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Studies to Determine Long-Term Effects of Acidic Atmospheres

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY

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Research Division

STUDIES TO DETERMINE LONG-TERM EFFECTS OF ACIDIC ATMOSPHERES

Final Report

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I. ABSTRACT.

To assess the potential for acid and oxidant air pollution in California to increase the risk of lung disease, Fisher 344/N rats were exposed to nitric acid vapor (HNO_3) and ozone (O_3) under conditions relevant to exposure of people to urban air pollution. The exposures included 1) a 1 month dose-response study of HNO_3 vapor at 50, 150, and 450 $\mu g/m^3$ and 2) a 9 month exposure to 50 $\mu g/m^3$ HNO_3 and 0.15 ppm O_3 alone and in combination. Exposures were 4 hours per day and 3 days per week, a pattern modeling the episodic exposure of people. The biological endpoints analyzed were related to induction of the pulmonary diseases, asthma, bronchitis, respiratory infection, and pulmonary fibrosis/emphysema, and these included a broad array of measurements of pulmonary function, respiratory tract morphometry, biochemical changes in lung tissues and bronchoalveolar lavage fluid, and pulmonary macrophage functions. In the 1 month HNO_3 dose-response exposure, there were significant alterations in lung morphology and defensive functions of pulmonary macrophages. In the 9 month exposure, endpoints related to asthma and bronchitis were not significantly altered. However, biological endpoints related to more general respiratory tract infections showed some significant effects including increased nasal epithelial permeability and shifts in nasal cell populations by the HNO_3 exposures and trends for slowing of upper respiratory tract particle clearance and acceleration of deep lung clearance in response to O_3 exposures. HNO_3 exposure also resulted in increased density of acid phosphatase in macrophages, O_3 exposure resulted in increased ß-glucuronidase in BAL fluid, and both acid and oxidant exposures resulted in a trend toward reduced phagocytic activity. The combination of effects, increased nasal permeability, decreased upper airway clearance, and altered macrophage function, suggests that HNO_3 exposure, and to a lesser extent, O_3 exposure, may be associated with increased incidence or risk of respiratory tract infection. Biological endpoints associated with pulmonary fibrosis/emphysema also showed some significant effects and trends including small changes in epithelial and interstitial tissues at the bronchiole-alveolar duct junctions in response to O_3 exposures and small changes in lung structure-elasticity characteristics in HNO_3 and O_3 exposure groups. Finally, there were significant responses by lung heat shock proteins and the cytochrome P450 monooxygenase system. The biological effects observed in 9 months exposure to 50 $\mu g/m^3$ HNO_3 and 0.15 ppm O_3 were generally small and there were no indications that HNO_3 and O_3 at the levels tested interacted synergistically on

biological responses. Many of the effects appeared as highly suggestive trends, which were not statistically significant, but fit a pattern along with other biological variables in the same tissues that suggested an early stage of disease process. The relationship of these biological effects to human pulmonary disease indicates that exposure to low concentrations of HNO_3 or O_3 may result in increased incidence of respiratory tract infections, and changes in elasticity and fine structure of the lung. The small effects observed in the present study of healthy animals suggests that more serious responses may occur in sensitive populations of humans or in humans exposed to additional gaseous and particulate air pollution.

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DISCLAIMER

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SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS.

The objective of this project was to evaluate the potential for chronic adverse health effects due to inhalation exposure of nitric acid vapor (HNO_3) under conditions relevant to exposure of people to urban air pollution in California. Because HNO_3 may alter the toxicity of co-existing pollutants or, in turn, the toxicity of HNO_3 may be altered by other air pollutants, we examined the toxic effects of HNO_3 and ozone (O_3) both alone and in combination exposure. O_3 is an important oxidant air pollutant in the Southern California urban air environment, and is hypothesized to have toxic interactions with inhaled acids. O_3 levels are also highly correlated the HNO_3 levels in ambient urban atmospheres. Laboratory rats were exposed in 1) a 1 month dose-response study to survey the toxic effects of HNO_3 vapor at 50, 150, and 450 $\mu g/m^3$ and select a concentration for study in 2) a 9 month exposure to 50 $\mu g/m^3$ HNO_3, 0.15 ppm O_3, and the combination 50 $\mu g/m^3$ HNO_3 + 0.15 ppm O_3. The exposures were 4 hours per day for 3 days per week, a pattern designed to model the episodic exposure of people to urban air pollution, and the concentrations of HNO_3 and O_3 selected for the exposures were based on urban pollutant levels experienced in the South Coast Air Basin. The project was designed to address hypotheses about the possible mechanisms for induction of chronic pulmonary disease by acids and oxidants. These pulmonary diseases included asthma, bronchitis, upper and lower airway infection, and pulmonary fibrosis/emphysema. A set of biological endpoints associated with these disease processes were selected for study that included measurements of pulmonary function, microscopic anatomy of the respiratory tract, biochemical changes in lung tissues and fluid lining the respiratory tract, and respiratory defensive functions against disease and foreign compounds.

In the 1 month HNO_3 dose-response exposure, there were significant alterations in lung morphometry and defensive functions of pulmonary macrophages and some of these changes were significant, or appeared as consistent trends, in the low concentration 50 $\mu g/m^3$ exposure. Therefore this concentration was selected for study in the long term 9 month exposure to HNO_3 and O_3 alone and in combination. The biological effects observed in this 9 month exposure were, however, generally small and not statistically significant. At the low concentrations tested, many of the effects appeared as highly suggestive trends, which were not statistically significant,

but fit a pattern of relationship to other biological variables affected in the same tissues. Endpoints potentially related to asthma included irritant breathing pattern responses, proliferation of mast cells in bronchi, and bronchoalveolar permeability; none were significantly altered by the exposures. Biological endpoints most specific to bronchitis were changes in the tracheobronchial airways, particularly those related to mucus production and secretion, including tracheal epithelial glycoprotein density, glycoprotein in bronchoalveolar lavage fluid, and epithelial cell turnover in the trachea and bronchi. These measures of airway injury and secretory response were not affected by the 9 month exposure, however glycoprotein in bronchoalveolar lavage fluid was elevated in a dose-dependent relation in response to the 1 month exposures to graded concentrations of HNO_3. Increased glycoprotein in airway fluids indicates that production and release was increased, and although this pattern was not observed in the extended 9 month exposure, the results showed that glycoprotein secretion into bronchoalveolar fluids is responsive to concentrations of HNO_3 at near ambient levels, and sensitive populations of people could respond more strongly to lower concentrations.

For the broad category of respiratory tract infection, relevant biological endpoints included mucociliary clearance which is the functional mechanism for removing foreign particles, including infectious agents, from the respiratory tract, epithelial permeability which measures the integrity of the epithelial barrier to movement of foreign compounds and organisms, and pulmonary macrophage function which determines the capacity of these immune system cells to defend the respiratory tract against foreign compounds and organisms. A few of these functions were affected by the 9 month acid and oxidant exposure that could result in increased sensitivity to respiratory infections. Nasal epithelial permeability was significantly increased by the 50 $\mu g/m^3$ HNO_3 exposures, and there were concomitant small shifts in cell composition of the nasal epithelium and an increase in epithelial glycoprotein density which were attributed to exposure to HNO_3 vapor. In addition, there were trends for slowing of early (upper respiratory tract) particle clearance and acceleration of late (deep lung) clearance in response to O_3 exposure. Pulmonary macrophage function showed several suggestive responses to the 9 month exposure. Lung macrophages demonstrated activation by HNO_3 exposure with increased cellular density of acid phosphatase, and ß-glucuronidase, which is released by injured phagocytic cells, was increased in lung fluids following exposures to the O_3-containing atmospheres. Although

macrophage phagocytic activity was not significantly changed by the exposures, there was a trend toward reduced phagocytosis in all the acid and oxidant exposure atmospheres after 9 months of exposure. Impaired macrophage function and elevated lung fluid ß-glucuronidase is consistent with a small degree of macrophage injury. A nasal epithelium with increased permeability allows inhaled foreign chemical compounds, particles, and pathogens greater access to underlying tissues and increases the risk of allergic responses or infection. A delay in upper airway clearance will retard the elimination of deposited foreign particles and micro-organisms, and increase the risk of infection. The combination of effects, increased nasal permeability, decreased upper airway clearance, and altered macrophage function suggests that HNO_3 exposure, and to a lesser extent, O_3 exposure, may be associated with increased incidence or risk of respiratory tract infection.

The fourth disease state examined was pulmonary fibrosis/emphysema, and the relevant biological endpoints were measures of lung structure and connective tissue and epithelial injury (morphometric analysis, collagen deposition and biochemistry, and epithelial cell turnover in the bronchioles and parenchyma) and measures of pulmonary function (quasi-static compliance, excised lung gas volume, and pulmonary irritant reflex breathing patterns). There were no major alterations in lung structure observed in morphometric analyses of the 9 month HNO_3-O_3 exposures. Detailed examination of the bronchiole-alveolar duct junctions indicated minimal, but occasionally significant changes in epithelial and interstitial tissues occurring in the 9 month O_3 exposures (both O_3 alone and O_3 + HNO_3). There were no significant changes in collagen deposition or collagen cross linking in the lungs of 9 month exposed animals, no significant induction of breathing pattern changes, nor was irritation of deep lung tissues sufficient to increase epithelial cell turnover. Nevertheless, quasi-static compliance was reduced by O_3 exposure following 3 months exposure, and the structure-elasticity characteristics of the lung were sufficiently altered to increase excised lung trapped gas volume in HNO_3 and HNO_3 + O_3 at 3 months and to produce a trend of increased trapped gas volume in all exposure groups at 9 months. Increases in excised lung gas volume result from changes in the point at which airways close as the lung collapses when the chest is opened and these volume increases as well as the changes in quasi-static compliance and morphometry of bronchiole-alveolar duct junctions indicate that there has been some change in the morphology or elastic properties of the small

airways in the acid and oxidant exposures.

Finally, there were significant responses to the 9 month single and combination HNO_3 and O_3 exposures by lung heat shock proteins (increase in HSP 70) and by the cytochrome P450 monooxygenase system (increased benzphetamine N-demethylation and benzo[a]pyrene metabolism). Neither of these responses can be directly related to human pulmonary disease, however the heat shock protein response indicates that the lung was exposed to a toxic stress sufficient to elicit heat shock protein synthesis, and up regulation of the cytochrome P450 monooxygenase system (enhancement of benzphetamine N-demethylation and benzo[a]pyrene reactions) could potentially increase carcinogenic action of inhaled protocarcinogenic compounds metabolized by the lung. However, at this stage, this potential risk cannot be evaluated.

The biological effects observed in 9 months exposure to the low concentrations of HNO_3 and O_3 tested were generally small and there were no indications that HNO_3 and O_3, at the levels tested, interacted synergistically on biological responses. Many of the effects appeared as highly suggestive trends, which were not statistically significant, but fit a pattern of relationship to other biological variables affected in the same tissues. The possible relationship of these biological effects to human pulmonary disease indicates that exposure to low concentrations of HNO_3 or O_3 may result in increased incidence of respiratory tract infections, and changes in elasticity and fine structure of the lung. The small effects observed in the present study of healthy animals may occur more readily in sensitive populations of humans or in humans exposed to additional gaseous and particulate air pollution. In view of recent epidemiological evidence implicating relationships between urban airborne fine particles and human mortality, fine particulate air pollution and its possible interaction with oxidant gases and acid compounds is likely a more important area for research and regulation than specific focus on nitric acid vapor.

I. INTRODUCTION

A. OBJECTIVE.

The objective of this project was to evaluate the potential for chronic adverse health effects due to nitric acid (HNO_3) under conditions relevant to exposure of people to urban air pollution in California. Because HNO_3 may alter the toxicity of co-existing pollutants or, in turn, the toxicity of HNO_3 may be altered by other air pollutants, we also examined the effects of 0.15 ppm O_3 alone and in combination with HNO_3.

B. BACKGROUND.

1. Acid Air Pollution in California.

Air pollution in communities of the South Coast Air Basin (SCAB) of California is frequently above the State and Federal ambient air quality standards. As summarized by the South Coast Air Quality Management District with data from Azusa, California, the State PM-10 standard of 50 $\mu g/m^3$ was exceeded in 1986 in about 80% of the 24-hour samples, and the State ozone (O_3) standard of 0.09 ppm was exceeded on 45% of the days. In addition, nitrogen dioxide (NO_2) peak 1-hour levels were about 0.2 ppm and maximum 24-hour average nitric acid (HNO_3) concentrations were about 20 $\mu g/m^3$ in nearby Upland (ARB Research Contract No. A4-144-32, California Institute of Technology, March, 1988). Russel et al., (1985) have reported that HNO_3 and peroxyacetylnitrate (PAN) are the major end products of nitrogen oxide (NO_x) emissions over a 24 hour period. During daylight, nitric acid formation is driven by the chain of photochemical reactions which form ozone (O_3), hence nitric acid concentration patterns during the day are similar to those of ozone. At night, however, nitric acid can continue to form via reaction of ozone with residual NO_2 (Russel et al., 1985).

The acidity of ambient air in Southern California is strongly influenced by airborne ammonia (NH_3). The major sources of NH_3 in the SCAB are dairy operations in and around the Chino area (Russel and Cass, 1986). Excess NH_3 will rapidly neutralize acid vapors and particles. NH_3 concentrations are dependent upon meteorological conditions and exhibit substantial fluctuations with shifts in wind speed and direction. Thus, although total nitrates in Riverside were approximately 6 times higher then those in Pasadena, the concentrations of nitric

acid vapor were about 10-fold lower (Munger et al., 1990). Measurements of HNO_3 made over a 6 month period (November 1987 through March 1988) in Riverside ranged from 0.5 to 4.8 $\mu g/m^3$ with an average of 1.8 $\mu g/m^3$, while during the same period, concentrations in Pasadena ranged from 0.8 to 56 $\mu g/m^3$, with an average of 17 $\mu g/m^3$ (Munger et al., 1990). Other acidic gases or vapors including hydrochloric, hydrofluoric, acetic and formic acids, have not been as well quantified in the SCAB, but they may contribute substantially to the aggregate ambient acidity (Solomon et al., 1988).

2. Epidemiological Evidence for Health Effects of Acid Air Pollution.

The long term health effects of chronic exposure to nitric acid in the atmosphere have received scant attention from epidemiologists, with the exception of a series of studies conducted in Chattanooga, Tennessee, in the vicinity of a TNT (trinitrotoluene) manufacturing plant. During the Vietnam War (1966) the plant was reopened and the surrounding residential neighborhood was exposed to elevated levels of oxides of nitrogen for more than 5 years. Annual mean NO_2 levels ranged from 50 to almost 200 $\mu g/m^3$ (Love et al., 1982) with annual mean suspended nitrate levels ranging from 1-9.5 $\mu g/m^3$ (Pearlman et al., 1971). Unfortunately, nitric acid levels were not monitored directly, however, the EPA determined that corrosion rates of steel, zinc, and nylon were markedly elevated in Chattanooga in 1967 and 1968. Given the concurrent NO_2 levels, this suggests that nitric acid levels were probably elevated as well. A 3 year study beginning in 1966, shortly after the plant reopened, indicated a significant increase in lower respiratory tract infections, as well as acute and chronic bronchitis in children who resided in the high and intermediate exposure areas for 2 or more years (Pearlman et al., 1971). There was no change in the prevalence of asthma associated with exposure. A follow up study (Love et al., 1982) was done in 1972-73 when NO_2 levels had decreased significantly compared to the late 1960's. In addition, a strike by plant workers in 1973 sharply reduced NO_2 levels for 3 months. There was a significant drop in upper respiratory tract illness in all age groups during the strike. However, illness rates for upper respiratory tract disease, lower respiratory tract disease, and acute respiratory disease continued to be associated with residence in what had historically been the high exposure area. This indicates a persistence of alterations of respiratory defense systems due to oxides of nitrogen. It is unfortunate that the effects of HNO_3

in this series of studies can not be separated from the effects of concurrent NO_2 or other pollutant exposure; however, this work does indicate that bronchitis and compromise of respiratory tract defenses are important foci for studies of chronic nitric acid exposure.

Although there is very limited evidence on the health effects of chronic nitric acid exposure, there is considerable literature linking various pulmonary diseases to chronic sulfuric acid exposure. As is the case for nitric acid, there are relatively few epidemiological studies in which H_2SO_4 was directly measured, even in the recent literature.

Thurston (1989) and co-workers have re-analyzed mortality data in London during the winters of 1963-1972 using direct acid measurements made in Central London during those same years. The analyses indicated that the log of acid aerosol concentration was more closely correlated with raw total mortality than British smoke shade or SO_2. The Harvard six cities study has recently begun monitoring H^+ concentration. Preliminary data from 4 of the cities (Speizer, 1989) indicate that prevalence of bronchitis among children correlated more closely with $[H^+]$ data reported by Spangler and co-workers (1989) for the four cities than with PM-15 levels. The magnitude of the potential health problem revealed by these data deserves emphasis. The prevalence of bronchitis in children 10-12 years of age was 10% in Kingston, TN, which had a mean H^+ concentration of 40 nmoles/m^3, whereas in Portage, WI the $[H^+]$ concentration was about 10 nmoles/m^3 and bronchitis prevalence was less than 4%. These bronchitis prevalence rates were measured in a non-susceptible segment of the population and thus the effects of $[H^+]$ may be even more striking in the aged and in individuals pre-disposed to bronchitis.

Raizenne et al. (1989) examined the relationship between air pollution episodes involving elevated O_3 and H_2SO_4 levels and acute changes in pulmonary function in 8-14 year old girls. Decreases in forced expiratory volume in 1 sec (FEV_1) and maximum expiratory flow rate of 3.5% and 7% respectively were associated with air pollution episodes. 40% of the study group had a positive response to methacholine (with no history of asthma) and this group tended to have a larger decrease in pulmonary function than non-responders. Although it is not possible to determine if the changes in flow rates were due to the effects of O_3, H_2SO_4 or both, this study does indicate that acid air pollution has the potential for increasing airway resistance even in a non-susceptible segment of the population.

3. Human Clinical and Animal Toxicologic Studies.

Most of the human clinical research on acids to date has been, directed towards the effects of acid sulfates (U.S. EPA Acid Aerosols issue paper, 1989). Utell (1985) summarized effects of acid exposures on pulmonary function in normal and asthmatic volunteers. In general, there was an absence of pulmonary function effects at concentrations below 100 $\mu g/m^3$. At concentrations of 350 $\mu g/m^3$ and greater there were increased respiratory symptoms (cough and throat irritation) and changes in pulmonary function variables. When subjects were examined 24 hr post-exposure, they exhibited significant increases in the bronchoconstrictor response to carbachol, suggesting a possible latent hyper-reactivity effect of H_2SO_4 inhalation. Asthmatic subjects may be more sensitive than normals, with young, extrinsic asthmatics comprising an especially sensitive subgroup. Leikauf et al. (1981) demonstrated significant changes in mucociliary clearance in ten normal subjects exposed to 110, 330, or 980 $\mu g/m^3$ of sulfuric acid aerosol (0.5 μm MMAD). Clearance was accelerated at the lower concentration and slowed at the higher concentration. The biphasic nature of this response suggests that care must be taken in interpreting the effects on clearance relative to the exposure concentrations used. The effects of inhaled sulfuric acid aerosol are mitigated by endogenous NH_3 production. Exercising asthmatic subjects showed greater changes in FEV_1 and maximum expiratory flow rates after acid aerosol inhalation at low oral NH_3 levels than when the same amount of acid was inhaled at high oral NH_3 levels (Mariglio et al., 1983)

The results of some laboratory animal studies and epidemiologic field studies have suggested that inhaled acids can amplify the toxic effects of ozone (Lippmann, 1989). Human clinical studies have not convincingly demonstrated significant differences between exposures to ozone alone and exposures to mixtures of ozone and sulfuric acid aerosol (Kleinman et al., 1981; Kulle et al., 1982; Stacy et al., 1983; Horvath et al., 1987). These clinical exposures were of relatively short duration (about 2 hrs) and the effects were assessed immediately post-exposure. Some differences might have been manifested if subjects were exposed for longer durations or if subject responses were followed up for longer periods of time. Lioy et al. (1985) reported a week-long baseline shift in maximum expiratory flow rate which was associated with both O_3 and H_2SO_4 during a four-day pollution episode.

Until recently, there have been few studies on the health effects of inhaled nitric acid.

Following an accidental and lethal exposure of three young men, their lungs were examined by electron microscopy and by using immunohistochemical techniques. These lungs exhibited altered neutrophils and necrotic endothelial cells in alveolar capillaries. Small and large serum proteins were also found in the lungs (Hajela et al., 1990), which might be related to increased lung permeability. Controlled laboratory low level exposures to sulfuric acid (68 $\mu g/m^3$) and to HNO_3 (128 $\mu g/m^3$) caused significant reductions in FEV_1 (6% and 4%, respectively) and increases in total respiratory resistance (12% and 23%, respectively) in nine allergic adolescent subjects (Koenig et al., 1989). The analyses of accidental exposures indicate that despite the very high water solubility of nitric acid vapor, penetration to lung parenchyma is possible. The accident was an explosion, and it is possible that some of the HNO_3 was in aerosol form, either as acid droplets or as acid adsorbed onto other debris particles. The Koenig study data were preliminary in that the experiment was not complete, however they demonstrated that administration of equivalent amounts of hydrogen ion (H^+) as either HNO_3 or sulfuric acid can cause similar effects. In contrast, Aris et al. (1993) found no significant effects of 500 $\mu g/m^3$ HNO_3 on pulmonary function tests or cellular and biochemical constituents of bronchoalveolar lavage fluid in subjects exposed during 4h of moderate exercise. Subjects exposed to HNO_3 in fog did not show increased sensitivity to subsequent exposure to 0.2 ppm O_3, in fact O_3 sensitivity was diminished following HNO_3 (Aris et al., 1991).

Fine et al. (1987) exposed eight asthmatics to acidic aerosols (HCl and H_2SO_4) at different pH's and different titratable acidities (using solutions buffered with glycine). Subjects exhibited no significant increases in specific airway resistance (SRaw) after exposures to unbuffered acid particles (pH 2.0) and significant increases after inhalation of buffered solutions with increased titratable acidity at the same pH. The inhalation of HCl caused greater increases in SRaw than inhalation of H_2SO_4. These studies suggest that the total titratable H^+ delivered to the lung is more important than the particle pH as a predictor of pulmonary function change. In addition, the chemical nature of the acid may also play a role. HCl, which has a higher vapor pressure than H_2SO_4 (as does HNO_3), caused greater responses than did H_2SO_4.

Nearly all animal model toxicology investigations of the effects of acid air pollutants on the respiratory system have concerned acid sulfates. Nitric acid studies have involved tracheal instillation or inhalation of large doses of HNO_3 which induce substantial lung damage (Fujita

et al., 1988; Kolhatkar et al., 1987; Christensen et al., 1988). Inhaled acid sulfates including H_2SO_4, $(NH_4)HSO_4$, and $(NH_4)_2SO_4$ have a variety of effects on the respiratory system including changes in pulmonary function, morphological changes in the lung parenchyma, bronchial airways, trachea, and nasal epithelium, and changes in pulmonary particle clearance, (Amdur et al., 1978 a,b; Wolff et al., 1986; Gearhart and Schlesinger, 1986; Juhos et al., 1978; Schwartz et., 1977; Phalen et al., 1980; Schlesinger and Gearhart, 1987; Kleinman et al., 1989).

Most of the reports of pulmonary function changes in response to acid sulfate exposure are increases in pulmonary resistance of guinea pigs (Amdur, et al., 1978a,b; Silbaugh, 1981). While the guinea pig model appears to be highly sensitive to the action of acid particles on the airways, the pattern of response to acid inhalation exposures among the different studies is not entirely consistent. A concentration related dose-response relationship was observed in some studies (Amdur et al., 1978 a,b) but other workers found large individual animal variation and threshold behavior of the pulmonary bronchoconstriction response (Wolff et al., 1979; Silbaugh et al., 1981). The time course of pulmonary function responses to acid inhalation also differs substantially among different studies (in some cases large particle size delayed onset of bronchoconstriction), and the bronchoconstriction response was not always directly related to particle acidity in comparisons of acid sulfate salts (Amdur et al., 1978b; Schaper et al., 1984; Silbaugh et al. 1981). The basis for these differences among different studies is not clear, but may be due to differences in animal strain and health or exposure differences that affect the dose of acid particles delivered to target tissues. Rats do not have the bronchoconstrictive sensitivity of guinea pigs and few studies of acid inhalation in rats have examined pulmonary resistance. Lewkowski et al. (1979) measured pulmonary resistance of rats exposed to 2.4 mg/m^3 H_2SO_4 for 14 weeks and found no effects.

Prominent among morphologic changes in the respiratory tract due to long term exposure to H_2SO_4 at concentrations < 2 mg/m^3 are hypertrophy and hyperplasia of epithelial secretory cells in bronchi and bronchioles (Schlesinger, et al., 1983; Gearhart and Schlesinger, 1987; Busch et al., 1984; Alarie, 1973). Higher concentrations of acid exposure result in involvement of the alveolar region in an inflammatory response with signs of edema, cellular infiltration, and epithelial desquamation and more severe effects on bronchi and trachea including lesions and thickening of mucus (Brownstein, 1980; Cavender et al., 1977a,b; Wolff et al., 1986; Juhos et

al., 1978; Schwartz et al., 1977). There are considerable differences in sensitivity of different animal species to morphologic effects of acid exposure. Guinea pigs appear to be particularly sensitive; however, it is possible that the sensitivity of bronchial epithelial structure in the guinea pig may be a secondary effect of hyper-responsive airways and bronchoconstriction rather than a direct effect of acid deposition on airway epithelia. The fact that acid aerosol exposure results in secretory cell hyperplasia and hypersecretion of mucus suggests a role of acid deposition in chronic bronchitis which is supported by epidemiological studies.

There are several lines of evidence indicating that exposure to acid air pollutants can compromise respiratory tract defense mechanisms. These defense mechanisms include mucociliary clearance which removes particles deposited in the airways, the integrity of the epithelial cell barrier to movement of foreign particles and molecules from the lungs to the circulation, and the functional capacity of pulmonary macrophages to mount an immune defense and clear particles from the alveolar zone and pulmonary airways.

The effects of inhaled acids on the rate of clearance of tracer particles from the respiratory tract has been extensively investigated over the past 15 years. In the earlier studies, brief exposure up to 14 mg/m^3 of airborne H_2SO_4 failed to demonstrate striking effects on short-term clearance (Sackner et al., 1978; Phalen et al., 1980), but more recently, clearance responses at lower concentrations have been reported. In our previous studies we have seen effects of acid inhalation on clearance. Late clearance was significantly delayed in rats exposed for 4 hours to 3.5 mg/m^3 of sulfuric acid at low relative humidity. A high humidity acid-exposed group had a change in the same direction but it was not statistically significant. A subsequent study in which rats were exposed 2 hours per day for 20 days to 1 mg/m^3 sulfuric acid failed to show a significant change in short or long-term clearance. A combination of propane soot (0.5 mg/m^3) and a mixture of nitric (0.35 mg/m^3) and sulfuric acid (0.22 mg/m^3) for 5 exposure time produced a significant delay in long-term clearance in the rat. These studies indicate that the rat clearance model can respond to acid inhalation. More than a dozen studies relating to the effects of sulfuric acid inhalation on clearance were reviewed in the U.S. EPA Acid Aerosols Issue Paper (1989). These studies indicate that bronchial clearance can be altered by repeated exposure to acidic droplets and that alveolar clearance can be altered by repeated exposures to sulfuric acid at 0.25 mg/m^3 (Schlesinger et al., 1979; Schlesinger and Gearhart, 1986). More recently,

Schlesinger (1990) has reported that repeated exposure of rabbits to 0.05 mg/m^3 sulfuric acid alters alveolar clearance.

The airway epithelium functions as a barrier to inhaled particles and prevents their entry into the tissues and circulation. Disruption of the epithelial permeability barrier of the lung by exposure to air pollutants has been observed in response to cigarette smoke (Jones et al., 1980; Hulbert et al., 1981; Mason et al., 1983) and oxidants including O_3 and NO_2 (Bhalla et al., 1986, 1987; Ranga et al., 1980; Selgrade et al., 1981). Studies in our laboratory have demonstrated increased permeability of both nasal and bronchoalveolar epithelia in rats immediately following acute exposures to single or mixed oxidant and acid air pollutants, O_3, O_3 + NO_2 or the complex atmosphere consisting of these gases plus SO_2, Fe_2O_3, $(NH_4)_2SO_4$, $Fe_2(SO_4)_3$, and $MnSO_4$. Atmosphere mixtures containing O_3 and NO_2 form HNO_3 vapor. The magnitude of increase in bronchoalveolar permeability was approximately the same after exposure to O_3 alone or its combination with other gases. However, a significant increase in the persistence of enhanced permeability occurred after exposure to O_3 + NO_2 or to the complex atmosphere but not after exposure to O_3 alone, suggesting extension of the duration of biologic effects by gas combinations containing HNO_3 and acid sulfate aerosol (Bhalla et al., 1987). In a recent study, increased permeability across both nasal and bronchoalveolar mucosa was observed after 4 weeks of exposure (4 hrs/day, 3 day/week) to atmospheres containing O_3, NO_2, HNO_3, and $(NH_4)HSO_4$ (Mautz et al., ARB Final Report, Contract A833-104, 1993). These studies indicate significant effects of acid-containing atmospheres on the upper respiratory tract in addition to the expected lower respiratory tract effects.

Pulmonary macrophages are a crucial defense against pulmonary infection. Exposure to sulfuric acid reduces the capacity of rabbit macrophages to adhere to glass (Nauman & Schlesinger, 1986) but does not suppress bacterial killing or reduce viability of macrophages recovered from mice (Coffin, 1972). Increased numbers of neutrophils and macrophages were lavaged from lungs of rabbits after aerosol exposure to H_2SO_4 (Nauman & Schlesinger, 1986), indicating induction of an inflammatory reaction. Aerosolized sulfuric acid produced transient mobilization of macrophages and neutrophils with little evidence of functional impairment (Schlesinger, 1985), in contrast to the effects of 0_3 or NO_2 (Gardner, 1985). More recently, Prasad et al., (1988) found that a 7-component atmosphere containing O_3 and NO_2 (and from

their interaction, HNO_3) tended to decrease phagocytosis and inhibit the Fc receptors of macrophages from exposed rats. In addition, macrophages from rats exposed to HNO_3 + H_2SO_4, or to the acid combination and diesel exhaust soot, exhibited significantly reduced phagocytic activity and Fc receptor binding (Prasad et al., 1990).

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. Exposures to combinations of O_3 and acid sulfate aerosols generally produce effects attributable to O_3 alone. However, the investigations of Last and co-workers have found evidence for synergistic interactions between O_3 and H_2SO_4 on collagen synthesis. These investigations have examined the interaction between ozone and aerosols containing either ammonium sulfate or sulfuric acid (Warren, Guth, and Last 1986, Last et al., 1986, Warren and Last, 1987). In general these studies indicated a synergistic interaction between ozone and acidic aerosols that resulted in lung damage at concentrations of O_3 and H_2SO_4 that had little or no effect by themselves. H_2SO_4 (and in some cases $(NH_4)_2SO_4$) potentiated ozone-induced increases in total lung protein, soluble proline, hydroxyproline (i.e., collagen) synthesis rate, and lung lesion size. The interaction between O_3 and H_2SO_4 was found to be dependent on the droplet size; H_2SO_4 at .02 μm MMAD had no synergistic effect with ozone whereas H_2SO_4 at 0.5 μm MMAD did potentiate ozone-induced lung injury. Given the size dependence of the H_2SO_4-O_3 synergistic interaction, it is difficult to predict whether vapor phase HNO_3 will interact in the same way as H_2SO_4 with O_3.

Interestingly, Warren and Last (1987) also found that H_2SO_4 alone at a concentration of 0.1 mg/m^3 (MMAD = 0.4 μm) resulted in a significant increase in lung collagen synthesis, but at higher (0.5 and 1.0 mg/m^3) or lower (.04 mg/m^3) concentrations, H_2SO_4 had no effect. Enhancement of the effects of O_3 on rat lung injury by H_2SO_4 in a complex mixture was observed by Kleinman et al. (1989) in single exercising exposures of rats. Mautz et al. (1988) observed synergistic interaction between 0.6 ppm NO_2 and 0.35 ppm O_3 (single exercising exposure) on lung tissue injury which may have been related to the formation of 50 μg/m^3 HNO_3 in this pollutant mixture.

C. Hypotheses.

This investigation of the health effects of HNO_3 and O_3 was organized around the following hypotheses:

1. Chronic, episodic exposure to ambient urban levels of HNO_3 has one or more adverse respiratory health effects including respiratory tract irritation, airway mucus hypersecretion, altered pulmonary connective tissue and airway epithelial structure, and impairment of pulmonary macrophage function and lung clearance.

2. HNO_3 will interact synergistically with O_3 to increase the severity of pulmonary function responses, pulmonary connective tissue and epithelial structural alterations, and effects on pulmonary defensive functions.

D. Experimental Design and Rationale.

1. Atmosphere Selection.

The composition of a polluted atmosphere and the concentration individual pollutants vary with location, by season, from day-to-day within a season, and with time within a day. In developing an exposure plan for this study, we considered these factors and chose a set of pollutant concentrations of O_3 and HNO_3 vapor based on their presence in substantial amounts in regions of the South Coast Air Basin and their potential to cause adverse health effects on exposed California residents.

Exposures in areas affected by urban air pollution are episodic in nature. Ozone episodes occur most frequently during the summer months, however episodes have occurred at other times of the year. Within each episode the number of consecutive days on which the State and Federal standards are exceeded is typically 1 to 6. Other pollutants also show episodic behavior. Recent nitric acid concentration data (Air Resources Board, information supplied as a personal communication, 1988) show a strong correlation between hourly O_3 and hourly HNO_3 concentrations. On a seasonal basis, Solomon et al., (1988) have shown that HNO_3, like O_3, has maximal concentrations during the summer.

The exposure pattern was selected based on variation in air pollutants in the San Gabriel

Valley (Table 1). The ARB reports a strong correlation between peak 1 hr and longer term averages for O_3 (ARB Staff Report, 1987). The levels for O_3 (Table 1) for exposures were set at 0.15 ppm, a level which was equalled or exceeded between 10-50% of days during the high pollution April-September period in cities of the San Gabriel Valley. Nitric acid was not measured at Azusa, but data were available for the cities of Claremont and Glendora. The few available data on peak hourly concentrations show an average of 60 $\mu g/m^3$ measured in Glendora over an 8-day period in August 1986; 24 hr samples showed lower concentrations (20 $\mu g/m^3$). Because there was so little background information available on toxicology of inhaled HNO_3 vapor, an initial 4 week dose-response exposure was planned that included measured ambient as well as higher concentrations of HNO_3 vapor (see section I.D.3. below). The rationale for such dose-response exposures is the expectation that one can clearly identify specific biological effects occurring at the higher concentrations and determine if these are detectable at the lower concentrations. The subchronic dose-response exposure was then used to select an HNO_3 concentration to use in the chronic 9 month exposure (see section I.D.3. below).

Azusa and the surrounding region of the San Gabriel Valley was selected as the environmental model for the episodic exposure pattern. A substantial amount of recent air monitoring data is available from that location. 1986 was chosen as a base year to take

Table 1. Concentrations of airborne components of air in the San Gabriel Valley and concentrations for subchronic and chronic exposures.

Pollutant Component	Measured in San Gabriel Valley	1 Month HNO_3 Dose-Response Exposure	9 Month HNO_3 and O_3 Exposure
O_3	0.31 ppm (peak 1 hour concentration)		0.15 ppm
HNO_3	60 $\mu g/m^3$ (peak 1 hour concentration)	50 $\mu g/m^3$ 150 $\mu g/m^3$ 450 $\mu g/m^3$	50 $\mu g/m^3$

advantage of the data available from an in-depth atmospheric sampling and analysis program in the SCAB. Ozone concentrations in Azusa exceeded the California 0.09 ppm ozone standard 45% of the days in 1986, which, when spread over the entire year, represents average episodes of about 3 days per week. Thus a laboratory toxicology exposure of 3 days per week was selected to reasonably represent ambient episodes. The daily ozone peak in California has its major intensity over about 2-4 hours, therefore a 4 hour per day exposure was selected.

2. Biological Endpoints.

The majority of biological endpoints selected for study were relevant to development of human pulmonary diseases. In choosing these biological endpoints we relied on the principle that the lung (regardless of species) responds to chronic insult and injury with a limited repertoire of responses. These responses are 1) airway mucus hypersecretion, 2) airway reactivity, 3) altered immune defenses, and 4) alterations in lung connective tissue and morphometry. In humans, these responses correspond to 1) chronic bronchitis, 2) asthma, 3) upper and lower respiratory tract infection, and 4) emphysema and fibrosis. We chose endpoints that are indices of these 4 responses of the lung as described below.

a. Biological Endpoints Related to Airway Irritation. Irritation of respiratory airways from exposure to air pollutants can result in reflex changes in breathing pattern, bronchoconstriction, and tissue injury, responses related to asthma. Reflex breathing pattern responses can be highly variable depending on the irritant agent. Pulmonary irritants, like O_3, invoke a rapid-shallow breathing pattern that is a vagally mediated reflex believed to originate in stimulation of lung C fibers (Alarie, 1973; Coleridge and Coleridge, 1984; McDonnell et al., 1983). Compounds classified as sensory or upper airway irritants, such as HNO_3 and formaldehyde, induce reflex responses mediated by trigeminal afferent pathways (Alarie, 1973), and generally involve a shift to slow-deep breathing patterns. Irritation of tracheobronchial tissues may invoke reflex bronchoconstriction, increasing pulmonary resistance. In addition, measurements of epithelial cell turnover rate and epithelial morphometry in the nose, trachea, and bronchi and measurements of broncho-alveolar permeability were evaluated to indicate whether the repeated, episodic exposures produced a continuing pattern of irritation and injury to the airways.

b. Biological Endpoints Related to Mucus Secretion. Methods evaluating changes in mucus secretion included morphometric determination of serous cell numbers, histochemical measures of epithelial glycoprotein density, measurement of glycoprotein in bronchoalveolar lavage fluid, and epithelial cell turnover in the trachea and bronchi. Measurement of serous cell numbers and epithelial glycoprotein density alone does not necessarily reflect the rate at which cells are synthesizing and secreting mucus. Thus, mucus (glycoprotein) production into the fluid lining the respiratory tract (recovered by broncho-alveolar lavage) was also measured.

c. Biological Endpoints Related to Pulmonary Defenses. Based on the epidemiological evidence linking increased respiratory tract infection rate to acid air pollution (Pope, 1989 and Dockery et. al., 1989) we assessed 6 parameters of respiratory tract defense: 1) mucociliary clearance, 2) epithelial permeability, 3) lavage cell differential, 4) lavage fluid biochemical indices of pulmonary macrophage function and injury, and 5) pulmonary macrophage viability, phagocytosis capacity, and Fc receptor binding capacity. The measurement of inhaled particle clearance efficiency has become recognized as an important biological effects assessment in toxicology evaluations. The particle clearance measurement was used to evaluate the ability of the lung to rid itself of insoluble tracer particles during the last month of the 9 month exposure. This measurement is important because failure of this self-cleaning mechanism would lead to a build-up of foreign matter in the respiratory tract. Epithelial permeability was measured in the nose and bronchoalveolar portions of the respiratory tract. These measurements were used to assess the integrity of the epithelial barrier in its capacity to resist movement of foreign particles and large molecules into deeper tissues and circulation. Disruption of the epithelial permeability barrier was expected to depress the capacity of the lung to resist infection. HNO_3 is highly soluble and was expected to injure the nasal epithelium, however the presence of O_3 may induce permeability changes in the deep lung and there may be toxic interactions between HNO_3 and O_3 on respiratory tract epithelia. The pulmonary macrophage functions assessed were important measures of the functional capacity of macrophages to defend the respiratory system against inhaled foreign particles and pathogens (Prasad et al., (1988).

d. Biological Endpoints Related to Connective Tissue Alteration. Alterations of normal connective tissue architecture are seen in two crippling lung diseases: emphysema and pulmonary fibrosis. Emphysema is thought to result from destruction of elastin in the small

airways, and pulmonary fibrosis involves proliferation of fibroblasts and collagen deposition. Both disease processes are associated with chronic injury and inflammation. Measurements used to examine lung inflammation and changes in connective tissue included: 1) pulmonary irritant breathing patterns, 2) measurement of quasi-static compliance, 3) excised lung gas volume, 4) lung morphometric analysis, 5) lung collagen deposition and biochemistry, 6) lavage fluid elastase inhibitory capacity,

and 7) epithelial cell turnover in the bronchioles and lung parenchyma. Reflex breathing pattern responses measured during exposure were used to assess whether the exposure atmospheres produced deep lung irritation (rapid-shallow breathing), and whether this was diminished or persisted with repeated, episodic exposures. Autoradiographic analysis of cell turnover rate in the lung tissues at the end of exposure was used to determine if the repeated pollutant exposures produced a continuing pattern of cell injury and turnover. Quasi-static compliance and excised lung gas volume assessed whether pollutant exposures induced changes in lung elasticity. Collagen deposition was determined histochemically as density of sirius red stain in lung sections and biochemically from lung tissue samples (Warren, Guth and Last 1986). In addition, collagen cross-linking were measured as an index of fibrosis. Lavage fluid elastase inhibitory capacity is a measure of the balance between enzymes that regulate connective tissue in the lung. Elastase inhibitory capacity has been found to be strongly increased by ozone exposure (Pickerell, 1987). The elastase inhibitors that are detected by this assay also inhibit other metallo-proteases such as collagenase. It is possible that collagenase inhibition prevents collagen remodeling after lung repair and thus may result in increased collagen deposition and fibrosis (Clark et al., 1987). Thus, the presence of elastase inhibitors in the lung lining layer may actually contribute to fibrogenesis. Morphometric analysis of lung tissues was used to evaluate changes in microscopic anatomy.

3. Subchronic and Chronic Exposure Studies.

A 1 month (4 week) exposure to a graded series of concentrations of HNO_3 (Table 1) was used to survey the biological effects of HNO_3 and evaluate dose-response relations. Based on the results of this exposure, 50 $\mu g/m^3$ HNO_3 was chosen for the 9 month (40 week) chronic study. The 9 month exposure included groups exposed to 0.15 ppm O_3 and 50 $\mu g/m^3$ alone and

in combination, a design that permitted analysis of interactions between the acid and oxidant on the biological endpoints. Additional biological endpoints were added to the 9 month exposure assessment including quasi-static compliance, excised lung gas volume, epithelial permeability, lung collagen histochemistry, lung heat shock proteins, and an analysis of changes to the lung cytochrome P450 monooxygenase system. The 9 month exposure included analysis points for certain subsets of biological endpoints at 1 month, 3 months, and 9 months of exposure. Exposure subjects were male Fisher 344/N rats. These animals have an extensive background in inhalation toxicology research, and for most biological endpoints, rats are a sensitive and economical animal model for study of respiratory system effects of air pollutants.

II. METHODS.

A. Exposure Atmosphere Generation and Characterization.

Nitric acid vapor was generated by passing purified air over a heated (30 C) aqueous solution of HNO_3. Ozone was generated by passing medical-grade oxygen through a corona-discharge ozonizer (Sander type III) and diluting the ozone into purified air. The resulting single or mixed HNO_3 and O_3 atmospheres were equilibrated with purified air to yield the appropriate concentrations of each constituent at 60% relative humidity, when measured at a nose-only exposure port (the breathing zone of exposed animals). HNO_3 vapor was measured using tandem collection filters and real time monitoring. A teflon-coated glass fiber filter (PallFlex Products Corp., Putnam CT) collected any particle phase nitrate (no detectable particle phase was observed) and preceded a nylon filter (Nylasorb, Gelman Sciences, Inc. Ann Arbor, MI) which collected HNO_3 vapor. Filter samples were extracted in a dilute buffer solution (0.03 M $NaHCO_3$ and 0.024 M $Na2CO_3$, pH 10.2), and the extract was analyzed by ion chromatography. Hydrogen ion concentrations of the extracts were measured by the method of Koutrakis et al. (1988) using a Beckman Model 4500 pH meter. HNO_3 concentrations were also monitored continuously during exposures by converting HNO_3 vapor to NO (Thermocon) and analyzing NO_X with a Monitor Laboratories Model 8840 nitrogen oxides analyzer. Concentrations of ozone were be monitored with a calibrated ultraviolet light absorption continuous monitor (Dasibi Environmental Corp., Model 1003-AH).

Ozone monitors were calibrated by the ARB at 6 month intervals, and the calibration was checked before the start of each exposure and at weekly intervals during the exposures using an ozone transfer standard (Meloy Labs Model CN0S-40 Ozone Calibrator). Ozone was sampled using fluorocarbon tubing in order to minimize sampling losses. Filter sampler flows were measured at the beginning and end of each sampling interval using a secondary flow-standard, which were periodically calibrated with primary air-flow standards (a soup film bubble meter and gas-tight syringe). The ion chromatograph was calibrated each morning using gravimetrically-prepared standard solutions containing nitrate ions, and the calibration was checked several times during the day with the standard solutions. Blank filters were analyzed each day in order to correct for background levels of contaminants present on the filters prior

to sampling. Exposures were controlled by measurement of near-continuous samples of O_3 and HNO_3. Daily integrated samples (2 filter samples per daily 4 h exposure) for determination of nitric acid, and total titratable acidity were taken and analyzed before the next day of exposure. Gas and vapor generators were adjusted to ensure that the running average of concentrations was on target.

B. Animal Housing, Quality Control, and Exposure.

Exposure subjects were male Fisher 344/N rats (Simonsen Laboratories, Inc., Gilroy, CA), barrier reared and maintained in laminar flow isolation units supplied with filtered air. Rats were purchased certified viral free, and health reports were provided for each shipment. 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 (1 month HNO_3 dose-response exposure) or every 4 weeks (9 month HNO_3-O_3 exposure). No animals sampled for quality control showed evidence of pulmonary disease or exposure to rodent pathogens. The rats were held in the laboratory for 1 week prior to exposures which began when the animals were 11 weeks old. 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. Exposure 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. They were housed in wire cages over pans of rock salt which collect feces and urine under desiccating conditions to prevent bacterial growth and ammonia production. Caging was changed twice weekly and cages were disinfected. 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. Vivarium facilities met the stringent requirements of the American Association for Accreditation of Laboratory Animal Care.

C. Pulmonary Function Measurements.

1. Breathing Pattern and Minute Ventilation.

Breathing pattern, (frequency and tidal volume) and minute ventilation were measured in rats at periodic intervals during exposures (Mautz and Bufalino, 1989). These intervals were weekly in 1 month HNO_3 dose-response exposure and every 4 weeks in the 9 month HNO_3-O_3 exposure. For each weekly episode measured, measurements were made on the first and third (last) day. Standard nose only exposure tubes were modified to function as flow plethysmographs for the measurements during exposure. A latex dental dam membrane was clamped between the aluminum nose cone and body tube fitting snugly around the rodent's head and separating the nose and mouth from the body. Thoracic displacement of air during respiration was measured with a pneumotachograph and differential pressure transducer connected between the body tube and a port in the aluminum nose cone opening at the rat's nose. The flow signal was electrically integrated and counted to display tidal volume and breath frequency on a chart recorder. Eight plethysmographs were plugged into ports of each 1 m^3 stainless steel exposure chamber. Within the chamber the exposure atmosphere was conducted through stainless steel ducts past the ports providing an individual nose-only exposure to each rat. 3 successive measurements from each rat at 20 min intervals were averaged to yield a value of each variable for each rat over a given hour of the exposure.

2. Excised Lung Gas Volume.

Excised lung gas volume was measured from rats immediately following sacrifice procedures described below (see part III D 1 below). The chest cavity was opened allowing the lungs to collapse and retain the volume of trapped gas behind the closed airways. The trachea was then ligated, and the lungs and trachea were removed. They were then tied to a weight and suspended under saline in a vessel attached to a balance. The volume of the trapped gas was then measured as buoyant force of the lungs on the tared vessel and ballast weight immersed in saline. Wet to dry mass ratios were measured from whole lungs or lobes when tissue was

available by blotting the fresh tissue, measuring wet mass, and drying to constant mass at 70 C.

3. Quasi-static Compliance.

Compliance was measured from lungs excised following bronchoalveolar lavage performed to supply fluid for other biological endpoints. Lungs were then degassed in vacuum, attached to a saline filled cannula, and immersed and sealed in a saline filled chamber. Transpulmonary pressure and volume displacement were monitored as the lungs were slowly and repeatedly inflated and deflated with saline. Compliance was measured as the slope of the linear portion of the volume vs. pressure curve during the second inflation when the values were most stable.

4. Particle Clearance.

Respiratory tract clearance was studied during the final month of the 9 month HNO_3-O_3 exposure. Polystyrene latex microspheres (1.8 μm MMAD, GSD $<$ 1.3) were radiolabeled with ^{51}Cr. One month prior to the end of the exposure, rats inhaled the radiolabeled particles for 30 min and the animals were immediately counted twice in a whole body counter in order to obtain an accurate determination of the initial total deposited dose. During the subsequent 48 hr, feces were collected from the rats at 8h, 14h, 24h and 48h to measure early (upper airway) clearance. At 48h post-deposition the rats were again counted in a whole body counter to 1) obtain an estimate of the particle fractions deposited in the deep lung region, and 2) provide a basis for normalizing lung count data obtained later to account for differing initial radiolabeled particle burdens in the long-term clearance measurement. To measure long-term clearance, rats were sacrificed at the end of the exposure, the left lungs, trachea, and larynx were excised (other lung tissues were committed to other biological endpoints) and counted using a well counter to measure uncleared activity.

5. Epithelial Permeability.

Epithelial permeability measurements were made following the 9 month HNO_3-O_3 exposure at 1 hour post-exposure. Lung permeability was measured as protein accumulation in

lavage fluid. Total protein in the lavage fluid was measured by a BCA procedure, and albumen was measured by ELISA. A set of protein standards of concentrations ranging from 0.05 mg/ml to 0.25 mg/ml were be prepared by diluting a stock solution of bovine serum albumin (BSA). For measuring permeability changes across the nasal mucosa, a tracheostomy was performed and the oropharynx filled with dental impression cream to block the posterior nares. A radiolabeled tracer solution containing ^{99m}Tc labeled diethylenetriaminepenta-acetate (^{99m}Tc-DTPA, mol wt 492) in 0.1 ml phosphate buffered saline was instilled into the nose. The tracer solution was instilled into the right naris until it passed across the nasal septum at the posterior end, filled the left nasal cavity, and emerged through the left naris. Heparin solution (20 units in 0.2 ml saline) was injected into the rats through carotid or femoral artery catheters prior to blood sampling. Blood samples, 0.1 ml each, were drawn at 6, 7, 8, 9, and 10 min after the start of instillation. Radioactivity in the blood samples was measured immediately after collection of the samples in a gamma counter. The results were expressed as the percent of inoculum present in the blood at 8 min after instillation into the nasal lumen.

D. Morphometry and Histopathology.

1. Preparation of Tissues for Analyses: Excised Gas Volume, Bronchopulmonary Lavage, and Histopathology.

Rats were anesthetized with sodium pentobarbital and killed by exsanguination via the abdominal aorta. Separate groups of rats provided lavage fluid for macrophage studies and tissue for morphometry as described below. The thoracic cavity was opened, and the trachea and lungs were exposed. A small opening was made in the trachea just craniad of the bifurcation and a cannula inserted and tied in place. The lung lobes were then lavaged via the cannula using phenol red-free Hank's balanced salt solution, without Ca^{++} or Mg^{++} (HBSS). Depending on the rat size, the lavage volume was between 6 and 10 ml. The HBSS was instilled and withdrawn twice, and saved separately. The lavage was repeated 3 more times, and these fluids were pooled. The lavage fluids were centrifuged to recover the cells, and the supernatant from the first lavage removed and frozen at -70° for later analysis of protein as described below. The cell pellets were pooled, and used to prepare slides for differential counts, phagocytosis assays, and Fc receptor assays.

For lung morphometry and histochemical assays, a cannula was placed in the trachea as above. The cranial portion of the trachea was removed and fixed in 10% buffered formalin. The entire lung with attached cannula was 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 fixed left lobe provided sections used for morphometry, histochemistry, and lung autoradiographic measures of cell turnover at UCI. Additional animals in the 9 month HNO_3-O_3 exposures were sacrificed for morphometry of the bronchiolar-alveolar duct junction at UC Davis, and lungs were fixed for 30 min at 20 cm hydrostatic pressure with 0.6% paraformaldehyde and 0.9% gluteraldehyde in cacodylate buffer (pH 7.4). In UCI analyses, the left lobe was cut longitudinally to expose the left main airway and major intrapulmonary airways. After embedding in paraffin, 5 μm sections were cut and mounted on glass slides. Measurements of section area after free hand cutting the slab, and again after mounting 5 μm sections was used to determine the degree of shrinkage during embedding and sectioning. Separate sections were stained for morphometry and autoradiography preparation.

The fixed trachea was embedded in paraffin and 5 μm cross-sections cut from the cranial, middle, and distal regions. The sections were prepared for measurement of epithelial glycoprotein density and autoradiography. For analysis of the nasal region, the head was skinned, external tissue and muscle removed, and the nasal section fixed by immersion in 10% buffered formalin. Decalcification was performed in 6% EDTA, followed by embedding in paraffin and sectioning at 5 μm for autoradiography and morphometry. Cross-sections were cut approximately midway between the nares and the eye to provide sections containing squamous, respiratory, and olfactory epithelium.

2. Lung Morphometry.

For morphometric analyses at UCI, the left lobe was analyzed using a computer image analysis system (Summagraphic digitizer interfaced with a PC/AT and using a proprietary software package from American Innovision). The subgross volume fractions of parenchyma and non-parenchyma (including large airways and large vessels) was 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. At higher magnification relative fractions of alveoli, alveolar ducts, bronchi, and bronchus associated lymphatic tissue (BALT) were determined. Standard formulae were used to estimate the lung volume fractions (Elias and Hyde, 1983). Mean chord length of lines randomly placed across alveoli was measured, and mean septal wall thickness at the points where lines cross septae was also determined.

For morphometric analyses performed at UC Davis by Dr. Kent Pinkerton, transverse slices of the left lobe were cut and embedded in paraffin, glycolmethacrylate, or araldite. Tissues embedded in paraffin were sectioned at 6 μm to qualitatively assess the preservation and histopathology of each lung for all animals. Tissues embedded in glycolmethacrylate were sectioned at 1.5 μm to morphometrically assess the general composition and architectural makeup of the lung. Tissues embedded in araldite were sectioned at 0.5 μm to define changes in the major tissue compartments of the alveolar septa. Glycolmethacrylate embedded sections were stained with toluidine blue or methylene blue-basic fuchsin. Araldite embedded tissue sections bearing bronchiole-alveolar duct junctions were identified for analysis.

3. Autoradiographic (ARG) Analysis of Cell Turnover Rate.

Rats were given tritiated thymidine by sub-cutaneous administration of the compound as a solution in sterile 0.9% NaCl. The dose was be 2 μCi/gm b.w. at a specific radioactivity of 50-60 Ci/mmole, as supplied by the vendor. Purity of the radiotracer was confirmed by paper chromatography using solvent systems recommended by the supplier. The concentration of label administered to the rats was 0.5 mCi/ml. The specific radioactivity was held constant within each experiment. The animals were given [^{3}H]-dThd 1 day prior to sacrifice. Autoradiographic analyses were performed on 5-μm paraffin sections of the nose, trachea, left lung lobar bronchus, segmental bronchi, and alveolar zone prepared as described above using liquid photographic emulsion (Kodak NTB-2) development in Kodak D-19, followed by staining with nuclear red, fast green. Labeled cell fractions were determined by direct counting of finished autoradiographic preparations at a magnification of 400X. 20 randomly-selected fields were scored for each tissue and section.

4. Epithelial Glycoprotein Staining and Tracheal Serous Cell Counts.

Sections of the trachea were prepared by paraffin embedding, and sections of the nasal region were prepared after decalcification with EDTA, as described above. The sections were stained using an alcian blue/PAS/hematoxylin method (Sheenan and Hrapchak, 1980). The relative density of stain in epithelial tissue was measured by image analysis and expressed per unit of epithelial tissue section area. Serous cell density in the trachea was determined as number of serous cells per field (unit airway epithelium length) in sections separately stained with Periodic Acid Schiff reagent and with Alcien Blue. 20 tracheal fields were counted at 600X per animal.

5. Pulmonary Macrophage Acid Phosphatase Density.

Acid phosphatase in pulmonary macrophages was measured in sections of lung stained with an acid phosphatase stain. Macrophages in random samples of the lung section were scored for 3 classes of stain intensity.

6. Collagen Density in Lung Sections.

Collagen in sections of lung was stained with Sirius Red. Stain molecules align with collagen fibers in the tissue and partially polarize transmitted light. Sections were viewed between crossed polarizing filters and areas showing bi-refringence were quantified by image analysis. Results were expressed as the ratio of birefringent area to total parenchymal tissue area analyzed.

E. Bronchoalveolar Lavage Fluid Analyses and Pulmonary Macrophage Function.

1. Bronchoalveolar Lavage Fluid Biochemical Analyses.

In the cell free supernate of bronchoalveolar lavage fluid, protein was analyzed as described in section II.C.5. Lactate dehydrogenase was analyzed spectrophotometrically by enzymatic conversion of lactate to pyruvate in the presence of NADH (Sigma Chemical). ß-glucuronidase was analyzed spectrophotometrically using a modification of the method of Fishbein et al. (1967). The sample containing ß-glucuronidase was incubated with a phenolphthalein glucuronic acid substrate, and the phenolphthalein released was measured at 550

nm. Fibronectin was analyzed using an ELISA method, as described by Driscoll et al. (1990). The sample containing fibronectin was reacted with an excess of goat anti-fibronectin antibody, the non-reacted antibody was quantitatively transferred to a well of a fibronectin-coated 96 well microculture plate, and after washing to remove non-adherant antibody, the residual was reacted with a peroxide-conjugated rabbit anti-goat IgG antibody and quantified by assaying for peroxidase.

Glycoprotein in lavage fluid was analyzed by Dr. C. Basbaum at UC San Francisco using an ELISA method (Finkbeiner et al., 1988; Steiger, et al., 1994). Samples were diluted in 0.05 M $NaHCO_3$ and applied to Immunon Dynatech 96 well plates in volumes of 50 μL per well. Samples were dried at 37 C, wells were blocked for 1 h at room temperature with 100 μL PBS containing 0.3% Triton and 1% normal goat serum. Plates were then rinsed in blocking buffer. 50 μL of monoclonal antibody 10G5 (1:2) (hybridoma supernatant diluted 1:5 in blocking buffer) was added to each well and plates were shaken at room temperature for 1 h. Plates were then washed 4X in blocking buffer. Next, biotinylated anti-mouse IgG, diluted 1:100 in blocking buffer, was added to the wells in 50 μL volumes. Plates were shaken at room temperature for 30 min prior to being washed 4X in blocking buffer. Next, 50 μL of Vectastain ABC reagent was added to each well. Plates were shaken at room temperature for 30 min prior to being washed 5X in blocking buffer. Next, 100 μL of substrate (p-nitrophenyl phosphate) was added to each well and plates were incubated at 37 C. Plates were read in a microplate reader at 10 min intervals for up to 1 h.

Elastase inhibitory capacity of lavage fluid (Pickrell et al., 1987) was analyzed by incubating 100, 200 and 300 μl aliquots of lavage fluid with lung of porcine pancreatic elastase at room temperature for five minutes followed by the addition of fluorescein-labeled elastin (2mg). The samples were incubated at 37°C for 30 minutes with constant mixing followed by centrifugation. The fluorescence of the supernatant was measured using a Perkin Elmer LS-5 Spectrofluorimeter at 495 excitation and 520 emission. A blank without elastase was used to determine non-specific release of fluorescein. Standards consisted of 100, 200 and 300 μg of HBSS plus 1 ug elastase and 2 mg fluorescien-elastin.

2. Differential Cell Count.

Cell counts in bronchoalveolar lavage fluid were determined using a bright line hemocytometer. 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 cytofuge, the cells were stained with Wright-Giemsa stain, and a differential count was made. The remaining cell suspension was aliquoted as described below.

3. Phagocytosis.

Phagocytic activity of pulmonary macrophages was measured by the method of Rao et al. (1980). In brief, 0.1 ml of each cell suspension was added to Lab-Tek chambers, containing 0.5 ml of culture medium, and incubated for 1 hr at 37°C. The chambers were then washed with the medium to remove the non-adherent cells. Then, 0.1 ml of a suspension of spherical polystyrene latex (PSL) microspheres (diameter 1.1 - 1.4 μm) was added to each chamber and incubated for 60 to 90 minutes. The cells were washed with calcium and magnesium-free HHBS to remove the free latex particles. The slides were air dried, the cell chambers dismantled and the cells stained with a Wright-Giemsa monochromatic cytological stain (Diff-Quick, Baxter). The slides were immersed in xylene for 2 min to remove the microspheres and phagocytized spheres were visualized as unstained "ghosts" in the cell cytoplasm. The percentage of PSL positive cells (defined as containing > 2 spheres/cell) was determined.

4. Fc Receptor Binding.

A rosette assay was used to determine the effect of exposures on Fc receptors. Lab-Tek chambers, each containing 1 x 10^5 cells in 0.1 ml of HBBS, were prepared with macrophages as described for the phagocytosis assay. The cells 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). For initial preparation of antibody, sheep red blood cells (SRBC) obtained in Alseviers solution was 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. For the assay, 0.1 ml of anti-SRBC at a concentration determined by trials prior to the experiment in RPMI medium 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 (1×10^7) was added to each chamber. The macrophages were incubated for 30 minutes at room temperature, unbound SRBCs were washed away gently, and the number of cells (out of 300 total) attached with three or more SRBC's were counted using an inverted microscope.

F. Additional Biochemical and Molecular Biology Analyses.

1. Biochemical Analysis of Lung Collagen.

Collagen content and collagen crosslinking was analyzed in rats in the 9 month HNO_3-O_3 exposure. A weighed sample of lung tissue was hydrolyzed in 6 N HCl at 110°C for 24 hours to liberate amino acids from peptide bonds. The resultant hydrolysate was neutralized with NaOH, then oxidized with freshly prepared chloramine T to convert 4-hydroxyproline to pyrollidone carboxylic acid, which was next converted to a pyrrole derivative by heating at 100°C. A colorimetric analysis (Woessner, 1961) was then used to calibrate the assay and fresh standards run with all unknowns to ensure that such standard curves accurately reflect the color yield for each batch of reagent used. Data were expressed as lung lobe collagen content by multiplying (mg of hydroxyproline found in a sample) X (lung lobe weight/sample weight) X 6.67. The factor of 6.67 arises from the 4-hydroxyproline content of lung collagen, 15% by weight (Gallop and Paz, 1975).

Tissue was prepared for crosslink analysis as follows: approximately 40 mg wet weight of lung tissue was minced into fine pieces and washed overnight in 5 mM phosphate buffer containing 0.9% sodium chloride (NaCl), pH 7.4. The next day, the wash fluid was removed with a Pasteur pipette, and the tissue was incubated in 3 ml of 0.1 M sodium phosphate, pH 7.4, for four hours at room temperature (about 25°C). The tissues were then thoroughly rinsed with distilled water, hydrolyzed in 6 N hydrochloric acid (HCl) for eighteen hours at 110°C, rotary evaporated to remove HCl, and filtered using a Rainin microfiltration apparatus. Hydroxyproline content of the hydrolysates was determined by the colorimetric assay described

above. An aliquot of lung hydrolysate containing about 5 mg of hydroxyproline was chromatographed on a 0.4 X 10 cm C_{18} reverse-phase column (Accupak Short-One). The elution solvent was 25% n-propanol in 0.1 M phosphate buffer, pH 2.83, containing 0.3% sodium dodecylsulfate. The flow rate was 0.8 ml/minute. A Hitachi 2000 adjustable wavelength fluorometer was used (excitation=295 nm, emission=395 nm) with a 12 μl flow cell to detect OHP in the eluent by its intrinsic fluorescence. The system was calibrated with a purified standard prepared from bovine Achilles tendon (Reiser and Last, 1983).

2. Lung Heat Shock Proteins (HSP 70).

Stress-inducible HSP 70 was examined in lung tissues of rats following the 9 month HNO_3-O_3 exposure. Tissue from the right apical lobe were homogenized and prepared for slot-blot analysis. Protein concentration was determined by the Bradford protein assay (Bradford, 1976), and slot blotting was performed followed by protein immunoblotting with a primary antibody (a monoclonal immunoglobulin G affinity- purified antibody to HeLa cell stress-inducible HSP 70, StressGen Corporation) and a secondary antibody (goat anti-mouse alkaline phosphatase, BioRad Co.). Bound antibody was detected using the BioRad chemiluminescence detection system, Immuno-lite, with Kodak XAR X-ray film, and computerized optical scanning.

3. Lung Cytochrome P450 Monooxygenase System.

The cytochrome P450 monooxygenase system was analyzed in rats following the 9 month HNO_3-O_3 exposure. Cardiac lobes of the lung were collected and processed for preparation of microsomes. Because of the small quantities of lung tissue available for analysis, tissues of 5 animals in each exposure group were pooled yielding 2 determinations per exposure group. Benzphetamine N-demethylase activity was determined as follows: the reaction mixture contained 100 mM Tris-HCl (pH 7.4 at 37°C), 2.5 mM benzphetamine, microsomes (approximately 0.75 mg protein), 1 mM NADPH, 0.5 units of glucose phosphate dehydrogenase, 10mM glucose-6-phosphate, and H_2O in a total volume of 0.5 ml. Formaldehyde formed in the clarified supernatants was estimated by the method of Werringloer (1978). Total metabolism of ^{3}H-benzo[a]pyrene was studied as described by DePierre et al.

(1975) and protein was measured as described by Lowry et al., (1951).

G. Statistical Analysis.

This project included a one month dose-response exposure to three different concentrations of HNO_3 and a 9 month exposure to HNO_3 and O_3 alone and in combination. Data for each biological endpoint variable were tested for significant effects of exposure atmosphere using analysis of variance. Significant differences among atmosphere group means were tested with *a posteriori* Tukey multiple comparisons. A primary purpose of the one month dose-response exposure was to identify an appropriate concentration for testing in the 9 month exposure. In the 9 month exposure, the questions of interest included whether the HNO_3 and the O_3 exposure resulted in significant effects on the biological variables and whether the effects of the mixture, HNO_3 + $_3$, differed from the effects of the single components alone. Thus, 5 comparisons were appropriate: pure air *vs.* HNO_3, pure air *vs.* O_3, pure air *vs.* mixture, HNO_3 *vs.* mixture, and O_3 *vs.* mixture. Because the 9 month exposure was a 2 X 2 design for 2 treatment variables: HNO_3 (present or absent) and O_3 (present or absent), a less conservative approach for detecting pollutant effects was a 2 way ANOVA for effect of HNO_3, effect of O_3, and interaction of HNO_3 and O_3. Tabulated data in the results section show the results of 1 way ANOVA and *a posteriori* multiple comparisons. Where significant effects were found, the 2 way ANOVA was performed and described in the text. Comparisons were two-tailed, and the significance level was set at $p < 0.05$.

III. RESULTS.

A. Exposure Atmospheres.

The concentrations of HNO_3 and O_3 measured in the breathing zone of rats exposed the 1 month HNO_3 dose-response and in the 9 month exposures are shown in Table 2. The 9 month exposure data include cumulative measures for rats sacrificed at 1 , 3, and 9 months. The rats were exposed 4h/day, 3 days/week, and the means are based on daily averages of each component over the exposure days of each study. There was good agreement between the target concentrations and the actual exposure concentrations measured.

Table 2. HNO_3 and O_3 concentrations generated for exposure groups in the 1 month dose-response and 9 month exposures. Data are mean ± SD, n of daily averages.

	Target Concentration	Measured Concentration
1 Month Dose-Response Exposure		
	50 ($\mu g/m^3$) HNO_3	51.7 ± 13.8, 12
	150 ($\mu g/m^3$) HNO_3	170.6 ± 55.9, 12
	450 ($\mu g/m^3$) HNO_3	460.5 ± 88.5, 12
9 Month Exposure		
1 Month Sacrifice Groups		
	1. 50 ($\mu g/m^3$) HNO_3	54.4 ± 8.7, 12
	2. 0.15 ppm O_3	0.153 ± 0.002, 12
	3. 50 ($\mu g/m^3$) HNO_3 + 0.15 ppm O_3	52.2 ± 12.6, 12 0.152 ± 0.003, 12
3 Month Sacrifice Groups		
	1. 50 ($\mu g/m^3$) HNO_3	52.9 ± 8.9, 36
	2. 0.15 ppm O_3	0.151 ± 0.003, 36
	3. 50 ($\mu g/m^3$) HNO_3 + 0.15 ppm O_3	50.1 ± 10.7, 36 0.153 ± 0.003, 36
9 Month Sacrifice Groups		
	1. 50 ($\mu g/m^3$) HNO_3	51.1 ± 7.4, 120
	2. 0.15 ppm O_3	0.151 ± 0.003, 120
	3. 50 ($\mu g/m^3$) HNO_3 + 0.15 ppm O_3	49.9 ± 7.0, 120 0.152 ± 0.003, 120

B. Pulmonary Function.

Pulmonary function variables measured for this study included breathing pattern and minute ventilation, excised lung gas volume, quasi-static compliance, particle clearance from the respiratory tract, and permeability of the respiratory tract epithelium. Table 3 shows breathing pattern and ventilation which were measured periodically during the exposures. The only significant change observed was an increase in tidal volume and $\dot{V}_E$ during the last hour of the 1 month dose-response exposure to 50 $\mu g/m^3$ HNO_3. This change was small, and among the many breathing pattern comparisons examined in the 1 and 9 month exposures with no observed significant effect, does not represent a systematic occurrence of respiratory tract irritation affecting breathing pattern.

Table 4 shows the volume of gas trapped in freshly excised lungs of exposed rats. Trapped gas volume was increased at the higher HNO_3 concentrations of the 1 month dose-response exposure and at the 3 month sacrifice point of the 9 month exposure. Two way ANOVA of 3 month sacrifice data showed significant main effects of O_3 ($F = 4.5$, $p < 0.05$) and HNO_3 ($F = 14.5$, $p < 0.001$). A trend of increased trapped gas volume was also present at the 9 month sacrifice point, but none of the differences were statistically significant. Limited fresh lung tissue was available to measure wet/dry lung tissue mass ratios (Table 4), and these data do not indicate presence of any pulmonary edema which could cause increased trapped gas volume. Quasi-static compliance (Table 5) was measured in the 9 month exposure experiment. A significant difference was present between exposure to HNO_3 alone and O_3 alone at the 3 month sacrifice point (Table 5), and two-way ANOVA showed a significant main effect of O_3 ($F = 7.3$, $p < 0.01$). Measures of excised lung gas volume and quasi-static compliance are consistent with the presence of a small alteration of lung elasticity induced by the acid and oxidant exposures at 3 months; a response which was subsequently diminished at 9 months.

Table 6 shows respiratory tract clearance analyzed at the end of the 9 month exposure experiment. Early clearance, as measured by appearance of labeled particles in feces over the first 14 h period following deposition showed a trend toward delayed clearance in the exposure atmospheres. Chest counts measured at 48 h post deposition and following the early phase of clearance of the tracheobronchial region were all similar among exposure groups indicating that deep lung deposition was not affected by the history of pollutant exposure of the rat groups.

Table 3. Breathing pattern and minute ventilation on hour 4 of the first and third days of the first and last weeks of exposures. Exposures were 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. Rats were exposed 4 h/day, 3 days/week. Data are mean ± SE, n=8.

1 Month Dose-Response Exposure				
	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Breath Frequency (min^{-1})				
Day 1	145±6	144±7	147±6	148±5
Day 3	153±6	147±8	161±8	162±7
Day 22	128±5	125±3	134±4	132±6
Day 24	141±5	134±4	133±5	139±5
Tidal Volume (ml)				
Day 1	1.08±0.09	1.10±0.10	1.34±0.06	1.24±0.09
Day 3	1.10±0.09	1.18±0.11	1.13±0.07	1.13±0.06
Day 22	1.27±0.09	1.19±0.05	1.19±0.08	0.94±0.08[A]
Day 24	1.01±0.07	1.36±0.05[BC]	1.22±0.06	1.13±0.02[C]
Minute Ventilation (ml/min)				
Day 1	155±11	159±15	195±7	184±14
Day 3	168±14	173±17	184±11	166±8
Day 22	164±15	147±4	159±10	124±12
Day 24	142±10	184±10[B]	159±5	157±7

9 Month Exposure				
	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3 + O_3
Breath Frequency (min^{-1})				
Day 1	149±4	150±10	148±6	149±4
Day 3	153±4	145±6	156±6	161±4
Day 274	131±8	132±6	137±5	136±6
Day 276	124±4	134±5	141±7	136±5
Tidal Volume (ml)				
Day 1	0.99±0.07	0.92±0.06	0.91±0.04	1.02±0.07
Day 3	0.92±0.02	0.90±0.03	0.87±0.04	0.81±0.05
Day 274	1.30±0.06	1.38±0.07	1.32±0.08	1.26±0.05
Day 276	1.36±0.06	1.37±0.10	1.29±0.06	1.42±0.07
Minute Ventilation (ml/min)				
Day 1	144±9	133±9	130±6	150±8
Day 3	135±3	129±4	133±5	129±8
Day 274	163±7	180±14	176±9	166±8
Day 276	165±8	177±10	177±10	189±10

[A]Significantly different from control, $p < 0.05$.
[B]Significantly different from control, $p < 0.01$.
[C]Means significantly different, $p < 0.05$.

Table 4. Excised lung gas volume and wet to dry ratio of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 3 and 9 months. Data are mean ± SE, n.

1 Month Dose-Response Exposure to HNO_3

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Excised Lung Gas Volume (ml)	0.391±0.018,7	0.375±0.026,8[A]	0.418±0.028,8	0.476±0.027,6[A]
Lung Wet to Dry Ratio	4.60±0.04,7	4.54±0.05,8	4.55±0.03,8	4.56±0.02,6

9 Month Exposure

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Excised Lung Gas Volume (ml)				
3 Month	0.377±0.032,8	0.467±0.015,10[B]	0.432±0.014,9	0.502±0.023,8[C]
9 Month	0.537±0.018,19	0.639±0.031,20	0.606±0.037,17	0.567±0.022,18
Lung Wet to Dry Ratio				
9 Month	4.79±0.08,8	4.71±0.05,10	4.68±0.07,9	4.70±0.03,10

[A]Means significantly different, $p < 0.05$.
[B]Significantly different from control, $p < 0.05$.
[C]Significantly different from control, $p < 0.01$

Table 5. Quasi-static compliance (ml/cm H_2O) measured during the second inflation cycle of lungs of rats exposed in the 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 3 and 9 months. Data are mean ± SE, n.

9 Month Exposure				
	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Analysis Time				
3 Month	2.19±0.08,11	2.27±0.08,11[A]	1.86±0.14,9[A]	2.07±0.09,11
9 Month	2.12±0.05,12	2.20±0.09,12	2.02±0.11,12	2.28±0.08,13

[A] Means significantly different ($p < 0.05$).

Table 6. Respiratory tract clearance of rats exposed 9 months to HNO_3 and O_3 alone and in combination. The measure of early clearance is the fraction of ^{51}Cr activity present in cumulative excreta at 14 h. Late clearance (A_{30}) is activity in the left lung, trachea, and larynx measured 30 days post-deposition and normalized to activity present (initial chest count) 48 h post-deposition. Group mean initial activity at 48 h post-deposition did not indicate any exposure related differences in deep lung deposition. Data are mean ± SE, n.

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Early Clearance				
Fecal count 14 h	0.19±0.03,30	0.16±0.03,30	0.12±0.02,30	0.11±0.03,30
Late Clearance				
Chest count 48 h	0.29±0.01,30	0.29±0.01,30	0.31±0.01,30	0.29±0.01,30
A_{30}	2.39±0.08,28	2.30±0.10,27	2.19±0.08,28	2.32±0.06,29

Activity present at 30 days and normalized to individual differences in deep lung deposition is a measure of late clearance. Late clearance showed a trend of delay in O_3 alone.

Permeability of the respiratory tract epithelium was significantly elevated in the nose in the exposure to HNO_3 alone and it was also elevated in the exposure to O_3 + HNO_3 (Table 7). Two-way ANOVA showed a significant main effect of HNO_3 ($F = 7.3$, $p < 0.01$). Measures of protein in bronchoalveolar lavage fluid were not different among exposure groups indicating no detectable changes in bronchoalveolar permeability in response to the exposures.

Table 7. Epithelial Permeability of rats exposed 9 months to HNO_3 and O_3 alone and in combination. Nasal permeability is measured as transfer of nasally instilled DTPA (^{99}Tc labeled diethylenetriaminepentaacetate, mol wt. 492d.) to the bloodstream at 8 min. Data are percent of inoculum transferred to the blood. Bronchoalveolar permeability is measured as total protein and albumen (mg/ml) in bronchoalveolar lavage fluid. Data are mean ± SE,n.

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Nasal Permeability (%)	0.137±0.058,9	1.724±0.656,9[A]	0.330±0.155,10	0.900±0.464,7
Bronchoalveolar Permeability				
Total Protein (mg/ml)	0.134±0.006,9	0.138±0.003,9	0.134±0.007,9	0.140±0.006,9
Albumen (mg/ml)	0.009±0.001,9	0.007±0.001,9	0.008±0.001,9	0.008±0.001,9

[A]Significantly different from control, $p < 0.05$.

C. Morphometry and Histopathology.

Morphometric analysis of lung tissues showed significant alterations in lung structure of rats in the 1 month dose-response exposure to HNO_3, but no changes in animals in the 9 month exposure to HNO_3 and O_3 alone and in combination. For the one month exposure (Table 8) showed a progressive dose-related change in alveolar mean chord length and septal wall thickness in concert with a decline in fixed lung volume. Animals in the 9 month exposure showed no such effects at any of the sacrifice analysis points including the 1 month exposure to 50 $\mu g/m^3$ HNO_3 which replicated an exposure group in the 1 month dose-response study. Apparently this exposure concentration was sufficiently close to the threshold region for detecting a response that batch variation in sensitivity became important in detecting a significant response. There were also no significant differences in volume fractions of different tissues of the lung in either the 1 month dose-response or 9 month HNO_3-O_3 exposure (Table 9 A and B).

Morphometric analysis of lung ventilatory units following the 9 month exposure was examined in greater detail by Dr. Kent Pinkerton at UC Davis. This analysis centered on the bronchiole-alveolar duct junction and adjacent pulmonary acinus which is a focus of oxidant injury to the lung. Morphometric analysis of terminal bronchioles and associated pairs of alveolar ducts from single pollutant exposures (HNO_3 alone and O_3 alone) embedded in glycolmethacrylate demonstrated subtle trends of change due to pollutant exposure. There was an increase in alveolar tissue volume normalized to the surface area of the alveoli within the first 200 μm region beyond the bronchiole-alveolar duct junction (BADJ) in animals exposed to O_3 alone (Fig 1A) and a trend toward elevation in the region 500 to 800 μm beyond the BADJ. This trend of change due to O_3 was studied at still greater resolution in tissues embedded in araldite and examined in thinner section (0.5 μm) for the O_3 exposed animals (O_3 and O_3 + HNO_3 groups). Analysis of epithelial and interstitial volume density of alveolar parenchyma did not demonstrate significant differences among these exposure groups and purified air control animals (Fig 1 B and C). There was a trend of increased epithelial volume per surface area in lungs of animals exposed to O_3 and O_3 + HNO_3 as far down as 400 μm into the alveolar duct, however the differences were not statistically significant. These results suggest minimal, but occasionally significant changes occur with exposure of Fisher 344 rats to 0.15 ppm O_3 and 0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3 for 9 months.

Table 8. Morphometric measures of lung tissues of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1, 3, and 9 months. Data are mean ± SE.

1 Month Dose-Response Exposure to HNO_3

	Purified Air n=10	HNO_3 Concentration 50 $\mu g/m^3$ n=9	150 $\mu g/m^3$ n=9	450 $\mu g/m^3$ n=10
Fixed Lung Volume (ml)	10.6±0.3	10.0±0.5	10.5±0.2	9.3±0.1[A]
Alveolar Mean Chord Length (μm)	48.9±0.7	42.7±1.2[B]	40.7±1.4[B]	40.2±0.9[B]
Septal Wall Thickness (μm)	4.56±0.11	5.53±0.17[B]	5.56±0.13[B]	6.56±0.23[B]

9 Month Exposure	Purified Air n=10	50 $\mu g/m^3$ HNO_3 n=10	0.15 ppm O_3 n=10	HNO_3+O_3 n=10
Fixed Lung Volume (ml)				
1 Month	11.9±0.2	12.2±0.3	11.7±0.4	11.8±0.2
3 Month	11.2±0.3	11.4±0.3	11.3±0.3	11.5±0.2
9 Month	13.3±0.2	13.9±0.3	13.7±0.2	13.9±0.2
Alveolar Mean Chord Length (μm)				
1 Month	43.8±1.5	45.7±1.3	41.5±0.9	44.4±1.0
3 Month	47.3±1.2	46.7±4.4	51.0±2.2	47.6±1.4
9 Month	48.4±1.2	47.7±1.6	48.4±1.4	49.5±0.9
Septal Wall Thickness (μm)				
1 Month	6.52±0.12	6.49±0.16	6.39±0.19	6.02±0.14
3 Month	5.73±0.25	6.00±0.21	6.10±0.19	5.71±0.31
9 Month	5.97±0.31	5.80±0.21	5.84±0.15	5.73±0.11

[A]Significantly different from control $p < 0.05$.
[B]Significantly different from control $p < 0.01$.

Table 9A. Volume fractions of lung tissues of rats exposed in 1 month HNO_3 dose-response exposure. Data are mean ± SE.

1 Month Dose-Response Exposure to HNO_3				
	Purified Air n=10	HNO_3 Concentration 50 $\mu g/m^3$ n=9	150 $\mu g/m^3$ n=9	450 $\mu g/m^3$ n=10
Parenchyma	0.816±0.013	0.812±0.008	0.801±0.024	0.807±0.013
Alveoli	0.430±0.028	0.432±0.015	0.390±0.021	0.405±0.009
Alveolar Ducts	0.338±0.016	0.323±0.014	0.334±0.023	0.350±0.014
Bronchioles	0.048±0.005	0.058±0.012	0.078±0.014	0.052±0.007
Lobar and Secondary Bronchi	0.140±0.014	0.147±0.011	0.147±0.025	0.145±0.009
Vascular Tissue	0.044±0.009	0.038±0.006	0.044±0.007	0.041±0.011
BALT	0.0	0.003±0.002	0.007±0.003	0.007±0.004

Table 9B. Volume fractions of lung tissues of rats in 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1, 3, and 9 months. Data are mean ± SE.

9 Month Exposure	Purified Air n=10	50 $\mu g/m^3$ HNO_3 n=10	0.15 ppm O_3 n=10	HNO_3+O_3 n=10
Parenchyma				
1 Month	0.845±0.028	0.865±0.014	0.867±0.016	0.872±0.019
3 Month	0.866±0.027	0.861±0.018	0.864±0.016	0.865±0.025
9 Month	0.911±0.019	0.857±0.019	0.865±0.017	0.923±0.020
Alveoli				
1 Month	0.509±0.024	0.502±0.012	0.490±0.017	0.519±0.027
3 Month	0.509±0.025	0.475±0.024	0.516±0.023	0.500±0.021
9 Month	0.578±0.020	0.554±0.026	0.537±0.019	0.563±0.023
Alveolar Ducts				
1 Month	0.288±0.021	0.323±0.009	0.295±0.012	0.300±0.020
3 Month	0.279±0.017	0.288±0.011	0.288±0.015	0.287±0.015
9 Month	0.271±0.024	0.238±0.016	0.258±0.014	0.284±0.026
Bronchioles				
1 Month	0.048±0.015	0.041±0.012	0.082±0.013	0.053±0.015
3 Month	0.078±0.018	0.098±0.015	0.060±0.010	0.078±0.017
9 Month	0.063±0.015	0.065±0.014	0.069±0.016	0.076±0.021
Lobar and Secondary Bronchi				
1 Month	0.073±0.021	0.066±0.020	0.069±0.014	0.056±0.013
3 Month	0.099±0.022	0.089±0.014	0.077±0.020	0.095±0.021
9 Month	0.061±0.018	0.078±0.021	0.073±0.019	0.046±0.015
Vascular Tissue				
1 Month	0.073±0.012	0.064±0.013	0.064±0.011	0.065±0.011
3 Month	0.032±0.008	0.049±0.014	0.056±0.011	0.033±0.007
9 Month	0.028±0.011	0.065±0.017	0.060±0.014	0.032±0.011
BALT				
1 Month	0.009±0.008	0.005±0.003	0.0	0.007±0.004
3 Month	0.002±0.002	0.0	0.004±0.004	0.008±0.005
9 Month	0.0	0.0	0.002±0.007	0.0

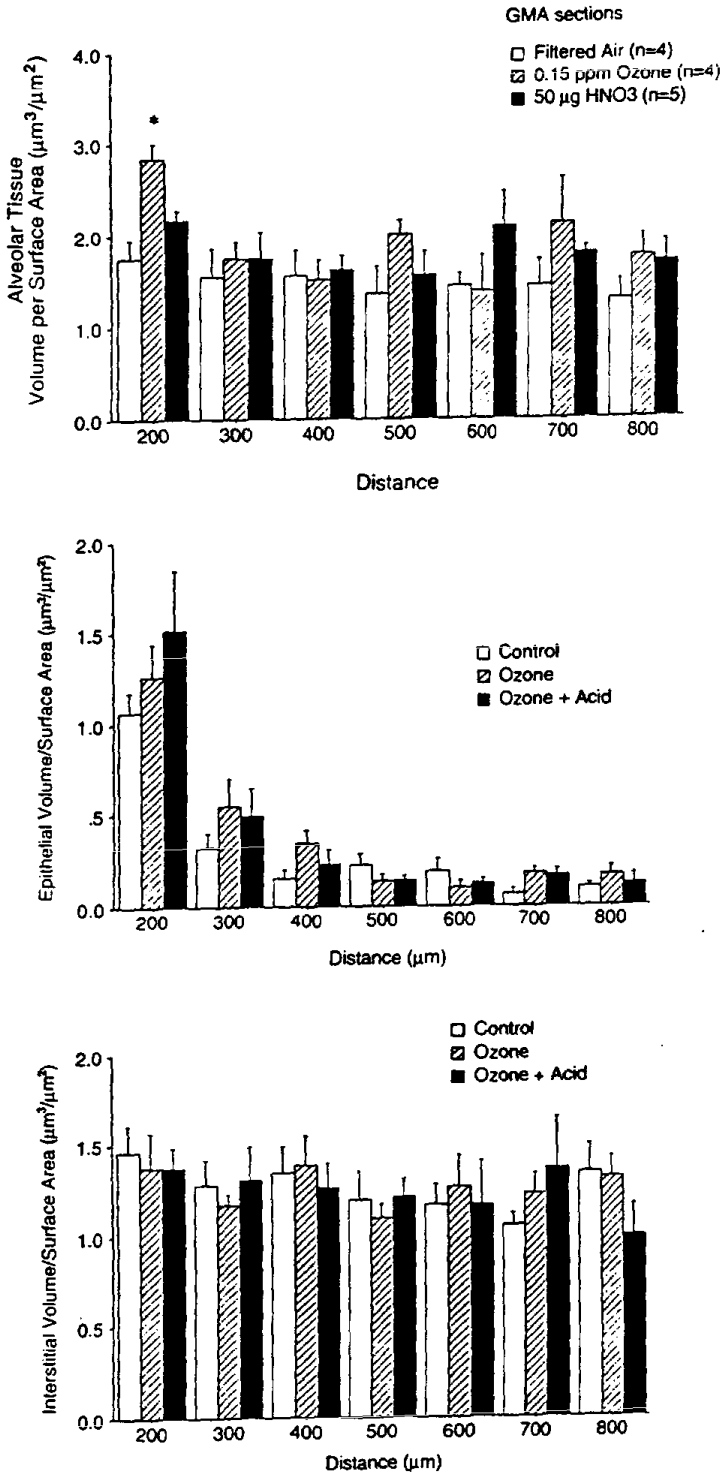

Figure 1 A. Alveolar tissue density of animals exposed to purified air, 0.15 ppm O_3, or 50 $\mu g/m^3$ HNO_3 for 9 months. Data are expressed as a function of distance down the alveolar duct path.

1 B. Epithelial volume per surface area within the ventilatory unit of lungs of animals exposed to purified air, 0.15 ppm O_3, or 0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3.

1 C. Interstitial volume density in the lungs of animals exposed to purified air, 0.15 ppm O_3, or 0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3.

Tables 10 A and B show cellular composition of the nasal epithelium of rats in the 1 month HNO_3 dose-response and 9 month HNO_3-O_3 exposures. There was a significant difference in representation of respiratory epithelium (Type R) basal cells in the higher concentrations of HNO_3 in the 1 month exposure, and an increase in secretory cells in the exposure to HNO_3 alone at the 9 month analysis point when compared to the combination HNO_3 + O_3 (Table 10B). However, these shifts were modest and did not involve large changes from the purified air control animals or changes that showed reciprocal significant changes in the other cell types of the same epithelial samples. In the R-1 or transitional epithelium tissue, there was a significant shift toward more ciliated and fewer secretory cells in ozone exposures (O_3 and HNO_3 + O_3) for the 1 month analysis period but this pattern was not present at later analysis points at 3 months and 9 months (Table 10B). Two-way ANOVA showed a significant main effect of O_3 for ciliated cells ($F = 14.9$, $p < 0.0005$) and for secretory cells ($F = 15.6$, $p < 0.0004$).

Table 10A. Cellular composition of the nasal epithelium of rats exposed in 1 month HNO_3 dose-response exposure. Data are mean ± SE, n.

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Type R epithelium cells				
Ciliated	0.328±0.009,10	0.354±0.008,10	0.350±0.005,9	0.346±0.009,10
Secretory	0.405±0.010,10	0.386±0.010,10	0.397±0.006,9	0.372±0.007,10
Basal	0.267±0.007,10	0.260±0.007,10	0.254±0.004,9[A]	0.282±0.005,10[A]
Type R-1 epithelium cells				
Ciliated	0.349±0.012,10	0.388±0.023,10	0.334±0.023,9	0.355±0.027,10
Secretory	0.419±0.012,10	0.407±0.016.10	0.436±0.018,9	0.402±0.021,10
Basal	0.232±0.011,10	0.205±0.010,10	0.230±0.013,9	0.243±0.014,10

[A]Means significantly different, $p < 0.05$.

Table 10B. Cellular composition of the nasal epithelium of rats exposed in 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1, 3, and 9 months. Data are mean ± SE, n.

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	$HNO_3 + O_3$
Type R epithelium cells				
1 Month				
Ciliated	0.336±0.009,9	0.334±0.009,10	0.345±0.010,9	0.308±0.011,9
Secretory	0.366±0.006,9	0.380±0.012,10	0.352±0.007,9	0.383±0.010,9
Basal	0.298±0.007,9	0.276±0.012,10	0.303±0.008,9	0.309±0.009,9
3 Month				
Ciliated	0.364±0.016,7	0.358±0.008,7	0.352±0.018,8	0.336±0.012,9
Secretory	0.366±0.009,7	0.376±0.009,7	0.366±0.021,8	0.387±0.016,9
Basal	0.270±0.014,7	0.267±0.011,7	0.282±0.009,8	0.277±0.012,9
9 Month				
Ciliated	0.372±0.009,9	0.363±0.007,10	0.373±0.008,9	0.379±0.011,10
Secretory	0.346±0.007,9	0.380±0.009,10[A]	0.371±0.012,9	0.343±0.009,10[A]
Basal	0.283±0.008,9	0.257±0.010,10	0.256±0.010,9	0.278±0.011,10
Type R-1 epithelium cells				
1 Month				
Ciliated	0.395±0.027,9	0.443±0.015,9	0.511±0.019,9[B]	0.481±0.017,9[C]
Secretory	0.357±0.020,9	0.309±0.015,9	0.245±0.018,9[B]	0.288±0.012,9[C]
Basal	0.249±0.011,9	0.248±0.009,9	0.245±0.013,9	0.231±0.009,9
3 Month				
Ciliated	0.461±0.013,7	0.461±0.014,7	0.453±0.012,9	0.475±0.015,9
Secretory	0.321±0.008,7	0.302±0.013,7	0.316±0.016,9	0.295±0.019,9
Basal	0.218±0.008,7	0.237±0.012,7	0.232±0.009,9	0.231±0.010,9
9 Month				
Ciliated	0.455±0.012,9	0.430±0.015,10	0.438±0.009,9	0.422±0.015,8
Secretory	0.246±0.013,9	0.285±0.015,10	0.271±0.011,9	0.299±0.018,8
Basal	0.299±0.006,9	0.285±0.013,10	0.291±0.010,9	0.279±0.011,8

[A]Means significantly different, $p < 0.05$.
[B]Significantly different from control, $p < 0.01$.
[C]Significantly different from control, $p < 0.05$.

Mast cell numbers in the main bronchi of lung lobes did not show a significant response to exposure to HNO_3 or O_3 in either the 1 month dose-response or the 9 month exposure at any of the analysis points (Table 11).

Table 11. Mast cells in the lobar bronchus of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1, 3, and 9 months. Data are mast cells as a fraction of epithelial cells (mean ± SE, n).

1 Month Dose-Response Exposure to HNO_3

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Mast Cells	0.023±0.002,10	0.025±0.002,9	0.028±0.002,10	0.026±0.002,10

9 Month Exposure

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Mast Cells				
1 Month	0.040±0.005,10	0.029±0.004,10	0.035±0.006,10	0.032±0.006,10
3 Month	0.025±0.002,10	0.036±0.005,10	0.037±0.008,8	0.036±0.005,10
9 Month	0.030±0.004,10	0.040±0.008,10	0.036±0.007,10	0.040±0.006,10

Density of glycoprotein stain in the respiratory tract epithelia was used as a measure of secretory activity of this tissue. No significant changes in this index were observed in the 1 month HNO_3 dose-response exposure or in the 9 month HNO_3-O_3 exposure with the exception of nasal R-1 respiratory epithelium analyzed at 9 months. Here, elevated glycoprotein density in the single exposures (HNO_3 alone and O_3 alone) was significantly different from the reduced glycoprotein density observed in the exposure to HNO_3 + O_3. Two-way ANOVA indicated a significant main effect of HNO_3 ($F = 4.7$, $p < 0.04$), and a highly significant interaction between HNO_3 exposure and O_3 exposure ($F = 12.7$, $p < 0.001$). The results suggest that

Table 12. Respiratory tract epithelial glycoprotein of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1, 3, and 9 months. Data are relative density of alcian blue-periodic acid schiff stain per 0.25 mm of epithelium (mean ± SE, n).

1 Month Dose-Response Exposure to HNO_3				
	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Nasal Respiratory Epithelium				
Type R	2.33±0.27,10	2.37±0.25,10	2.32±0.24,9	2.04±0.10,10
Type R-1	0.160±0.019,10	0.156±0.013,10	0.134±0.022,8	0.141±0.008,10
Trachea	0.277±0.038,9	0.397±0.059,9	0.339±0.035,8	0.242±0.055,10

9 Month Exposure				
	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Nasal Respiratory Epithelium				
Type R				
1 month	1.13±0.15,10	1.07±0.15,10	1.17±0.07,10	1.37±0.11,9
3 month	1.07±0.07,9	0.96±0.13,7	1.11±0.13,10	1.24±0.91,9
9 month	1.17±0.11,6	1.27±0.16,10	0.93±.013,10	0.97±0.09,7
Type R-1				
1 month	0.340±0.036,10	0.377±0.038,9	0.291±0.042,10	0.373±0.028,9
3 month	0.267±0.022,9	0.204±0.020,7	0.209±0.029,10	0.187±0.016,10
9 month	0.319±0.033,6	0.356±0.016,10[A]	0.382±0.026,10[B]	0.231±0.031,7[AB]
Trachea				
1 month	0.152±0.027,9	0.140±0.032,9	0.143±0.042,10	0.127±0.022,9
3 month	0.237±0.042,10	0.307±0.051,9	0.300±0.057,9	0.198±0.053,8
9 month	0.332±0.063,7	0.379±0.062,9	0.389±0.079,9	0.348±0.049,9

[A]Means significantly different, $p < 0.05$.
[B]Means significantly different, $p < 0.01$.

exposure to the single pollutants resulted in accumulation of secretory glycoprotein products, while exposure to the combination resulted in depletion of glycoproteins. While no statistically significant effects of the 9 month exposure on tracheal glycoprotein were observed, there was a similar trend at 3 and 9 month analysis points for single HNO_3 or O_3 exposures toward increased density, while the combination exposure reduced density or returned it to near control levels. Such a trend was also present in the 1 month dose-response exposure in which tracheal glycoprotein density was elevated at intermediate HNO_3 concentrations, but depressed at the highest concentration (Table 12). Serous cell counts in the trachea showed no significant effects in the 9 month HNO_3-O_3 exposure (Table 13). Trends in epithelial glycoprotein stain density appear to have resulted from changes in cell secretory activity rather than from changes in serous cell proliferation.

Table 13. Serous cell density in the tracheal epithelium of rats exposed in the 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 3 and 9 months and counts of cells stained with Periodic Acid Schiff (PAS) reagent and Alcian Blue (AB). Data are numbers of serous cells per unit linear epithelial field (mean $\pm$ SE, n).

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Serous cells				
PAS Stain				
3 month	3.81±1.46,7	3.10±0.81,10	2.97±0.58,10	3.94±1.28,10
9 month	2.74±0.87,9	1.73±0.6,10	3.42±1.21,10	1.38±0.43,9
AB Stain				
3 month	0.58±0.14,7	0.76±0.11,10	0.69±0.13,10	0.75±0.11,10
9 month	0.42±0.12,9	0.60±0.22,10	0.17±0.05,10	0.37±0.10,9

Cell turnover in respiratory tract epithelia as measured by incorporation of tritium labeled thymidine into DNA of replicating cells is shown in Table 14. In the 1 month HNO_3 dose-response exposure, there were trends for increased cell labeling rates in the upper respiratory tract (nose and trachea) and in the lung parenchyma, and the increase was significant in nasal R epithelium exposed to 450 $\mu g/m^3$. In the 9 month HNO_3-O_3 exposure, analyses at 1 and 3 months showed no indications of exposure related effects on numbers of labeled cells. In the 9 month analysis point, the cell turnover rate in the older rats was apparently so low, that the labeling index could not be resolved.

Pulmonary macrophages stained for acid phosphatase activity showed a shift toward more dense acid phosphatase (higher stain intensity class numbers) in intermediate concentrations of the 1 month HNO_3 dose-response exposure (Table 15). This pattern of change was not present in the 1 and 3 month analysis points of the 9 month HNO_3-O_3 exposure. However, by 9 months of exposure, there were significant shifts in stain class representation toward increased numbers in class III for exposure to HNO_3 + O_3 exposure. Two way ANOVA showed a significant interaction between HNO_3 and O_3 exposure for class II stain density ($F = 6.3$, $p < 0.02$) and a significant main effect of O_3 on class III stain density ($F = 10.2$, $p < 0.003$).

Collagen deposition in the lung parenchyma was examined by image analysis of sirius red stain density in histological sections and by biochemical analysis of collagen in lung tissue samples. The results of histochemical stain analysis are shown in Table 16, and the biochemical analysis results are described below under section III.E. The histochemical stain analysis showed no significant effects of the 9 month HNO_3-O_3 exposures analyzed at 9 months.

Table 14. Epithelial cell turnover in the respiratory tract of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1 and 3 months; animals sacrificed at 9 months had a cell turnover rate too low to be measured by this method. Data are percent of cells labeled with ^3H-Thymidine (mean ± SE, n).

1 Month Dose-Response Exposure to HNO_3				
	Purified	HNO_3 Concentration		
Nasal Respiratory	Air	50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Epithelium				
Type R	0.347±0.043,10	0.503±0.082,10	0.653±0.169,9	0.767±0.131,10[A]
Type R-1	0.335±0.113,10	0.229±0.085,10	0.491±0.122,10	0.318±0.080,10
Trachea	0.650±0.172,9	1.014±0.381,9	0.797±0.244,8	1.171±0.364,9
Lobar				
Bronchus	0.102±0.073,10	0.227±0.093,9	0.090±0.061,9	0.033±0.033,9
Terminal				
Bronchiole	0.093±0.041,10	0.160±0.084,9	0.175±0.110,9	0.039±0.026,9
Lung				
Parenchyma	0.441±0.109,10	0.775±0.149,9	0.555±0.147,9	0.716±0.181,9

9 Month Exposure				
	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Nasal Respiratory				
Epithelium				
Type R				
1 Month	1.081±0.130,9	1.011±0.477,8	0.896±0.215,9	1.078±0.400,9
3 Month	0.992±0.234,9	1.000±0.658,6	0.771±0.196,9	1.179±0.757,8
Type R-1				
1 Month	0.802±0.302,9	1.794±0.767,8	1.337±0.249,9	0.937±0.435,10
3 Month	0.282±0.091,9	0.795±0.384,6	0.537±0.276,10	0.491±0.164,8
Trachea				
1 Month	2.363±0.288,10	2.487±0.318,10	1.510±0.135,10	2.429±0.277,10
3 Month	2.242±0.238,10	2.910±0.692,10	3.124±0.411,10	2.107±0.288,10
Lobar Bronchus				
1 Month	0.756±0.164,10	0.902±0.190,9	0.931±0.189,10	0.774±0.177,9
3 Month	0.794±0.190,10	0.623±0.184,10	0.566±0.108,10	0.426±0.146,10
Terminal Bronchiole				
1 Month	0.423±0.119,10	0.507±0.195,9	0.466±0.253,10	0.349±0.123,9
3 Month	0.292±0.113,10	0.506±0.299,10	0.248±0.089,10	0.319±0.107,10
Lung Parenchyma				
1 Month	1.566±0.152,10	2.170±0.429,9	1.694±0.418,10	1.306±0.210,9
3 Month	1.587±0.268,10	1.182±0.207,10	1.610±0.313,10	1.105±0.216,10

[A]Significantly different from control, $p < 0.05$.

Table 15. Acid phosphatase activity in pulmonary macrophages of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1, 3, and 9 months. Activity is quantified by intensity of acid phosphatase staining, and staining intensity in a macrophage is classified into 3 levels. Data are mean ± SE.

1 Month Dose-Response Exposure to HNO_3

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Stain Intensity Class				
I	0.769±0.049,9	0.581±0.062,9[AB]	0.637±0.046,9	0.815±0.031,10[B]
II	0.216±0.047,9	0.339±0.044,9[C]	0.298±0.036,9	0.170±0.033,10[C]
III	0.015±0.010,9	0.080±0.028,9	0.066±0.027,9	0.015±0.010,10

9 Month Exposure

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Stain Intensity Class				
1 Month				
I	0.648±0.051,10	0.572±0.073,10	0.717±0.080,10	0.606±0.052,10
II	0.329±0.043,10	0.403±0.068,10	0.266±0.073,10	0.367±0.043,10
III	0.023±0.016,10	0.026±0.018,10	0.018±0.013,10	0.027±0.020,10
3 Month				
I	0.570±0.049,10	0.490±0.060,10	0.444±0.046,10	0.566±0.066,10
II	0.395±0.041,10	0.422±0.046,10	0.463±0.044,10	0.382±0.050,10
III	0.035±0.025,10	0.088±0.032,10	0.093±0.032,10	0.053±0.024,10
9 Month				
I	0.296±0.047,10	0.269±0.040,10	0.233±0.034,10	0.232±0.056,10
II	0.536±0.047,10	0.614±0.035,10[D]	0.556±0.027,10	0.458±0.025,10[D]
III	0.167±0.051,10	0.117±0.034,10[E]	0.211±0.033,10	0.310±0.047,10[E]

[A]Significantly different from control, $p < 0.05$.
[BE]Means significantly different, $p < 0.01$.
[CD]Means significantly different, $p < 0.05$.

Table 16. Collagen deposition in the lungs of rats exposed in 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 1, 3, and 9 months. Collagen is measured as density of sirius red stain in parenchymal portions of sections of lung. Data are mean ± SE, n.

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	$HNO_3 + O_3$
Stain Density				
1 Month	0.028±0.003,10	0.030±0.002,10	0.026±0.002,10	0.030±0.003,10
3 Month	0.038±0.003,10	0.037±0.002,10	0.039±0.003,10	0.037±0.003,10
9 Month	0.066±0.004,10	0.071±0.009,10	0.072±0.008,10	0.071±0.005,10

D. Bronchoalveolar Lavage Fluid Analyses and Pulmonary Macrophage Function.

Bronchoalveolar lavage fluid was analyzed for a variety of compounds reflecting respiratory tract injury or macrophage function including: total protein and albumen (Table 7); lactate dehydrogenase, ß-glucuronidase, and fibronectin (Table 17); glycoprotein (Table 18); and elastase inhibitory capacity (Table 19). No significant changes in BAL protein concentrations were observed (Table 7 and Table 17), indicating that, under the exposure conditions of the 1 month HNO_3 dose-response and the 9 month HNO_3-O_3 exposures, lung epithelial permeability was not increased. Consistent with this result, the exposures did not cause changes in BAL concentrations of LDH, a biochemical marker of tissue injury. ß-glucuronidase concentrations increased following 9 month exposures to the ozone-containing atmospheres (O_3 main effect, $F = 5.7$, $p < 0.02$) (Table 17). Fibronectin concentrations were analyzed in the 9 month HNO_3-O_3 exposures and did not show significant changes.

In addition to analysis for markers of pulmonary injury, BAL fluid was analyzed for glycoprotein to examine the hypothesis that acid and oxidant exposures increase secretory activity of tracheobronchial epithelia. In the 1 month HNO_3 dose-response exposure (Table 18) there was a strong dose-related increase in BAL glycoprotein. However, at the end of the 9 month HNO_3-O_3 exposure, there were no exposure-related differences in BAL glycoprotein.

Elastase inhibitory capacity of BAL was analyzed to test the hypothesis that the balance of enzymes influencing lung connective tissue was altered by the 9 month HNO_3-O_3 exposures (Table 19). There were no significant differences due to the HNO_3 or O_3 exposures.

Differential cell counts in samples of BAL fluid bronchoalveolar lavage are shown in Table 20. The majority (93 to 98%) of the cells in the BAL were macrophages or monocytes. Lymphocytes and polymorphonuclear leukocytes (PMN) were typically present and represented about 5% and 1% of the remaining cells respectively. Other cell types, including epithelial cells or giant cells, and were present infrequently and sporadically (less than 1%). There were no significant differences in cell types among exposure groups in the 1 month HNO_3 dose-response or 9 month HNO_3-O_3 exposures.

Pulmonary macrophages were obtained by bronchoalveolar lavage immediately post-exposure. The functional properties of macrophages examined included viability (measured by

Table 17. Bronchopulmonary lavage fluid biochemical assays of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 3 and 9 months. Data are mean ± SE, n.

1 Month Dose-Response Exposure to HNO_3

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Protein Content (mg/ml)	0.175±0.015,12	0.166±0.012,12	0.190±0.012,12	0.174±0.010,12
Lactate Dehydrogenase (activity units)	6.00±0.24,12	5.75±0.30,12	6.37±0.35,12	6.01±0.37,12
ß-Glucuronidase (activity units)	3.73±0.41,12	3.88±0.33,12	3.03±0.43,12	4.12±0.54,12

9 Month Exposure

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Protein Content	(see Table 7)			
Lactate Dehydrogenase (activity units)				
3 Month	3.48±0.18,12	3.40±0.07,12	3.37±0.15,12	3.49±0.17,11
9 Month	3.34±0.20,12	3.55±0.15,12	3.66±0.21,12	3.84±0.40,12
ß-Glucuronidase (activity units)				
3 Month	2.10±0.29,11	1.78±0.24,12	2.28±0.31,12	2.38±0.35,11
9 Month	1.50±0.18,12	2.02±0.28,10	2.12±0.26,11	2.70±0.35,12[A]
Fibronectin (activity units)				
3 Month	3.56±0.61,12	2.57±0.45,11	3.29±0.53,11	2.04±0.40,9
9 Month	1.36±0.38,9	2.96±0.92,8	2.08±0.56,10	1.60±0.36,11

[A]Significantly different from control, $p < 0.05$.

Table 18. Glycoprotein in bronchopulmonary lavage fluid from rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. Mucin was analyzed by ELISA and data are mean ± SE, n absorbance units.

1 Month Dose-Response Exposure to HNO_3

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
BAL Glycoprotein (absorbance units)	0.064±0.003,10	0.158±0.007,10[ABC]	0.211±0.010,10[ABD]	0.278±0.019,10[ACD]

9 Month Exposure

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
BAL Glycoprotein (absorbance units)	0.157±0.017,10	0.146±0.007,10	0.144±0.012,10	0.155±0.017,10

[A]Significantly different from control, $p < 0.01$.
[B]Means significantly different, $p < 0.05$.
[CD]Means significantly different, $p < 0.01$.

Table 19. Elastase inhibitory capacity of BAL fluid from rats in the 9 month exposure to HNO_3 and O_3 alone and in combination. Animals were analyzed following 3 and 9 months of exposure. Data are percent inhibition of pancreatic elastase (mean ± SE, n).

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Elastase Inhibitory Capacity				
3 month	70.1±6.1,10	77.1±5.3,10	72.6±4.2,10	74.4±5.2,10
9 month	60.4±5.8,10	56.4±5.1,10	55.0±4.3,10	54.6±3.6,10

Table 20. Cellular composition (%) of bronchopulmonary lavage fluid of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 3 and 9 months. Data are mean ± SE, n.

1 Month Dose-Response Exposure to HNO_3

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Monocytes	97.1±0.7,12	96.3±0.7,12	96.4±0.6,12	96.6±0.8,12
Lymphocytes	1.8±0.7,12	2.3±0.5,12	2.5±0.5,12	1.9±0.7,12
Polymorphonuclear Monocytes	0.5±0.1,12	0.7±0.2,12	0.6±0.2,12	0.6±0.1,12
Other	0.5±0.1,12	0.6±0.2,12	0.5±0.5,12	0.9±0.2,12

9 Month Exposure

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Monocytes				
3 Month	94.7±1.1,12	93.5±1.7,12	94.5±1.5,12	95.4±0.6,12
9 Month	97.7±0.5,10	97.7±0.4,10	96.6±0.7,10	97.7±0.5,8
Lymphocytes				
3 Month	4.6±3.5,12	6.0±1.7,12	4.7±1.4,12	3.9±2.0,12
9 Month	2.2±0.5,10	2.2±0.4,10	2.8±0.8,10	2.0±3.7,8
Polymorphonuclear Monocytes				
3 Month	0.8±0.2,12	0.5±0.2,12	0.8±0.2,12	0.7±0.2,12
9 Month	0.1±0.1,10	0.0,10	0.5±0.3,10	0.2±0.2,8
Other				
3 Month	0.0,12	0.0,12	0.0,12	0.0,12
9 Month	0.0,10	0.0,10	0.1±0.1,10	0.0,10

Trypan Blue exclusion), the capacity for antibody-directed binding of antigenic material at surface Fc receptors (measured by a rosette assay), and the ability to phagocytize polystyrene latex particles (2 μm diameter). Viability was not significantly reduced by any of the exposures. FcR binding was significantly reduced by the high concentration HNO_3 exposure in the 1 month HNO_3 dose-response study. In the 9 month HNO_3-O_3 exposures, there were no significant changes in FcR binding. Phagocytic activity indexed by consumption of 2 or more particles was not significantly affected by the 1 month HNO_3 dose-response exposure, however there were significant reductions in numbers of macrophages ingesting large numbers ($>$ 7) particles. In the 9 month HNO_3-O_3 exposures, there was a trend toward decreased phagocytosis in all exposure groups for both small and large particle number indices at both 3 month and 9 month analysis points, but the group mean values were not statistically significantly different.

Table 21. Pulmonary macrophage function of rats exposed in 1 month HNO_3 dose-response and 9 month exposure to HNO_3 and O_3 alone and in combination. The 9 month exposure includes groups of animals analyzed at 3 and 9 months. Data are mean ± SE, n.

1 Month Dose-Response Exposure to HNO_3

	Purified Air	HNO_3 Concentration 50 $\mu g/m^3$	150 $\mu g/m^3$	450 $\mu g/m^3$
Macrophage Viability (%)	92.2±0.8,12	90.5±1.2,12	92.2±1.1,12	93.6±0.7,12
Fc Receptor Binding Capacity (% Rosettes)	43.0±2.8,12	42.8±1.9,12	42.6±2.6,11	36.2±1.6,11[A]
Phagocytosis (% of cells)				
>2 particles	85.9±3.4,11	82.1±6.1,11	87.4±4.4,12	86.8±5.3,12
>7 particles	16.75±5.05,11	6.27±1.50,11[A]	16.92±3.36,12	9.00±1.84,12[A]

9 Month Exposure

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Macrophage Viability (%)				
3 Month	91.1±0.9,12	88.9±0.9,12	88.8±1.2,12	88.0±1.3,12
9 Month	93.2±0.7,12	93.2±0.5,12	92.3±0.8,12	90.4±0.9,12
Fc Receptor Binding Capacity (% Rosettes)				
3 Month	47.4±1.9,11	46.1±2.3,11	52.0±2.3,11	49.7±2.1,10
9 Month	56.8±2.4,12	56.5±2.9,12	56.7±2.9,12	56.6±3.2,12
Phagocytosis (% of cells)				
3 Month				
>2 particles	44.3±4.8,9	43.7±2.9,11	42.5±3.5,12	33.8±1.6,11
>4 particles	17.9±3.7,9	17.7±1.8,11	19.9±3.6,12	12.0±1.2,11
9 Month				
>2 particles	74.2±6.8,9	72.4±4.6,10	66.2±6.5,8	63.7±6.6,9
>4 particles	54.9±9.4,9	50.0±5.8,10	40.9±9.2,8	38.2±6.2,9

[A]Significantly different from purified air control ($p<0.05$).

E. Biochemical and Molecular Biology Analyses.

Additional investigations of the effects of the 9 month HNO_3-O_3 exposure included analysis of lung collagen and collagen cross linking performed by Dr. J. Last at UC Davis, analysis of heat shock stress protein response in the lung performed by Dr. C. Wong at UC Irvine, and analysis of changes in the lung cytochrome P450 monooxygenase system performed by Dr. Y. Kikkawa and Dr. R. Sindhu at UC Irvine.

Lung collagen content was measured as content of 4-hydroxyproline and collagen crosslinking was measured as hydroxypyridinium to collagen ratio. These data are shown in Figure 2 A and B. There were no statistically significant differences among the 9 month HNO_3-O_3 exposure groups. These results are consistent with histochemical analysis of collagen deposition in sections of lung (Table 16).

Heat shock protein 70 showed a strong response to 9 months of exposure to HNO_3 and O_3 alone and in combination (Table 22). All exposure groups were significantly elevated over purified air control values, and the combination HNO_3 + O_3 exposure resulted in a significantly smaller heat shock protein response than either of the single HNO_3 or O_3 exposures.

The 9 month HNO_3-O_3 exposures also significantly modified the cytochrome P450 monooxygenase system (Table 23). Cytochrome P450 monooxygenase reactions studied included benzphetamine N-demethylation and benzo[a]pyrene metabolism. Benzphetamine N-demethylation was significantly depressed by exposure to HNO_3 alone, but enhanced by exposure to O_3 or HNO_3 + O_3. Benzo[a]pyrene metabolism was significantly elevated by all the acid and oxidant exposures, and the effect of combined HNO_3 and O_3 exposure was additive.

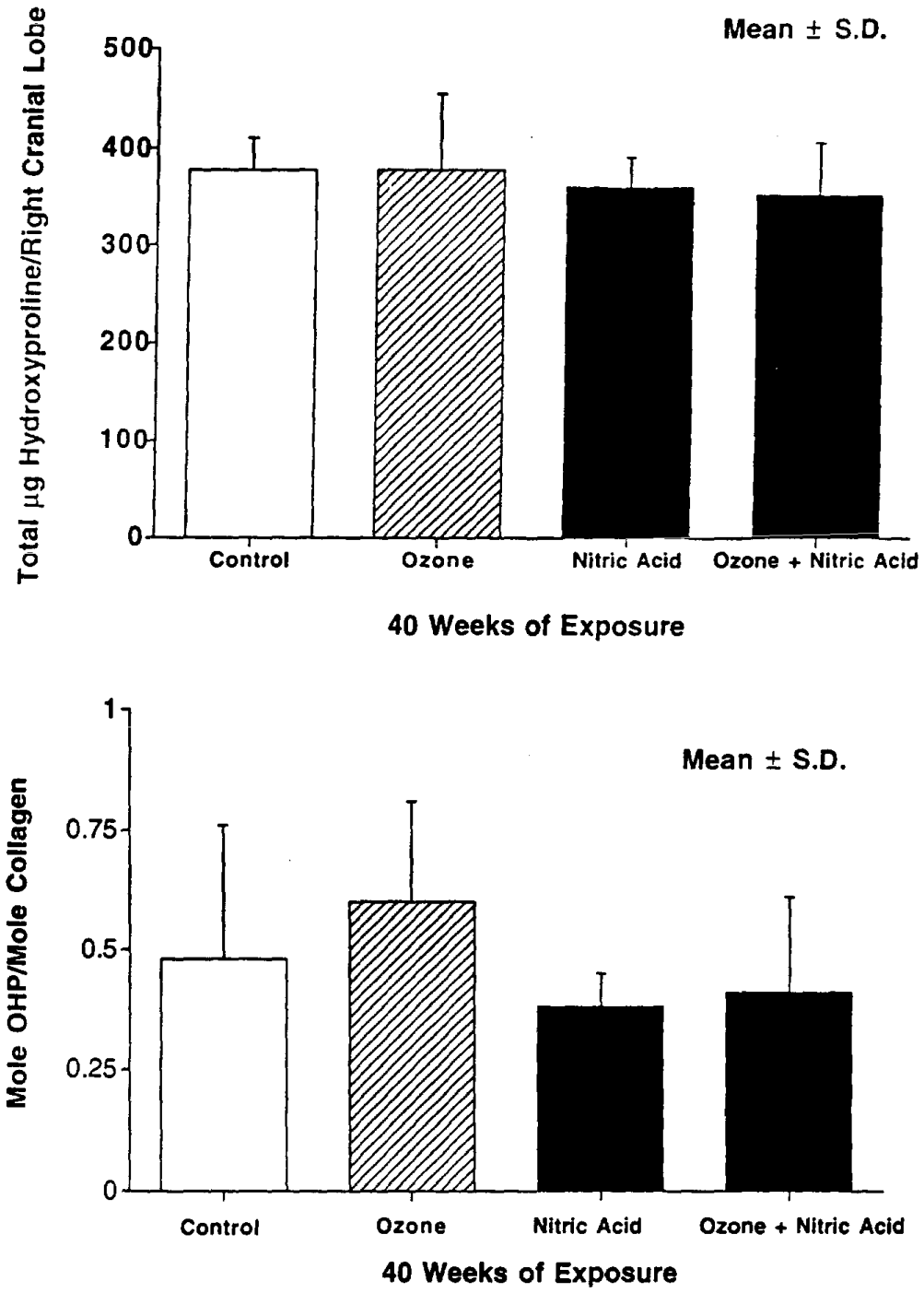

Figure 2A. Measure of hydroxyproline content in the right cranial lung lobe.

2B. Moles of hydroxypyridinium per mole of collagen in the right cranial lung lobe.

Table 22. Heat shock proteins in lungs of rats exposed 9 months to HNO_3 and O_3 alone and in combination. HSP 70 was analyzed by ELISA and data are mean ± SE, n optical density units. Data are mean ± SE,n.

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
HSP 70	37.5±4.3,10	82.8±2.8,11[ABC]	104.0±2.3,10[ABD]	66.2±3.7,9[ACD]

[A]Significantly different from control, $p < 0.01$.
[BCD]Means significantly different, $p < 0.01$.

Table 23. Changes in the cytochrome P450 monooxygenase system in lung of rats exposed 9 months to HNO_3 and O_3 alone and in combination. Measurements were made from microsomes pooled from 5 animals (n = 2 pool groups per exposure). Benzphetamine N-demethylation was measured as nmol of formaldehyde formed per mg protein per minute. ^{3}H-benzo[a]pyrene metabolism was measured as pmol ^{3}H-benzo[a]pyrene metabolized per mg protein per min. Data are mean ± SE.

	Purified Air	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3	HNO_3+O_3
Benzphetamine N-demethylation	0.144±0.002	0.125±0.001[ABC]	0.249±0.001[ABD]	0.270±0.001[ACD]
^{3}H-benzo[a]pyrene metabolism	3.4±0.5	16.9±1.0[EF]	9.5±0.9[AFG]	20.9±0.3[AG]

[A]Significantly different from control, $p < 0.01$.
[BCDG]Means significantly different, $p < 0.01$.
[E]Significantly different from control, $p < 0.05$.
[F]Means significantly different, $p < 0.01$.

IV. DISCUSSION

This investigation of the effects of HNO_3 and O_3 on the respiratory system was designed to address several hypotheses about the possible mechanisms for induction of chronic pulmonary disease by acids and oxidants. These chronic diseases included asthma, bronchitis, upper and lower airway infection, and pulmonary fibrosis/emphysema. The biological endpoints associated with these disease processes that were selected for study are described in the introduction. The concentrations of HNO_3 and O_3 selected for the exposures were based on urban pollutant levels experienced in the South Coast Air Basin (Table 1). Using such concentrations has the advantage of added relevance; the concentrations in the animal exposure study are the same as concentrations to which the human population is exposed. However, this strategy has the disadvantage that mechanistic effects may be difficult to resolve with the small sample sizes of animals that can be practically studied compared to the many thousands of people exposed in the urban population. Furthermore, the test animal population represents subjects in normative healthy condition, whereas there may be sensitive subgroups of the human population that are at significant risk. Studies to identify toxic mechanisms leading to disease usually, therefore, use higher concentrations to resolve mechanistic relationships. In order to strike a balance between these competing goals of experimental design in the present study, several features were incorporated to enhance the resolution of toxic effects. The exposure pattern was episodic (4h/day, 3 days/week) which was hypothesized to interfere with patterns of attenuated sensitivity that can develop with continuous or repeated daily exposures, and which is additionally more relevant to the variable exposure pattern of the human population. The exposure was extended (9 months) which represents a major portion of the rat's lifetime and allows potential chronic biological effects time to develop and be expressed. A large battery of biological endpoints relevant to each disease state was assessed to reduce the chance that important processes might not be resolved. Finally, an initial 1 month exposure to a graded series of HNO_3 concentrations was performed to identify a concentration that was likely to induce detectable effects. Table 24 is a summary of the results of the exposure experiments listing the biological endpoints analyzed and whether statistically significant effects or suggestive trends were observed. The discussion that follows is organized around the disease models hypothesized to be affected by HNO_3 and O_3 exposure.

Table 24. Summary of the effects of the 1 month dose-response HNO_3 (Part A) and the 9 month HNO_3 and O_3 (Part B) exposures. In the 9 month exposure, some endpoints were additionally measured following 1 and 3 months of exposure. + indicates statistically significant effects observed, - indicates no significant effects observed, ± indicates marginally significant effects or trends observed or a mixture of significant and non-significant effects observed among several measurements of endpoints in the category. Blanks indicate no data available.

Part A. One Month HNO_3 Dose-Response Exposure Biological Endpoint Category	Effect of Graded Doses of HNO_3 Vapor (50 - 450 $\mu g/m^3$)
Pulmonary Function	
Breathing Pattern	±
Excised Lung Gas Volume	+
Lung Wet/Dry Mass Ratio	-
Lung Morphometry	
Fixed Lung Volume	+
Alveolar Mean Chord Length	+
Septal Wall Thickness	+
Volume fraction of Lung Tissue Compartments	-
Respiratory Tract Epithelia	
Nasal Epithelium Cellular Composition	±
Mast Cell Numbers in the Lobar Bronchus	-
Epithelial Glycoprotein	-
Cell Turnover	
Nose	+
Trachea, Lobar Bronchus, Terminal Bronchiole, Lung Parenchyma	-
Acid Phosphatase Activity in Pulmonary Macrophages	±
Bronchopulmonary Lavage Fluid and Macrophage Function	
Protein Content	-
Lactate Dehydrogenase	-
ß-Glucuronidase	-
Glycoprotein in Lavage Fluid	+
Lavage Fluid Cellular Composition	-
Macrophage Viability	-
Macrophage Fc Receptor Binding Capacity	+
Macrophage Phagocytosis	±

Table 24 continued next page

Table 24 (continued)

Part B. Nine Month Exposure to HNO_3 (50 $\mu g/m^3$) and O_3 (0.15 ppm) Alone or in Combination

Biological Endpoint Category	Compound HNO_3	O_3	HNO_3+O_3
Pulmonary Function			
Breathing Pattern	-	-	-
Excised Lung Gas Volume			
3 month analysis	+	-	+
9 month analysis	-	-	-
Lung Wet/Dry Mass Ratio (9 month analysis)	-	-	-
Lung Quasi-static Compliance			
3 month analysis	+	+	-
9 month analysis	-	-	-
Respiratory Tract Clearance (9 month analysis)			
Early Clearance	-	±	±
Late Clearance	-	-	-
Epithelial Permeability			
Nasal	+	-	±
Bronchoalveolar	-	-	-
Lung Morphometry (1, 3, and 9 month analyses)			
Fixed Lung Volume	-	-	-
Alveolar Mean Chord Length	-	-	-
Septal Wall Thickness	-	-	-
Volume fraction of Lung Tissue Compartments	-	-	-
Alveolar Tissue Density	-	±	
Alveolar Epithelial Volume Per Unit Surface Area		-	-
Interstitial Volume Density		-	-
Respiratory Tract Epithelia			
Nasal Epithelium Cellular Composition			
1 and 3 month analyses	-	-	-
9 month analysis	±	-	-
Mast Cell Numbers in the Lobar Bronchus			
(1, 3, and 9 month analyses)	-	-	-
Epithelial Glycoprotein Stain Density			
1 and 3 month analyses	-	-	-
9 month analysis	±	±	-
Tracheal Serous Cell Density (3 and 9 month analyses)	-	-	-
Cell Turnover (Nose, Trachea, Lobar Bronchus, Terminal			
Bronchiole, and Lung Parenchyma at 1 and 3 months)	-	-	-

(Table 24, Part B continued next page)

Table 24, Part B continued

Acid Phosphatase Stain Density in Pulmonary Macrophages			
1 and 3 month analyses	-	-	-
9 month analysis	±	-	+
Collagen Deposition (Stain Density) in Lung Parenchyma (1, 3, and 9 month analyses)	-	-	-
Lung Collagen as 4-hydroxyproline and Collagen Crosslinks (9 month analysis)	-	-	-
Bronchopulmonary Lavage Fluid and Macrophage Function			
Protein Content (see above as bronchoalveolar permeability)			
Lactate Dehydrogenase and Fibronectin (3 and 9 month analyses)	-	-	-
ß-Glucuronidase	-	-	+
Glycoprotein in Lavage Fluid (9 month analysis)	-	-	-
Elastase Inhibitory Capacity (3 and 9 month analyses)	-	-	-
Lavage Fluid Cellular Composition (3 and 9 month analyses)	-	-	-
Macrophage Viability, Macrophage Fc Receptor Binding Capacity and Macrophage Phagocytosis (3 and 9 month analyses)	-	-	-
Lung Heat Shock Protein (HSP 70) Content (9 month analysis)	+	+	+
Lung Cytochrome P450 Activation as Benzphetamine N-demethylation and as ^{3}H-benzo[a]pyrene metabolism (9 month analysis)	+	+	+

Endpoints potentially related to asthma included irritant breathing pattern responses, proliferation of mast cells in bronchi, and bronchoalveolar epithelial permeability. None of these measures were responsive to the HNO_3 and O_3 exposures (Tables 3, 7, and 11). In short term exposures of human subjects to 500 $\mu g/m^3$ HNO_3, HNO_3 also did not induce pulmonary function changes, nor did it significantly modify the effects of 0.2 ppm O_3 in combination exposure (Aris et al., 1993). When tested in allergic subjects 0.05 ppm HNO_3 (129 $\mu g/m^3$)

increased respiratory tract resistance to air flow by 23% and decreased FEV_1 by 4% (Koenig et al., 1989). An epidemiological investigation has shown that while asthmatic symptoms in the Denver area were associated with airborne H^+, nitric acid and nitrates were not significantly associated the responses. It appears that sensitive populations could be responsive to nitric acid vapor in urban air pollution, however the responses are likely to be related to overall acidity rather than specific to nitric acid.

Bronchitis is a subcategory of the broader issue of upper and lower airway infection, and the endpoints most specific to bronchitis were changes in the tracheobronchial airways, particularly those related to mucus production and secretion. These include tracheal serous cell number density (Table 13), epithelial glycoprotein density (Table 12), glycoprotein in bronchoalveolar lavage fluid (Table 18), and epithelial cell turnover in the trachea and bronchi (Table 14). Epithelial glycoprotein density and serous cell density in the trachea were not significantly affected by the HNO_3 or O_3 exposures, however BAL glycoprotein was elevated in a dose-dependent relation in response to the 1 month exposures to HNO_3 (Table 18). Epithelial glycoprotein density represents an integrated measure of the quantity stored in secretory cells and numbers of secretory cells present. Increased glycoprotein in BAL indicates that production and release was increased without substantially changing epithelial stores present. Epithelial cell turnover was not significantly elevated, thus the tracheal epithelium was not substantially injured, nor was there significant secretory cell metaplasia such as is observed in instillation exposures to large doses of acids such as 0.08 N HNO_3 (Christensen et al., 1988). Nevertheless, the inhalation exposures in the present study show that glycoprotein secretion into BAL is responsive to concentrations of HNO_3 near ambient levels, and sensitive populations could respond more strongly to lower concentrations.

For the broad category of respiratory tract infection, biological endpoints included mucociliary clearance which is the functional mechanism for removing foreign particles including infectious agents from the respiratory tract, epithelial permeability which measures the integrity of the epithelial barrier to movement of foreign compounds and organisms, and pulmonary macrophage function which determines the capacity of these cells to defend the respiratory tract against foreign compounds and organisms. A few of the biological endpoints showed significant changes in response to the acid and oxidant exposures that could result in

increased sensitivity to respiratory infections. In the 9 month exposure, nasal epithelial permeability was significantly increased by the 50 $\mu g/m^3$ HNO_3 exposures (Table 7). A nasal epithelium with increased permeability allows inhaled foreign chemical compounds, particles, and pathogens greater access to underlying tissues and increases the risk of allergic responses or infection. In addition to the nasal epithelial permeability change, there were also significant shifts in epithelial cell composition in nasal respiratory epithelium (Table 10B), and there was an increase in epithelial glycoprotein density as a main effect of HNO_3 in the 9 month exposures (Table 12).

Although there were no significant effects of the 9 month exposure on short and long term respiratory tract particle clearance, there were trends for slowing of early (upper respiratory tract) clearance in response to O_3 exposures and acceleration of late (deep lung) clearance in response to the exposure to O_3 alone (Table 6). Dr. R. Phalen who developed and preformed this clearance measurement believes that the standard for accepting statistical significance ($p < 0.05$) adopted for this project is too conservative for clearance measurements and that $p < 0.10$ is preferable. If the latter standard were adopted, then there was a significant main effect of O_3 on depression of upper airway clearance ($F = 3.9$, $P < 0.06$). This pattern of response to O_3 is expected based on other studies of O_3 (Kenoyer et al., 1981). A delay in upper airway clearance will retard the elimination of deposited foreign particles and micro-organisms, and increase the risk of infection.

Pulmonary macrophage function showed several suggestive responses to the 9 month exposure. Populations of macrophages in lungs of rats in HNO_3 exposures (HNO_3 alone and in combination with O_3) were shifted toward greater cellular density of acid phosphatase (Table 15), indicating that the macrophages were being activated by the acid exposure. ß-glucuronidase is present in phagocytic cells and is released by injured phagocytes. There was a trend for ß-glucuronidase concentrations to increase following exposures to the O_3-containing atmospheres, and the increase was significant for the group exposed to the $HNO_3 + O_3$ at the 9 month analysis point (Table 17). Although phagocytosis was not significantly changed by the exposures, there was a trend toward reduced phagocytosis in all the acid and oxidant exposure atmospheres after 9 months of exposure (Table 21). Reduced macrophage phagocytic function and elevated BAL ß-glucuronidase is consistent with a small degree of macrophage injury. The

combination of effects, increased nasal permeability, decreased upper airway clearance, and altered macrophage function suggests that HNO_3 exposure may be associated with increased incidence or risk of respiratory tract infection.

The fourth disease state examined was pulmonary fibrosis/emphysema, and biological endpoints related to the development of such lung disease were primarily measures of lung structure but including some measures of pulmonary function: quasi-static compliance, excised lung gas volume, pulmonary irritant reflex breathing patterns, lung morphometric analysis, collagen deposition and biochemistry, lavage fluid elastase inhibitory capacity, and epithelial cell turnover in the bronchioles and parenchyma. There were no major alterations in lung structure observed in morphometric analyses of the 9 month HNO_3-O_3 exposures. Detailed examination of the bronchiole-alveolar duct junctions indicated minimal, but occasionally significant changes occurring in the 9 month HNO_3-O_3 exposures (Fig. 1). There was an increase in alveolar tissue volume per unit surface area within the first 200 μm region beyond the bronchiole-alveolar duct junction in animals exposed to O_3. Although other differences were not significant, there was a trend toward elevation of alveolar tissue density in the 500 to 800 μm range beyond the BADJ. There were also trends of increased epithelial volume per surface area in animals exposed to O_3 and HNO_3 + O_3 as far as 400 μm into the alveolar duct (Fig 1B). There were no significant changes in elastase inhibitory capacity (Table 19) or in collagen deposition or collagen cross linking in the lungs of 9 month exposed animals (Table 16, Fig. 2). Lung tissues were not altered sufficiently to induce significant breathing pattern changes (Table 3), nor was irritation of deep lung tissues sufficient to increase epithelial cell turnover (Table 14). Nevertheless, quasi-static compliance was reduced with a significant main effect of O_3 at the 3 month analysis point (Table 5), and the structure-elasticity characteristics of the lung were sufficiently altered to increase excised lung gas volume in HNO_3 and HNO_3 + O_3 at 3 months and to produce a trend of increased trapped gas volume in all exposure groups at 9 months (Table 4). Increases in excised lung gas volume result from changes in the point at which airways close as the lung collapses when the chest is opened and these increases indicate that there has been some change in the morphology or elastic properties of the small airways. Presence of mild pulmonary edema can cause early airway closure. Limited fresh lung tissue was available to measure wet/dry lung tissue mass ratios (Table 4), and there was no indication that edema contributed to

the response. Thus the observed increases in excised lung gas volume are likely due to either or both changes in small airway morphology or changes in tissue elasticity.

Finally, there were strongly significant responses to the 9 month HNO_3 and O_3 exposures by lung heat shock proteins (HSP 70) and by the cytochrome P450 monooxygenase system. Neither of these responses is directly related to human pulmonary disease. Heat shock proteins respond to generalized states of physical or chemical stress in addition to thermal stress for which they were originally named. The responses observed in the present study (Table 22) indicate that the lung was exposed to a toxic stress sufficient to elicit heat shock protein synthesis. At the present state of understanding, It is not known if this response is related to development of pulmonary disease. The cytochrome P450 monooxygenase system is instrumental in metabolizing and detoxifying foreign chemical compounds. Under some conditions foreign chemical compounds are metabolized to more toxic intermediate compounds in these reactions. The Benzphetamine N-demethylation and benzo[a]pyrene reactions that were enhanced by the 9 month HNO_3 and O_3 exposures (Table 23) indicate that the cytochrome P450 monooxygenase system was up-regulated, and this could potentially increase carcinogenic action of inhaled proto-carcinogen compounds metabolized by the lung. However, at this stage, this potential risk cannot be evaluated.

In parallel with the present study, a companion HNO_3 and O_3 exposure study was performed at New York University using rabbits as experimental subjects (Schlesinger, 1995). The exposure compounds, concentrations, and exposure schedules of the two investigations were identical; there was a 1 month (4 week) dose-response exposure to nitric acid vapor at 50, 150, and 450 $\mu g/m^3$ and a 9 month (40 week) exposure to 50 $\mu g/m^3$ HNO_3 and 0.15 ppm O_3 alone and in combination. The animals were exposed episodically for 4 h/day, 3 days/week. The biological endpoints analyzed in the New York University study included bronchoalveolar lavage assays (total soluble protein, lactate dehydrogenase, and lavage fluid cell composition), pulmonary macrophage function assays (cell viability, phagocytosis capacity, superoxide ion production, and tumor necrosis factor (TNF) production); lung morphometry (septal wall intercept and mean alveolar chord length); and tracheal smooth muscle responsivity to constrictor agents. Among the endpoints shared between the exposures of rabbits at New York University and rats at the University of California, Irvine, the results were generally similar

although there were some notable differences. Bronchoalveolar lavage assays measured in both animal models (total protein, lactate dehydrogenase, and cell composition) were not significantly affected by either exposure, although there was a significant effect of 9 months exposure of rats at UCI to the HNO_3-O_3 combination on ß-Glucuronidase (an indicator of injured or activated phagocytic cells) which was not analyzed in the exposures of rabbits. Pulmonary macrophage functions were altered in both the rat and rabbit HNO_3 dose-response exposures (Fc receptor binding capacity in rats and superoxide production and TNF production in rabbits), and these effects were not present in the subsequent 9 month exposures to HNO_3 and O_3 alone and in combination. These particular endpoints were measured in one of but not both the UCI and NYU studies, and macrophage endpoints common to both studies (viability and phagocytosis capacity) were not significantly affected by the exposures. Rats in the 1 month dose-response exposure showed pronounced changes in lung morphometry (increased septal wall intercept and decreased alveolar chord length and fixed lung volume). These changes were not observed in the rabbit dose-response exposure and were not present in rats or rabbits in the 9 month HNO_3-O_3 exposures. The more detailed morphometric analysis performed at UC Davis on the rats exposed at UCI showed only minor trends of change in 9 months of exposure to O_3. The final major biological assay performed in the rabbit exposures was an *in vitro* assay of bronchial responsivity to smooth muscle constrictor challenge. There were significant effects of 1 month exposure to HNO_3 concentrations of 150 and 450 $\mu g/m^3$ and 9 months exposure to 50 $\mu g/m^3$ HNO_3. This endpoint was not measured in rats, however the UCI study did find a significant effect of the exposures in the bronchi. Glycoprotein (secreted largely by bronchial epithelium) in rats was increased in dose-dependent response to HNO_3 concentration in the 1 month exposure. In summary, both exposure studies using rats and rabbits found significant effects of HNO_3 exposure in the deep lung that include alteration of macrophage functions and bronchial epithelia.

In conclusion, the biological effects observed in the 9 month exposure to 50 $\mu g/m^3$ HNO_3, 0.15 ppm O_3, and HNO_3 + O_3 were generally small, and there were no indications that HNO_3 and O_3 at the levels tested interacted synergistically on biological responses. At the low concentrations tested, many of the effects appeared as highly suggestive trends, which were not statistically significant, but fit a pattern of relationship to other biological variables affected in

the same tissues. The relationship of these biological effects to human pulmonary disease indicates that exposure to low concentrations of HNO_3 or O_3 may be most expected to involve increased incidence of respiratory tract infections, and changes in elasticity and fine structure of the lung. The small effects observed in the present study of healthy animals may occur more readily in sensitive populations of humans or in humans exposed to additional gaseous and particulate air pollution.

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13. ABSTRACT *(Maximum 200 Words)*

To assess the potential for acid and oxidant air pollution in California to increase the risk of lung disease, Fischer 344/N rats were exposed to nitric acid vapor (HNO_3) and ozone (O_3). The exposures included 1)a 1 month dose-response study of HNO_3 vapor at 50, 150, and 450 $\mu g/m^3$ and 2) a 9 month exposure to 50 $\mu g/m^3$ HNO_3 and 0.15ppm O_3 alone and in combination. There were significant alterations in lung morphology and defensive functions of pulmonary macrophages in the 1 month HNO_3 dose-response exposure. In the 9 month exposure, increased nasal epithelial permeability and a shift in nasal cell populations was seen with the HNO_3 exposures and trends for slowing upper respiratory tract particle clearance and acceleration of deep lung clearance were seen with O_3 exposure. HNO_3 exposure also resulted in increased density of acid phosphatase in macrophages, O_3 exposure resulted in increased β-glucuronidase in lung lavage fluid, and both acid and oxidant exposures resulted in a trend toward reduced phagocytic activity. These results suggest that HNO_3 and to a lesser extent, O_3 exposure, may be associated with increased risk of respiratory tract infection, and changes in elasticity and fine structure of the lung. The small effects observed in the present study in healthy animals suggest that more serious and lasting responses may occur in sensitive populations of humans or in humans exposed to additional more complex gaseous and particulate pollution mixtures.

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