology of these three inhaled compounds at relatively low concentrations, alone and in mixtures. The greatest amount of data was collected on rats exposed for 1 month. Several of the endpoints measured showed changes which could be biologically important, even though the changes were not statistically significant. In order to summarize these changes, we set a criterion for determining whether exposure to a given atmosphere increased or decreased the group mean response relative to that of the group exposed to purified air by 1 SE (computed as the pooled SE for the atmosphere). These trends are presented in Table 13. The main effects of atmosphere, and where appropriate, time, obtained from ANOVAs for each endpoint are shown in the Table, as well.

**Table 13.** Summary of Observed Biological Responses (Changes vs. Purified Air) in Rats After 12 Weeks of Exposure.

| | Main Effects | | Atmospheres | | | |
|---|---|---|---|---|---|---|
| Endpoint | Atmos. | Time | $O_3$ | Ac + $O_3$ | Ac + C | Ac+$O_3$+C |
| Permeability | N.S. | p≤0.0001 | | | | |
| Excised Lung Vol. | N.S. | | | I | | |
| Morphometry | | | | | | |
| Lung Vol. | N.S. | | I | | I | |
| Chord Length | N.S. | | | | I | D |
| Septal Wall | N.S. | | | D | I | D |
| % Alveoli | N.S. | | | | D | D |
| %Alv. Ducts + Bronchioles | N.S. | | I | I | I | I |
| Nasal Cells | | | | | | |
| Ciliated | N.S. | | D | | | D |
| Secretory | N.S. | | | | D | |
| Sec. Cell Activity | N.S. | | | I | I | |
| Mucin Glycoprotein | | | | | | |
| 2 week | N.S. | | | I | I | I |
| 4 week | N.S. | | | | D* | D* |
| Macrophage Funct. | | | | | | |
| Yield | N.S. | p≤0.0001 | | | D | |
| Acid Phos. Act. | N.S. | N.S. | | | D | |
| FcR Binding | N.S. | N.S. | | | | |
| Phagocyt. | N.S. | p≤0.0001 | | | | |
| Unstim. Resp. Bur. | N.S. | N.S. | I | | | |
| Stim. Resp. Bur. | N.S. | N.S. | I | | | |

Note: D = 1 SE decrease; I = 1 SE increase; * = $p \leq 0.05$

### *Atmosphere Constituents*

For this study, carbon was used as a representative particle. The carbon was generated by nebulization of a carbon suspension. In ambient air carbon, is generated by combustion, and there may be some differences between ambient carbon and the carbon to which our rats were exposed,

possibly with respect to the surface structure. However, carbon in the air is often incorporated into droplet aerosols and therefore under conditions favorable to formation of droplets, ambient carbon may exist in a form similar to that used in our study. The mass median aerodynamic diameter of ambient carbon aerosols is about 0.3 μm; we designed our generation system to produce particles of 0.3 μm, as well. The advantage to the nebulized carbon used in this study is its purity. The carbon was of high purity to begin with and was prewashed with dilute nitric acid before use which would have removed adsorbed contaminants, as well as providing an acidified surface. If a combustion-generated carbon had been used, the results could have been more difficult to interpret since the generation process would produce gaseous contaminants including carbon monoxide, nitrogen oxides, and organic vapors and particles, in addition to the carbon particles.

There is a substantial body of literature dealing with the health effects of $O_3$, and a fair amount is known about the health effects of carbon particles. By comparison, there is relatively little known about the effects of $HNO_3$ on the respiratory system The data which have been published, were obtained at considerably higher concentrations than those used in this study. The concentrations of $HNO_3$ and C used in this study were based on measurements made in the San Gabriel Valley in the Los Angeles Basin, and were estimated to represent peak hourly concentrations during a period of high urban pollution. Even so, the concentrations (50 $\mu g/m^3$ $HNO_3$; 50 $\mu g/m^3$ C; 0.15 ppm $O_3$) are low compared to those used in other toxicological studies of the health effects of these compounds. An episodic exposure pattern (4 hr/ day, 3 days/week, for up to 4 weeks) was employed rather than a continuous daily protocol. The episodic exposure was hypothesized to reduce the potential for response attenuation which has been reported for continual, repeated exposures to oxidant gases.

### *Morphometry and Emphysema*

Morphologic and histopathologic changes in the lung are probably the most definitive indicators of pulmonary disease processes. Although the changes observed in this study, and in a companion study performed by Mautz et al. (1995), were small and often were not statistically significant, a pattern of changes has emerged which suggests that $HNO_3$, alone and in combination with co-pollutants should continue to be studied.

Respiratory tract morphometry was examined in rats exposed for 1 month (12 total days); 3 hr per day, 3 days per week. We did not observe any changes in fixed lung volume. We did observe an increase in alveolar mean chord length and septal wall thickness in rats exposed to $HNO_3$ (50 $\mu g/m^3$) + C (50 $\mu g/m^3$). Ozone tended to decrease septal wall thickness, alone and in mixtures with $HNO_3 \pm C$. This study did not include $HNO_3$ alone among the exposure atmospheres. A companion study (Mautz et al., 1995) which used the same concentrations, rats , and inhalation exposure regimen as used in this study did include $HNO_3$ alone. $HNO_3$ alone did not cause changes in either chord length or septal wall thickness, but $O_3$, in combination with $HNO_3$, again decreased alveolar wall thickness. These findings may suggest a loss of lung elasticity. Peters and Hyatt (1986) induced airway injury in dogs that inhaled nebulized 1% $HNO_3$ that was characterized by bronchial and small airways inflammation. They observed a significant increase in pulmonary closing volume (CV) and closing capacity (CC). These are parameters that are sensitive to changes in lung elasticity; an increase in CC suggestive of a loss of elasticity (Bates, 1989). Smokers often exhibit increased CC, compared to non-smokers. Cigarette smoke contains substantial amounts of $NO_2$, and it has been postulated that the health effects of $NO_2$ are attributable in part to the $HNO_3$ formed when $NO_2$ dissolves in lung fluids (Goldstein et al., 1977). Pulmonary functions were not measured in the present study but quasi-static compliance was measured by Mautz et al. (1995) in rats exposed to 50 $\mu g/m^3$ $HNO_3$ and was observed to increase slightly. This, again, supports the idea that $HNO_3$ exposure may cause loss of elasticity of the lung. Excised lung gas volumes (ELGV) were measured in both this and in Mautz's studies. ELGV is a measure of the volume of gas trapped in a collapsed lung, hence should be correlated with static compliance, CC, and CV. ELGV was significantly increased in rats exposed to 450 $\mu g/m^3$ $HNO_3$ for 1 month and in rats exposed to 50 $\mu g/m^3$ $HNO_3$ for 3 and 9 months, but not for 1 month.

If $HNO_3$ exposures cause a loss of elasticity, this may be mediated by stimulated production of elastase, or an imbalance of the protease/antiprotease ratios. Nadziejko et al. (1992) reported an increase in lung elastase inhibitory capacity in rats exposed to 250 $\mu g/m^3$ $HNO_3$, but did not measure elastase production. This increase could have been a defense response to an acid-induced increase in elastase production within the lung, but it could also have reflected infiltration of serum-borne elastase inhibitors due to increased lung permeability. Since

there was no concomitant increase in BAL protein in her study, Nadziejko et al. concluded that permeability was probably not increased. Elastase inhibitory capacity was increased, but not significantly, in rats exposed to 50 $\mu g/m^3$ $HNO_3$ for 3 months, but not in rats exposed for 9 months (Mautz et al., 1995). It would be of interest in future studies to determine if elastase inhibitory capacity changes in response to pollutant-initiated effluxes of proteases as a defensive response.

### *Tissue Remodeling, Mucus Secretion and Bronchitis*

The volume fractions of lung tissues were measured and no statistically significant changes were observed. The volume fraction of alveoli (Table 5) was reduced and there was a corresponding increase the volume fractions of alveolar ducts and bronchioles. The changes were most pronounced in the lungs of rats exposed to C-containing acid atmospheres. Changes in the morphometric characteristics of small airways is consistent with results of inhalation of nebulized 1% $HNO_3$ (Peters and Hyatt, 1986). "Bronchiolization" or remodeling of terminal airways has been reported in animals chronically exposed to oxidant gases (Boorman et al., 1980; Barr et al., 1988) and cigarette smoke (Petty et al., 1983). Gearhart and Schlesinger (1988) reported an increase in the numbers of small airways in rabbits exposed to 250 $\mu g/m^3$ sulfuric acid aerosol, which they attributed to remodeling of alveoli and alveolar ducts. These morphological changes suggest that inhalation of acids, in conjunction with particles, could play a role in the etiology of bronchitis.

A hallmark of bronchitis is changes in mucus secretion. Several studies have documented that parameters associated with mucus secretion, such as numbers of secretory cells in the lung (Schlesinger et al., 1983; Gearhart and Schlesinger, 1986; Schlesinger et al., 1992) concentrations of sialic acid, a mucus-related compound (Wolff et al., 1986), and structure of the mucus layer (Wolff et al., 1986) are altered by acid inhalation. Parameters related to mucus secretion were investigated in the present study. We demonstrated no consistent changes in the numbers of secretory cells in nasal respiratory epithelia (Table 6), however secretory activity in nasal respiratory epithelia tended to be increased by the exposures (Table 7). On the other hand, secretory activity in tracheal epithelium (Table 7) tended to decrease in rats exposed to acid-containing atmospheres. The decrease in tracheal secretory activity (measured in rats exposed for 4 weeks to the Ac + C and the Ac + C + $O_3$ atmospheres) was accompanied by a significant

decrease in the mucin glycoprotein content of BAL (Table 9). However, in rats exposed for 2 weeks, mucin glycoprotein in BAL was increased in rats exposed to the acid-containing atmospheres. With only 2 timepoints it is not possible to determine whether or not there are time-sensitive parameters which cause increased mucus secretion during short term exposures and then cause decreases during extended exposures. Alternatively, variations in the storage of expressed mucin could play a role.

### *Macrophage Functions and Risk of Infection*

Changes in macrophage functions were studied in rats exposed for 1, 2, 3, 6, 9, and 12 days. The results did not demonstrate any statistically significant changes or coherent patterns of response in macrophage viability, Fc receptor binding or phagocytic activity. Respiratory burst activity in macrophages from rats exposed to $O_3$ increased with increasing days of exposure. The rate of increase was statistically significant for unstimulated macrophages and for macrophages stimulated with PMA. Respiratory burst activity in macrophages from rats exposed to purified air or to $HNO_3$-containing atmospheres with or without $O_3$ did not show an increasing trend. It is of interest to note that macrophage respiratory burst activity demonstrated a cumulative effect of $O_3$ exposure, and that this pattern was not seen after adding $HNO_3$ to the atmospheric mixture.

### *Conclusions*

In summary, all of the biological effects observed in this study were small, which is consistent with the low pollutant concentrations employed. Very few of the biological responses observed achieved statistical significance. The trends observed in morphometric, biochemical and histopathologic endpoints follow patterns which are consistent in direction with significant effects of acid-containing atmospheres administered at higher concentrations, and suggest that acid exposures may be associated with lung changes similar to those seen in cigarette smokers who are at risk of developing emphysema and chronic bronchitis.

At the outset, we sought to evaluate: (1) whether or not a particulate co-pollutant would exacerbate the effects of $HNO_3$; and (2) whether or not there would be differences in responses between acute and longer term exposures. There were too few statistically significant changes to answer these questions definitively. However, we can, on the basis of scoring the number of biologically important changes in Table 13 (group mean changes $\geq$ 1 SE from the control mean)

assess the relative potency of the atmospheres. By this criterion, atmospheres are ranked as follows: Ac+C ≥ Ac+C+$O_3$ ≥ (Ac+$O_3$ = $O_3$). Data suggest that effects seen after short term (≤ 2 week) exposures may be different from those observed after longer exposures. Furthermore there appears to be a statistically significant, cumulative effect of $O_3$ on respiratory burst activity, which has not been previously reported. If confirmed in other studies, this could provide a link between chronic $O_3$ exposures and the etiology of chronic pulmonary diseases. The pollutant concentrations used were within the range of ambient exposures. Humans are exposed to ambient pollutants over periods of time which are much longer than those tested under controlled laboratory conditions. We conclude that the results of this study can not rule out the possibility that exposure to acid vapors and acid-associated particles under ambient conditions can increase the risk of lung diseases and that acid+particle-containing atmospheres may be more potent than acid atmospheres without particles.

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