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Toxicity of Mixed Air Pollutants:

Oxidant, Acid, and Fine Particles

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY
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TOXICITY OF MIXED AIR POLLUTANTS: OXIDANT, ACID, AND FINE PARTICLES

Final Report A833-104

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ABSTRACT

To determine whether the effects of long-term ozone (O_3) exposure are substantially modified by acidic and particulate co-pollutants, groups of Fischer 344/N rats were exposed nose-only 4 h/day, 3 consecutive days/week for 6 months to purified air; 0.3 ppm O_3; and to a mixture of 0.3 ppm O_3, 0.2 ppm NO_2, 0.1 mg/m^3 NH_4HSO_4, 0.05 mg/m^3 HNO_3, and 0.06 mg/m^3 carbon particles. An initial 1 month mixture dose-response exposure experiment included this mixture at component concentration factors of 0.5 (Low), 1.0 (Med), and 2.0 (High) of the levels used in the 6 month exposure. The mixture and exposure protocol were based on air monitoring data in a heavily polluted region in South Coast Air Basin of California. At the end of the 1 month dose-response exposure, at the end of the 6 months exposure, and 1 month following the 6 months exposure, the biological effects on a variety of respiratory tract structural and functional measures were analyzed.

In the 1 mo exposure, rapid-shallow irritant breathing pattern responses were present at first exposure in Med and High groups and, with successive exposures, these showed diminished response in Med and exacerbated response in High groups. There were significant concentration related changes in lung morphometric variables and exposure to High was associated with presence of pulmonary lesions. Other significant relationships between affected biological variables and mixture concentration included, change in nasal epithelial cell composition; decreased secretory (glycoprotein) stain density in nasal epithelia and increased stain density in tracheal epithelia; increased acid phosphatase stain density in pulmonary macrophages; increased epithelial cell proliferation in the nose, trachea, terminal bronchiole, and lung parenchyma; increased bronchoalveolar epithelial permeability; decreased macrophage FcR binding capacity; and decreased macrophage particle phagocytosis. The changes in lung morphometry were suggestive of decreased tissue compliance or stiffening of the lung, which could be a mechanism for reduction of pulmonary function observed in humans breathing polluted air. Changes in secretory product densities in upper airway epithelia imply that mucus production was altered, but further study is required to establish the chain of relationship between cell secretory density and production rate. Increased acid phosphatase in pulmonary macrophages suggests that cell immunological functions were activated, and increased epithelial cell proliferation is

indicative of persistent irritation and cell death in the respiratory tract epithelium. Increased bronchoalveolar epithelial permeability could result in increased sensitivity to inhaled allergens and the changes in macrophage FCR binding and phagocytosis suggest presence of reduced defenses against respiratory infections.

6 months exposure to the Med level mixture induced an elevation of breath frequency on the third days of episodes, and at end-exposure, there was a depression of secretory stain density in tracheal epithelium, increased numbers of mast cells in lobar bronchi, elevated cell proliferation in the terminal bronchioles and alveoli, depression of pulmonary macrophage Fc receptor binding capacity, and trends of increased permeability of the nasal epithelium and decrease in long-term tracer particle clearance rate. O_3 alone did not significantly alter these variables. Both the mixture and O_3 alone produced trends of change in morphometric measures of lung fine structure and in alterations of fixed lung volume. All of the significant effects of the mixture showed recovery at 1 month post-exposure except for elevated mast cell numbers in lobar bronchi and cell proliferation in the alveolar zone. Trends of change in lung morphometric variables in the mixture and O_3 exposure were still present at 1 month post-exposure. Significant effects of the mixture on biological variables were almost always associated with mean values for O_3 exposure groups lying intermediate to mixture and control means and no significant differences between the mixture and O_3 groups. This suggests that a small O_3 effect was present, and that the mixture components with O_3 induced a larger effect. These results suggest that continued episodic exposure to a mixture of air pollutants similar in composition and concentration to urban ambient pollution can result in continued irritation of lung tissues and can compromise pulmonary defenses possibly leading to increased sensitivity to inhaled allergens and increased susceptibility to respiratory infections.

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CONCLUSIONS AND RECOMMENDATIONS

The purpose of this project was to examine the potential for development of cumulative respiratory tract injury in people who are repeatedly exposed to mixtures of air pollutants containing oxidants, acids, and fine particulate material. People living in the city of Azusa and surrounding communities in the South Coast Air Basin of southern California are frequently exposed to air pollution at concentrations above the State and Federal ambient air quality standards. The atmospheres in these communities contain complex mixtures, both in terms of size and chemical composition of the ambient aerosol and the concentrations and ratios of gas phase photochemically active compounds such as nitric acid, nitrogen dioxide and ozone. Epidemiological studies of populations in this region have demonstrated possible chronic health effects, including increased rates of mortality (especially in individuals with pre-existing lung or cardiovascular diseases), increased numbers of hospital visits (especially for asthmatic individuals), and increased reports of respiratory symptoms. These findings are significantly associated with either ozone or with the particulate fraction of urban pollution, however it is not always possible to separate out the effects of the oxidant gases (ozone and nitrogen dioxide) from those of the particulate phase. In addition, it is not well established which of the many chemical species in the complex mixture comprising urban aerosols are associated with the observed effects on human health. This study was undertaken to examine, under controlled conditions, the effects of 6 months exposure to an atmosphere modeling the ambient atmosphere in or near the city of Azusa, California. Azusa is notable for frequently exceeding both the federal and state ambient air standards for ozone and particulate matter.

Laboratory rats (male, barrier-reared and specific pathogen free Fischer 344/N) were exposed to a mixture containing ozone (0.3 ppm), nitrogen dioxide (0.2 ppm), nitric acid vapor (0.05 mg/m^3), ammonium bisulfate aerosol (0.10 mg/m^3, 0.3 μm mass median aerodynamic diameter [MMAD]), and carbon particles (0.066 mg/m^3, 0.3 μm MMAD). Groups of rats were also exposed to 0.3 ppm ozone alone, and, as controls, to purified air. The exposure pattern was in episodes of 4 h/day, 3 days/week. An initial 1 month dose-response exposure was performed using this 5 component mixture and including mixture

component concentration factors of 0.5, 1.0, and 2.0 of the levels used in the 6 month exposure. Biological endpoints expected to be indicative of processes leading to the development of lung diseases were measured immediately following the end of the 1 month dose-response exposure and at the end of and one month following the 6 month exposure.

The 6 month exposure to the mixture induced an elevation of breath frequency on the third days of episodes, and at end-exposure, there was a depression of mucus (glycoprotein) density in tracheal epithelium, increased numbers of mast cells in lobar bronchi, elevated cell proliferation in the terminal bronchioles and alveoli, depression of pulmonary macrophage Fc receptor binding capacity, trends of increased permeability of the nasal epithelium, and decrease in long-term particle clearance rate. Exposure to O_3 alone did not significantly alter these variables. Both the mixture and O_3 alone produced trends of change in morphometric measures of lung fine structure and in alterations of fixed lung volume. All of the significant effects of the mixture showed recovery at 1 month post-exposure except for elevated mast cell numbers in lobar bronchi and cell proliferation in the alveolar zone. Trends of change in lung morphometric variables in the mixture and O_3 exposure were still present at 1 month post-exposure. Although comparisons of effects of O_3 alone to purified air controls showed few significant effects, significant effects of the mixture on biological variables were associated with mean values for O_3 exposure groups lying intermediate to mixture and purified air control means. In these cases there was nearly always no significant difference between the mixture and O_3 groups. This pattern of results suggests that a small O_3 effect was present, and the mixture components with O_3 induced a larger effect. The changes observed suggest that continued episodic exposure to the mixture can result in continued irritation of lung tissues (breath frequency and lung cell proliferation changes) and can compromise pulmonary defenses including the barrier to infectious organisms and allergens (epithelial permeability changes), immune defense (depression of macrophage receptor binding and increase in airway mast cells), particle clearance (decrease in tracer particle clearance and altered tracheal epithelial glycoprotein density). This is consistent with hypotheses relating pollutant exposures to increased risks of lung infections, stiffening of the lung tissues (which could be an early indication of development of lung fibrosis), and release into the lung of inflammatory mediators and other serum factors.

These events could aggravate asthma and could, in turn, promote chronic lung inflammation which, in humans, is strongly related to the development of chronic bronchitis and emphysema. In previous studies we have demonstrated acute effects of ozone alone on respiratory structure and function. The results of the present study suggest that longer term exposure to ozone alone does not produce large changes in the biological endpoints measured, but a multicomponent mixture of oxidants, acids, and particles can produce a set of adverse biological effects on the respiratory system. Although the present study did not include additional groups of animals exposed to all the separate subcomponent gas and particle compounds of the multicomponent mixture, recent epidemiological studies suggest that particulate air pollution is a critical factor the effect of air pollution on human mortality and morbidity, and future studies should investigate the toxicology of particulate air pollution. While the composition of particulate air pollution is complex, and the interaction of components in mixtures of compounds may be of toxicological importance, future investigations should include single component and simple mixtures of components so that the interpretation of results can determine which components are producing toxic effects.

I. INTRODUCTION

A. Background.

People living in many regions of the South Coast Air Basin of Southern California are frequently exposed to levels of gaseous and particulate air pollutants in excess of the ambient air quality standards set by the federal Environmental Protection Agency and the State of California. These air quality standards are health based and are designed to protect sensitive individuals in the exposed populations from pollutant-related adverse health effects. Oxidant gases which are formed by photochemical processes in the atmosphere are known to adversely affect human health. Ozone, which is probably the most important of the photochemically derived oxidants, from the standpoint of human health effects, has been shown to significantly impair pulmonary function and lung mechanics, aggravate asthma (Whittemore and Korn, 1980; Abbey et al., 1991), and increase respiratory symptoms (for example see Hall et al., 1991). Although most epidemiological studies have not demonstrated significant associations between ozone exposures and mortality rates, Kinney and Ozkaynak (1991) report a statistically significant association between daily mortality and daily ozone concentrations in Los Angeles. Airborne particles have also been associated with human health problems, including increased rates of bronchitis and other respiratory ailments, losses of lung function, increased risk of lung cancer, and increased mortality. Studies have demonstrated associations between daily particulate levels and hospital admissions and emergency room visits for respiratory illnesses (Martin 1964, Greenberg et al. 1967, Knight et al. 1989), and between asthma attacks and airborne particle levels (Whittemore and Korn, 1980). Elevated levels of particles less than 10 μm aerodynamic diameter (PM10) have been linked with reduced pulmonary function in adults (Pope et al., 1991) and children (Stern et al., 1989). Ozkaynak and Spengler (1985) and Ozkaynak and Thurston (1987) analyzed the 1980 U.S. vital statistics and available ambient air pollution data bases for sulfates, fine inhalable particles, and total suspended particles and found significant associations between particle exposures and total mortality. Of the independent mortality predictors considered, particle exposure measures related to the respirable and/or toxic fraction of the aerosols, such as fine particles and sulfates, were most consistently and

significantly associated with reported total annual mortality rates.

Detels et al. (1987) studied never-smoking residents of Los Angeles, some of whom lived in a community with moderate levels of photochemical pollution and low levels of other pollutants, and a second group which lived in a community with very high levels of photochemical oxidant and relatively high levels of sulfates and particulates. In the more polluted area there were significantly worse lung function test results for both men and women at baseline and significantly more rapid decline in lung function in adults over a five-year study period, suggesting that chronic exposures to a mix of photochemical oxidants, sulfates and particulates are associated with increased losses of lung function.

The associations between air pollutant exposures and human health effects derived from these, and earlier, epidemiological studies provide suggestive relationships, but it is difficult to establish causal relationships. A better understanding of dose-response characteristics and quantitative measurements of the direct effects of specific components of ambient pollution were needed. We therefore designed a controlled laboratory exposure study which could explicitly examine the health impacts of exposures to a pollutant mixture similar, in several important respects, to that which residents of California are exposed. We developed an exposure protocol based upon observed patterns of air pollution concentrations, and we used pollutant concentrations which were linked to levels of components observed in the South Coast Air Basin of Southern California.

The principal objectives of this study were to 1) apply disease-related endpoints in order to assess the potential of selected air pollution components for injuring human populations, and 2) evaluate the toxicity of a pollutant mixture (based on concentrations measured in or near the city of Azusa, CA) both alone and in combination with ozone. In order to fulfill these objectives, a series of inhalation exposures was undertaken using laboratory rats.

B. Ambient Air Quality Standards.

Both California and the federal government have established annual and 24 hour standards for PM10, 1 hr maximum concentrations for ozone, and 1 hr average concentrations for nitrogen dioxide (Table 1). The State of California has a standard for

Table 1. Ambient Air Quality Standards

	PM10 Standard($\mu g/m^3$)		Ozone(ppm)	Nitrogen Dioxide(ppm)	
	Annual	24 H Average	1 Hr.	Annual	1 Hr Average
California	30	50	0.09	0.25	
Federal	50	150	0.12		0.053

sulfate (0.25 $\mu g/m^3$) but there is no corresponding federal standard.

In selecting the particulate components of PM10 which would be included in this toxicological study, we depended heavily on the results of intensive air sampling campaigns which were conducted in Southern California with the sponsorship of the California Air Resources Board, on the air monitoring reports published by the South Coast Air Quality Management District, and on published information on the chemical characterization of ambient air pollutants.

PM10 was sampled in Claremont, CA, as part of the South Coast Air Quality Sampling (SCAQS) program in the summer of 1987 (Wolff et al., 1991). The results are shown in Table 2. Nitrates and sulfates, together, represent about 30% of PM10, and elemental carbon, represents about 10% of the fine particle fraction of PM10. The chemical form of sulfate in ambient air is variable depending upon several factors. Most of the sulfate is derived from the atmospheric conversion of sulfur dioxide to sulfuric acid. The sulfuric acid can be rapidly neutralized by reaction with ammonia, which is often a common biogenic constituent of ambient air. The relative proportions of the neutralization products of sulfuric acid, ammonium sulfate (relatively non-acidic) and ammonium bisulfate (acidic) will depend to some extent upon the concentration of ammonia in the air. In planning our study we elected to model the case that the sulfates were only partially neutralized, and used ammonium bisulfate for our exposures. Nitrate, on the other hand is primarily derived from nitric acid which in turn is generated in the atmosphere by photochemical processes involving nitrogen dioxide. Because airborne acidity has been implicated in the epidemiological associations of air pollution and health effects, and since nitric acid vapor

Table 2. Concentrations ($\mu g/m^3$) of Particulate Components in Claremont, California. Data are based on 12 h samples (Wolff et al., 1991).

	Mean ± SD	Max	Mean % of PM10
PM10	60 ± 24	120	-
Fine Part. Matter (<2.5 μm)	36 ± 17	91	60
Coarse Part. Matter (>2.5 μm)	23 ± 10	47	38
Sulfate	6.5 ± 3.8	18	10.8
Nitrate	12.4 ± 6.5	28	20.7
Carbon (elemental and organic < 10 μm)	11.8 ± 5.5	40	20.0

is an important acidic constituent of the air in Southern California (perhaps in contrast to many other parts of the U.S. where acid sulfates are the dominant acidic form) we included nitric acid as the form of nitrate to be studied. The particle size of the sulfate-carbon aerosols was 0.3μm, based upon sizes reported in the literature for these components.

The concentrations of each particulate component used were selected to be representative of estimated peak 4 hr concentrations, based on extrapolations from ambient air data, and the exposure pattern was selected after analysis of reported ambient air seasonal and diurnal patterns. 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 standard is exceeded is typically 1 to 6. Other

pollutants also show episodic behavior. Recent nitric acid concentration data (California 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. Nitrogen dioxide shows some seasonal variation but the patterns are less well defined and it is difficult to state when concentrations will be highest (SCAQMD, Seasonal & Diurnal Variation in Air Quality - undated report). Sulfate aerosol has been measured at Azusa, and the State standard (25 $\mu g/m^3$ for a 24 hr average) was not exceeded in 1986 (the maximum 24 hr concentration was 15 $\mu g/m^3$). On a seasonal basis, sulfate concentrations appear highest in the S.C.A.B. in summer months (Solomon et al., 1988). Elemental carbon concentrations, also reported by Solomon et al. (1988), do not show striking seasonal variations. Because variation in O_3 concentration is a dominant factor in the structure of episodes in the S.C.A.B., the selected exposure scenario was keyed to O_3 concentration fluctuations.

Table 3 shows the peak 1 hr O_3 and NO_2 concentrations (0.31 and 0.21 ppm, respectively) measured at Azusa during 1986. The CARB reports a strong correlation between peak 1 hr and longer term averages for O_3 (CARB Staff Report, 1987), thus the medium dose levels for O_3 and NO_2 (Table 3) for the sub-chronic exposures were set at 0.3 and 0.2 ppm, respectively. 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 0.06 mg/m^3 measured in Glendora over an 8-day period in August 1986; concentrations averaged over 24 hr were lower (0.02 mg/m^3). Ammonium acid sulfate ($NH_4 HSO_4$) has not been measured in ambient air on a regular basis, but in atmospheric mixtures that contain ammonium sulfate and sulfuric acid as droplet aerosols, ammonium acid sulfate is a reasonably postulated reaction product. The maximum 24 hr average inorganic sulfate concentration reported at Azusa was about 0.02 mg/m^3. If as a worst-case scenario, we assume all sulfate is present as ammonium acid sulfate, and this is reasonable given that measured sulfuric acid concentration is the S.C.A.B. range for 0.001 to 0.01 mg/m^3 and that 0.01 mg/m^3 sulfuric acid plus 0.01 mg/m^3 ammonium sulfate would yield 0.02 mg/m^3 ammonium acid sulfate at equilibrium. Because the ratio of 2-4 h peaks

Table 3. Air pollutant concentrations for 1 month and 6 month exposures. Also shown are maximum concentrations of air pollutants in Azusa, CA and adjacent cities in 1986. In Azusa PM10 consists of organic material, carbon, nitrate, sulfate and other species. In our exposures, carbon and ammonium acid sulfate were present in the PM10 fraction. The 1 month exposure compared the low-, med- and high-dose levels and clean air control. The 6 month exposure was at the medium dose of the 3 dose levels and included purified air control and O_3 alone exposure.

	O_3 (ppm)	NO_2 (ppm)	HNO_3* (mg/m^3)	NH_4HSO_4** (mg/m^3)	Carbon*** (mg/m^3)	TSP (mg/m^3)	PM10 (mg/m^3)
Azusa, CA****	0.31	0.21	0.06	0.02	0.01	0.276	0.183
Low-Dose Level	0.15	0.1	0.025	0.05	0.03	0.08	0.08
Med-Dose Level	0.3	0.2	0.05	0.10	0.06	0.16	0.16
High-Dose Level	0.6	0.4	0.10	0.20	0.12	0.32	0.32

Notes:

* Nitric acid was not measured at Azusa. The value reported represents peak HNO_3 concentrations at Glendora, CA.

** Ammonium acid sulfate was estimated from 24 hr maximum sulfate concentrations at Azusa. The medium dose level of peak ammonium acid sulfate concentration was computed using a 6:1 ratio of peak 1 hr to 24 hr averages based on sulfuric acid measured at Claremont, CA.

** Carbon was estimated from 24 hr average measurements in the South Coast Air Basin during 1986. A 6:1 peak 1 hr to 24 hr average ratio (as per the sulfate computation) was used to derive the 0.06 mg/m^3 medium dose level.

**** Data from the South Coast Air Quality Management District for Azusa for O_3 and NO_2 are peak 1 hr concentrations and TSP PM10 are maximum 24 hr concentrations.

to 24 hr average sulfuric acid concentrations in S.C.A.B. cities is about 6:1, a mid-range ammonium acid sulfate concentration of 0.1 mg/m^3 was chosen. Peak 24 hr concentrations of elemental carbon are not reported for Azusa but based on data for other S.C.A.B. cities are about 0.01 mg/m^3. Assuming a 4 hr to 24 hr concentration ratio for carbon similar to

that for sulfuric acid (6:1), a mid-range concentration of 0.06 mg/m^3 was selected. The low-dose and high-dose concentrations selected were 1/2 and 2 times the medium dose levels. The 6 month exposure concentrations were performed at the medium dose levels in Table 3.

Azusa was selected as the environmental model because it is impacted by multiple pollutants with high peak concentrations and it has a large surrounding population. Fortunately, a substantial amount of recent air monitoring data is available from that location. As a base year, 1986 was chosen to take advantage of the data available from an in-depth atmospheric sampling and analysis program. Ozone concentrations in Azusa exceeded the California 0.09 ppm ozone standard 45% of the days in 1986. Elevated levels of air pollution are not isolated, single day events, but tend to occur in aggregates of repeated daily excursions driven by meteorological patterns which tend to persist over periods of days (Blumenthal, et al., 1978). Furthermore, human and animal toxicology studies have shown that repeated daily exposure to O_3 results in an initial increase in severity of symptoms followed by an attenuation of symptom severity. This attenuation or adaptation of responses to O_3 observed in repeated daily exposure may not develop when exposures occur in episodes of several days separated by intervals of days with low levels of exposure. Such an episodic pattern is more representative of ambient conditions, and the exposure pattern for this study was selected to challenge the attenuation response to repeated exposure and to better represent variability of urban air pollution levels. In repeated daily exposures to O_3, pulmonary function symptoms are typically most pronounced on the second or third day of exposure and then show an attenuation response on succeeding days of exposure. Following cessation of exposures, O_3 sensitivity begins to reappear after 4 days and is substantially recovered somewhere between 7 and 20 days. We therefore selected 3 days of successive daily exposure for the episodes with 4 days intervening between episodes for the exposure pattern for this study. The daily ozone peak in California has its major intensity over about 2-4 hours, therefore a 4 hour per day exposure was selected.

C. Exposure Plan.

The planned study included a preliminary experiment to compare the effects of ozone on rats exposed during the day (normally inactive phase) to rats on a reversed day-night cycle exposed during their "nighttime" (normally active) phase. Next, a sub-chronic dose-response exposure (4 hours/day, 3 day/week, for 4 weeks) was performed, and the data obtained were used to guide the selection of exposure concentrations in the subsequent 6 month exposure experiment (4 hours/day, 3 days/week for 26 weeks). The 6 month exposure period was about 17% of the 2.5-3.5 year life span of laboratory rats. The exposures represented periodic, elevated concentration human exposures. Two kinds of dose-response information were obtained. The 1 month exposure at 3 concentration levels provided a variable dose series based on concentration, and the 6 month exposure, containing an end exposure sacrifice analyses and a 1 month post-exposure sacrifice analysis (in concert with the 1 month sub-chronic exposures) provided information on time-duration effects of the exposure.

Exposure subjects were Fischer 244/N rats, barrier reared and maintained and specific pathogen free. All exposures were nose-only.

D. Biological Endpoints.

1. Lung Morphometry and Histopathology. Respiratory tract injury from exposure to a variety of air pollutant compounds alters the fine structure of the lung tissues in a manner that can be quantified by morphometric analysis (Barr et al., 1988, 1990; Tyler et al., 1987; Mauderly et al., 1987; Hyde et al., 1992; Chang et al., 1991, 1992; Pino et al., 1992; Jakab and Bassett, 1990; Gehr et al., 1993). Morphometric analyses for this study included measurement of volume fractions of lung parenchyma tissue compartments (Gehr et al., 1993), and measures of fixed lung volume, average chord length of alveoli, and average septal wall intercept. These measures are sensitive to pulmonary injury and inflammation associated with foci of interstitial infiltration and to changes in inflation of peripheral lung tissues associated with changes in tissue compliance. Nasal epithelial tissues are the front

line site for exposure and deposition of inhaled irritants and particles. Sites of particular high sensitivity or high deposition include respiratory and transition epithelia (Buckley et al., 1984, Morgan et al., 1986a,b; Mautz et al., 1988; George et al., 1993). Long-term exposure may alter the distribution of cell types in these epithelia, and morphometric analyses of nasal epithelial tissues for this study quantified relative numbers of the dominant cell type categories.

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

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

Inhalation of respiratory irritants such as oxidants, acids and particles injures respiratory tract epithelia and is often followed by cellular replication in the epithelium of the respiratory tract in a repair response to that damage. By using DNA-specific tracers, such as tritiated thymidine, we quantified the relative differences in cell replication in epithelia. Past studies using pulse-labeling following acute oxidant gas exposures (Mautz et al., 1988) have indicated that the maximum labeling (i.e., maximum fraction of cells labeled or labeling index) occurs 24-72 hours after the initial exposure. With continued long-term exposure, the labeling index may remain above normal in those areas experiencing continuing damage, and the labeling index provides a measure of persistent irritation to the respiratory tract epithelia.

2. Breathing Pattern and Minute Ventilation. Inhalation of respiratory irritants produces reflex respiratory changes in breathing pattern and can induce asthmatic attacks. Changes in frequency, tidal volume, and minute ventilation provide a relative measure of irritancy and an indication of whether the site of irritation is primarily in the upper or lower

respiratory tract (Alarie, 1973; Coggins et al., 1982; Chang et al., 1981; Mautz et al., 1985; Dallas et al., 1986). 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; Lee et al., 1979, 1980; Adams et al., 1981; Coleridge and Coleridge, 1984; McDonnell et al., 1983; Schelegle et al., 1993). Compounds classified as sensory or upper airway irritants, such as formaldehyde (HCHO) and airborne acids, induce reflex responses believed to be mediated by trigeminal afferent pathways (Alarie, 1973; Ulrich et al., 1972) and generally involve a decrease in frequency accompanied by depression of ventilation or a compensatory increase in tidal volume to slow-deep breathing (Chang et al., 1981; Davis et al., 1967; Mautz et al., 1983).

3. Particle Clearance. The accumulation over long periods of insoluble particles in the lung is the mechanism for the "pneumoconioses" or dust-lung-diseases seen in mining and other dusty trades (Abraham, 1992). It is still not known if such diseases may be acquired non-occupationally. In addition, foci of inflammation are seen to occur around particle accumulation in the lungs of laboratory animals exposed to inhaled insoluble airborne particles such as carbon (Mauderly, 1992). Uncleared material of any type appears to be a potential health problem. It is presumed that as uncleared material in the deep lung increases, the probability of developing fibrosis will increase. Recently, Gerrity et al. (1993) found that acute exposures of humans to 0.4 ppm ozone did not alter mucociliary clearance of radiolabeled tracer particles. Interestingly, this concentration is the apparent threshold at which ozone changes clearance in the rat (Phalen et al., in press). On the other hand, Laube et al. (1993) exposed humans to very acidic fog (MMAD 10 μm; pH=2.0) for 1 hour (20 min were exercise) and concluded that the clearance of radiolabeled particles was stimulated. Again, this is similar to our observations in rats (Phalen et al., in press).

4. Epithelial Permeability. A number of studies have shown pulmonary changes following inhalation exposure to air pollutants. An increase in airway permeability appears to be a sensitive indicator of pulmonary effects following exposure to airborne pollutants. In guinea pigs, Hu et al. (1982) found an increase in permeability as indicated by

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

5. Pulmonary Macrophage Function and Lavage Fluid Analysis. Pulmonary macrophages were evaluated to determine if this crucial element of pulmonary defense is compromised. Epidemiological findings indicate that pollutant exposure can result in increased rates of respiratory infections; this is borne out by studies with laboratory animals which demonstrate increased susceptibility to infection after exposure to acids and oxidant pollutants (Rose et al., 1988). Changes in macrophage function resulting from pollutant exposure are indicative of these adverse effects of the pollutants.

Macrophages possess receptors on their surfaces for the Fc portion of immunoglobulin (IgG) which help in the recognition of foreign antigens and pathogens (Gaafar et al., 1971). These receptors also mediate phagocytosis and lysis by the macrophages (Boltz-Nitalescu et al., 1981). The binding of these receptors with immune complexes facilitates the phagocytosis of IgG-coated particles (such as bacteria), stimulates both the secretion of the reactive oxygen intermediates and the release of lysosomal hydrolases, and also mediates antibody dependent cellular cytotoxicity (Johnston et al., 1985). Damage to the macrophage cell membrane can also result in leakage of lysosomal enzymes, proteases and Ca^{+2} ion. Modification of the cells Ca^{+2} balance can result in abnormal releases of oxygen free radicals. These factors may be related to the development of chronic lung disease in that when they are released in abnormal quantities they may adversely affect

surrounding cells and tissues.

The ability of macrophages to engulf foreign particles (polystyrene latex microspheres) by phagocytosis was measured in macrophages recovered from lungs of exposed and control rats (Prasad et al., 1988). The function of the macrophage Fc receptor in antigen-antibody response was assessed by measuring the capacity for binding sheep red blood cells in a rosette assay (Rao et al., 1980; Prasad et al., 1988; Kleinman et al., 1993).

Several analyses were performed on bronchoalveolar lavage fluid. Cytological smears of the lavage fluid were made and numbers of monocytes (including mature and immature macrophages), PMN's and, when present, eosinophils, basophils, and lymphocytes were counted. Total protein concentration in cell free lavage fluid was measured to provide additional estimates of permeability changes.

II. METHODS

A. Exposure Atmosphere Generation and Characterization.

Carbon aerosol was generated by nebulizing freshly prepared, ultrasonically agitated suspensions of carbon black (Monarch 120, Cabot Chemical). The aerosolized droplets were passed through a ^{85}Kr aerosol charge neutralizer and then diluted and equilibrated with purified air at 60% relative humidity. Ammonium bisulphate was nebulized from a dilute solution, discharged through a ^{85}Kr neutralizer, and then diluted and equilibrated with purified air at 60% relative humidity.

Vapor phase nitric acid was formed by reaction of O_3 and NO_2. Ozone was generated by passing medical-grade oxygen through a corona-discharge ozonizer (Sander type III) and diluting the ozone into purified air. Nitrogen dioxide (1% in nitrogen; Matheson) was metered into purified air and mixed with diluted ozone in a fluorocarbon tubing delay line, to allow for a dynamic equilibrium to be reached between the ozone and nitrogen dioxide precursors of nitric acid vapor. The resulting oxidant gas and nitric acid vapor mixture was

equilibrated with the test atmospheres to yield the appropriate concentrations of each constituent at 60% relative humidity, when measured at the rat's breathing zone.

Concentrations of ozone were monitored with a calibrated ultraviolet light absorption continuous monitor (Dasibi Environmental Corp., Model 1003-AH). Nitrogen dioxide was measured by chemiluminescence using a Monitor Labs Model 8440 detector.

The size distributions of the sulfate and carbon particles were measured using an eight-stage cascade impactor (Anderson Model 210). Total aerosol sample was collected on acid-washed and distilled-water-rinsed quartz fiber filters and weighed on a Cahn electrobalance. The insoluble carbon content was measured by combustion of the filter in oxygen and measurement of evolved CO_2. Samples were also collected using 2 filters in tandem, a prefilter (used to determine the concentrations of $SO_4^=$, NO_3 and H^+ in the collected aerosols) and a nylon backup filter (used to collect nitric acid vapor). Filter and impactor samples were extracted in aqueous media and aliquots of each extract were analyzed by ion chromatography. Hydrogen ion concentrations on the pre-filter extracts were measured by the method of Koutrakis et al. (1988) using a Beckman Model 4500 pH meter.

B. Animal Housing, Quality Control, and Exposure.

Exposure subjects were male Fischer 344/N rats that were barrier reared and specific pathogen free (Simonsen Laboratories, Inc., Gilroy, CA) and permanently marked for identification with tattoos. Rats were housed in wire bottomed cages (Hoeltge, Inc., Cincinnati, OH) over rock salt to absorb moisture from excreta and prevent ammonia formation from bacterial decomposition. Cages were maintained in front of laminar flow isolation units supplied with filtered air. The animals were supplied with dry laboratory chow (Wayne Lab Blox, Western Research Products, Orange, CA) and water ad lib. 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 exposure) or every 4 weeks (6 month exposure). 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. Exposures were 4 hours per day on 3 consecutive days per week. Animals were handled by personnel wearing clean lab coats, surgical masks, head covers, shoe covers, and gloves in order to prevent the spread of infections to the rats. Exposure tubes were thoroughly machine-washed in hot soapy water and disinfected with bleach daily. Animal quarters were cleaned twice weekly.

C. Respiratory Tract Morphometry and Histopathology.

Rats were anesthetized with sodium pentobarbital and killed by exsanguination via the abdominal aorta. The cranial portion of the trachea was removed and fixed in 10% buffered formalin. The remaining trachea was cannulated, and the attached lungs were fixed by instillation with 10% neutral buffered formalin at a pressure of 30 cm of water for 72 hr in preparation for morphometric studies (McClure et al., 1982). After fixation, fixed lung volume was determined by the method of Scherle (1970). The left lobe provided all sections used for morphometry and lung autoradiography (ARG) analysis. The lobe was cut longitudinally to expose the left main airway and major intrapulmonary airways. The area of the exposed surface was digitized and stored using a computerized image analysis system (American Innovision system). After embedding in paraffin, 5 μm sections were cut en face, mounted on glass slides, and again digitized to determine the degree of shrinkage during embedding and sectioning. Separate sections were be stained for morphometry, ARG preparation, acid phosphatase, or carbon particle content of macrophages. The fixed trachea was embedded in paraffin and 5 μm cross-sections cut from the cranial, middle, and distal regions. Sections were either stained with Alcian Blue/Periodic Acid Schiff stain or prepared for ARG.

For analysis of the nasal region, the head was skinned, external tissue and muscle

removed, and the nasal section fixed by immersion with vacuum degassing in 10% buffered formalin. Decalcification was performed in 6% EDTA, followed by embedding in paraffin and sectioning at 5 μm for autoradiography and morphometry. Cross-sections were cut approximately midway between the nares and the eye to provide sections containing squamous, transition, respiratory, and olfactory epithelium (Level 1 of Young, 1981).

Morphometric measurements of the lung were made using a computer image analysis system (American Innovision). The subgross volume fractions of parenchyma and nonparenchyma, including large airways, large vessels, and other tissues were estimated using a lattice system consisting of a 10 x 10 grid based on the methods described by Weibel (1966, 1979). A computer-generated lattice was laid over a video image of the lung section. Intersections (points) falling on structures of interest were scored separately and used to calculate the relative volume fractions of each. The same general approach was used at higher magnification to estimate the relative fractions of alveoli, alveolar ducts, and terminal bronchioles. Standard formulae were used to estimate the lung volume fractions (Elias and Hyde, 1983). Data from the subgross level were used to correct data from point counts for parenchymal fractions so the final data were expressed as volume fractions of the whole lung.

Autoradiographic analysis of relative cell turnover rate was determined by giving rats a sub-cutaneous administration of tritiated thymidine ([^{3}H]-dThd) as a solution in sterile 0.9% NaCl (2 μCi/g body mass). The animals were given [^{3}H]-dThd 1 day following the last exposure and 1 day prior to sacrifice. This allowed for optimal resolution of events in the nose and lung and allowed for complete metabolism of the tracer (Cleaver, 1967). Autoradiographic analyses were performed on 5 μm paraffin sections of the nose, trachea, left lung lobar bronchus, and terminal bronchioles, prepared as described above using liquid photographic emulsion (Kodak NTB-2) development in Kodak D-19, followed by staining with hematoxylin and eosin. Labeled cell fractions were determined by direct counting of finished autoradiographs at a magnification of 400X. In the lung, 20 randomly-selected fields were scored for each section and tissue type including lung parenchyma and bronchial and terminal bronchiolar epithelia. Separate sections of nose and trachea were used for ARG analysis of epithelia.

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

D. Breathing Pattern and Minute Ventilation.

Breathing pattern, (frequency and tidal volume) and minute ventilation were measured periodically during exposures. In the 1 month exposure, breathing pattern and minute ventilation were measured on the first day of exposure and thereafter on the third day of each of the 4 weeks of exposure. In the 6 month exposure breathing pattern and minute ventilation were measured on the first and third days of weekly episodes every fourth week. 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 fit snugly around the rat's head and separated 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 a 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. Eight rats were exposed simultaneously and 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.

E. Airway Epithelial Permeability.

Procedures for permeability measurements were similar to those described previously (Bhalla et al., 1986). In animals anesthetized with sodium pentobarbital, polyethylene tubing

(PE-90) was placed in the trachea and a polyethylene catheter (P-10) was placed in the femoral artery. For measuring the permeability in the nose, the oropharynx in tracheostomized rats was filled with dental impression cream to block the posterior nares. A radiolabeled tracer inoculum containing ^{99m}Tc labeled diethylenetriaminepentaacetate (^{99m}Tc-DTPA, mol wt 492) in 0.1 ml phosphate buffered saline (PBS) 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. Blood samples of 0.10 ml were drawn from a femoral artery at 6, 7, 8, 9, and 10 minutes after the start of instillation. Blood samples were counted for ^{99m}Tc radioactivity in a gamma counter. Isotope counts for the two molecules were expressed as the percent of the inoculum transferred from the site of application to the entire blood volume of each rat. The index of epithelial permeability was obtained by interpolation of a regression of fraction of label transferred at the mid time point, T_i, for the 5 samples. Differences in the fractions of each labeled molecule transferred to the blood at time, T_i, in the control and the pollutant exposed groups reflected differences in epithelial permeability among the groups.

For measuring tracheobronchial permeability, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, IL), 5 mg/100 g body weight. The abdominal aorta was severed and a polyethylene catheter (ID 0.05 in; OD 0.09 in) was placed in the trachea and tied in place. An incision was made in the diaphragm to allow lung expansion during the lavage. Lungs were lavaged by introduction of 6 ml phosphate-buffered saline (PBS) through the tracheostomy tube followed by withdrawal of the fluid. The process of introduction and withdrawal was repeated 3 times and 3 ml of lavage fluid was recovered. The lavage fluid from each animal was centrifuged at 1000 rpm for 10 min. The supernatant was stored in liquid nitrogen for protein analyses at a later time. Total protein in the BAL was determined by a bicinchoninic acid (BCA) procedure (Smith et al., 1985) using a set of protein standards of bovine serum albumin (BSA) and Pierce BCA Protein Assay Reagents (Pierce Chemical CO, Rockford, IL).

F. Particle Clearance.

Respiratory tract clearance was measured at the end of the 1 month subchronic exposure. Early clearance was measured over the first 50 h following respiratory tract deposition of ^{51}Cr labeled polystyrene latex particles by the appearance of labeled particles in feces as they are cleared from the respiratory tract and swallowed. This measurement reflected primarily muco-ciliary clearance of particles from the upper respiratory tract. Late clearance was measured by counting lungs for ^{51}Cr content 30 days post exposure. This measure primarily reflected clearance of particles from the deep lung by macrophages.

6 mo exposure.

For the 6 month exposure, the protocol for respiratory tract clearance was modified to evaluate long term clearance over the course of the 6 month exposure period. Rats for the clearance measurement were shared with the epithelial permeability and macrophage function animal groups for an atmosphere exposure group sample size of 30. Rats were deposition labeled with ^{54}Mn polystyrene latex (PSL) particles 5 days before the beginning of the exposure. The uncleared lung burden of PSL was determined at the end of the 6 month exposure. ^{54}Mn was distinguished from ^{99}Tc for permeability measurements by their different emission energies. PSL appearing in lavaged macrophages after 6 months exposure was not detectable.

G. Bronchoalveolar Lavage Analysis and Macrophage Function.

Rats were anesthetized with sodium pentobarbital and killed by exsanguination via the abdominal aorta. The trachea was exposed, a small opening was made in the trachea just craniad of the bifurcation, and a cannula was inserted and tied in place. The lung lobes were then lavaged via the cannula using 7 ml of phenol red-free Hank's balanced salt solution, without Ca^{++} or Mg^{++} (HBSS). The HBSS was instilled and withdrawn 3 times and saved. The lavage procedure was repeated with a new aliquot of fluid and the fluids were pooled. The lavage was repeated a third time and the fluid was saved separately. The lavage fluids were centrifuged to recover the cells, and the supernatant from the first 2 lavages removed and frozen at -70° for later analysis of protein. The cell pellets were pooled and used to prepare slides for differential counts, phagocytosis assays, and Fc

receptor assays.

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

Phagocytic activity of pulmonary macrophages was measured by a suspension assay. In brief, 0.5 ml of each cell suspension was added to a polypropylene tube containing 2.0 ml of culture medium and 5 X 10^8 polystyrene latex spheres. The tubes were incubated with gentle agitation for 60 min. One ml of the suspension was pelleted onto a slide using a cytocentrifuge. The slides were fixed with methanol and immersed in xylene for 8 hr to remove excess PSL microspheres. The slides were stained with a Wright-Giemsa monochromatic stain (Diff-Quick) 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.

A rosette assay was used to determine the effect of exposure on Fc receptors. Lab-Tek chambers, each containing 1 x 10^5 cells in 0.1 ml of HBBS, were incubated for 1 hr at 37°C and non-adherent cells removed by washing with medium. Fc receptor binding ability of the macrophages was measured by rosette assay (Rao et al., 1980; Prasad et al., 1988; Kleinman et al., 1993). In brief, 0.1 ml of anti-Sheep Red Blood Cells (SRBC) antibody was added to each of the chambers and incubated for 30 minutes at 37°C. After incubation, the macrophages were washed gently to remove excess antibody and 0.1 ml of SRBC (1 x 10^7) was added to each chamber. The macrophages were then incubated with the SRBC for 30 minutes at 37 C, unbound SRBCs were washed away gently and the number of cells (out of a total sample of 300) attached with three or more SRBC's were counted using an inverted microscope. The antibody was prepared as follows. 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.

In the cell free supernate of bronchoalveolar lavage fluid, protein was analyzed as described in section II.E. Lactate dehydrogenase was analyzed spectrophotometrically by enzymatic conversion of lactate to pyruvate in the presence of NADH (Sigma Chemical). In the 1 month exposure, Interleukin-1 was determined by bioassay (Gery, 1980). Briefly, macrophages (10^6) were cultured overnight in RPMI-1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Samples were incubated with and without 5 ng/ml lipopolysaccharide (Sigma Chemical) as an activating agent. The macrophage conditioned medium (MCM) was dialyzed against 40 volumes of RPMI 1640 medium. The mitogenic activity of IL-1 was determined by adding dilutions of the MCM to fresh preparations of mouse thymus cells in the presence of actinomycin. After 24 h the preparations were pulse-labeled with tritiated thymidine. the cells were lysed and the DNA collected on glass fiber media. The samples were then counted in a liquid scintillation counter and the t-cell mitogenic activity of IL-1 was expressed as % of control. Prostaglandin E2 was determined by ELISA (Cayman Chemical) on MCM from preparations which were not stimulated by lipopolysaccharide.

H. Statistical Analysis.

This project included a one month exposure to 3 concentrations of mixture components and purified air control and a 6 month exposure to the medium level concentration, the O_3 component alone, and purified air control. Data for each biological endpoint variable were tested for a significant effects of exposure atmosphere using analysis of variance. Significant differences among atmosphere group means were tested with *a posteriori* multiple comparisons using Bonferroni adjustment of critical values. A primary purpose of the one month exposure to graded concentrations of the mixture was to identify an appropriate concentration for testing in the 6 month exposure. Multiple comparison testing therefore consisted of 3 comparisons: each mixture concentration to purified air control. In the 6 month exposure, the questions of interest included whether the mixture

or O_3 had significant effects and whether the effects of the mixture differed from the effects of the O_3 component alone. Thus, 3 comparisons were appropriate: pure air *vs.* mixture, pure air *vs.* O_3, and mixture *vs.* O_3. Comparisons were two-tailed, and the significance level was set at $p < 0.05$.

III. PRELIMINARY STUDIES

Two exposure experiments were performed in the preliminary stages of this project. The first was a test for circadian sensitivity differences to acute 4 h 0.6 ppm O_3 exposure to determine 1) whether there were pronounced day vs. night differences in O_3 sensitivity that should be considered in an exposure of rats which are nocturnally active and 2) whether such a difference in sensitivity, if present, could be clock-shifted 180 degrees in phase by reversing the light-dark cycle. The second exposure experiment performed prior to the 6 month exposure was a 2 month exposure to the 5 component mixture and to O_3 alone.

Both exposures were impacted by presence of pneumonitis in approximately half of the population of test animals which interfered with our ability to quantify pollutant exposure related lung injury. The results of these experiments were, nevertheless, vital to the successful performance of the project. Confining analyses to animals that did not show pneumonitis in histopathologic analysis, the circadian periodicity experiment did not show significant differences in breathing pattern between animals exposed in nose-only tubes during the dark *vs.* light phase, and oxidant induced lesions in the lungs were greater in animals exposed during the light phase. Exposure of rats in nose-only tubes may remove the effects of day-night activity differences in influencing inhaled dose of O_3. In any case, the absence of indications of increased O_3 sensitivity during the dark phase supported the plan to conduct the exposure experiments during the light phase. Discovery of pneumonitis in the circadian periodicity exposure and in the 2 month mixture exposure prompted the laboratory to switch rodent vendors and intensify animal quality control screening and barrier area laboratory protocols. These procedures substantially contributed to the success of the

1 month mixture dose-response and 6 month mixture-ozone comparison exposures.

IV. RESULTS

A. Exposure Atmosphere Characterization.

The concentrations of gas and particle components measured in the rats' breathing zone for the 1 month and 6 month exposures are shown in Tables 4 and 5. Rats were exposed 4h/day, 3 days/week in both exposures. The means are based on daily averages of each component over the exposure days of each study, and there was good agreement between the target and actual exposure concentrations.

Table 4. Component concentrations of air pollutant compounds in the 1 month exposure. Data are means and standard deviations of daily average values. ABS is NH_4HSO_4.

Exposure Group	Component Pollutant	Target Concentration	Exposure Concentration (Mean ± SD)
HIGH	O_3 (ppm)	0.60	0.59 ± 0.04
	NO_2 (ppm)	0.40	0.39 ± 0.01
	ABS (mg/m^3)	0.20	0.22 ± 0.05
	C (mg/m^3)	0.12	0.10 ± 0.02
	HNO_3 (mg/m^3)	0.10	0.11 ± 0.03
	Aerosol Size (μM MMAD)	0.3	0.31 ± 2.1 GSD
MEDIUM	O_3 (ppm)	0.30	0.30 ± 0.03
	NO_2 (ppm)	0.20	0.21 ± 0.02
	ABS (mg/m^3)	0.10	0.10 ± 0.02
	C (mg/m^3)	0.06	0.06 ± 0.02
	HNO_3 (mg/m^3)	0.05	0.04 ± 0.01
	Aerosol Size (μM MMAD)	0.3	0.31 ± 1.4 GSD
LOW	O_3 (ppm)	0.15	0.16 ± 0.02
	NO_2 (ppm)	0.10	0.11 ± 0.01
	ABS (mg/m^3)	0.05	0.05 ± 0.02
	C (mg/m^3)	0.03	0.03 ± 0.01
	HNO_3 (mg/m^3)	0.025	0.020±0.005
	Aerosol Size (μM MMAD)	0.3	0.29 ± 1.6 GSD

ALL GROUPS: Relative Humidity (%) 60.5 ± 0.3
Temperature (°C) 23.5 ± 0.8

Table 5. Component concentrations of air pollutant compounds in the 6 month exposure. Data are means of daily average values. ABS is NH_4HSO_4.

Exposure Group	Component Pollutant	Target Concentration	Exposure Concentration (Mean ± SD)
MIXTURE	O_3 (ppm)	0.30	0.29 ± 0.01
	NO_2 (ppm)	0.20	0.20 ± 0.01
	ABS (mg/m^3)	0.10	0.10 ± 0.02
	C (mg/m^3)	0.06	0.06 ± 0.01
	HNO_3 (mg/m^3)	0.05	0.05 ± 0.02
	Aerosol Size (μM MMAD)	0.3	0.42 ± 2.4 GSD
O_3 ALONE	O_3 (ppm)	0.30	0.30 ± 0.01

ALL GROUPS: Relative humidity (%) 58.9 ± 1.8
Temperature (°C) 23.1 ± 0.7

B. Respiratory Tract Morphometry and Histopathology

1. Lung Morphometry.

1 month exposure to 3 concentrations of the 5 component mixture resulted in significant reductions of alveolar chord length but no significant change in fixed lung volume or septal wall intercept (Table 6). To test for the possibility that trends of change in fixed lung volume influenced alveolar chord length, the ratio of chord length to the cube root of fixed lung volume was computed. This index also showed highly significant effects of exposure concentration (Table 6), and changes in fixed lung volume did not explain the differences in alveolar chord length.

Lung morphometric data for the 6 month exposure are shown in Table 7. There were trends for a decrease in fixed lung volume in exposure groups that were significant at

Table 6. Lung morphometry of rats in 1 month exposure to 3 concentrations of the multicomponent mixture. Data are mean ± SE, n.

	Purified Air	Low	Medium	High
Fixed lung volume, right lobes (ml)	6.47±0.10,10	6.85±0.07, 10	6.57±0.14, 10	6.25±0.16, 10
Alveolar chord length (μm)	34.7±1.3, 9	30.4±1.2, 8[a]	28.3±1.1, 9[b]	25.6±0.8, 9[c]
$\frac{\text{Chord length}}{\text{Fixed Volume}^{1/3}}$	18.7±0.8, 9	16.0±0.6, 8[d]	15.1±0.5, 9[e]	13.8±0.4, 9[f]
Septal wall intercept (μm)	6.94±0.31, 9	6.92±0.38, 8	7.21±0.24, 9	7.58±0.26, 9

[a] Mean significantly different from control, $t=2.7$, $p<0.05$.
[b] Mean significantly different from control, $t=4.1$, $p<0.001$.
[c] Mean significantly different from control, $t=5.9$, $p<0.001$.
[d] Mean significantly different from control, $t=3.2$, $p<0.05$.
[e] Mean significantly different from control, $t=4.3$, $p<0.001$.
[f] Mean significantly different from control, $t=5.9$, $p<0.001$.

the 1 month post exposure analysis. There were also trends for decreased alveolar chord length and increased septal wall intercept, but these differences were not statistically significant. Point count morphometry of lung tissue volume fractions of alveolar duct and bronchioles are shown in Table 8 and all data including other compartments are shown in Appendix Tables 1 and 2. In the 1 month exposure to the medium mixture concentration, there was a significant shift of tissue representation to the bronchiolar compartment. In the 6 month exposure to the mixture, there was increased representation of the alveolar duct compartment and decreased representation of alveolar and bronchiole compartments

Table 7. Lung morphometry in 6 month exposure to the 5 component mixture and O_3 alone. Data are mean ± SE, n.

END EXPOSURE	Purified air	Mixture	Ozone
Fixed lung volume (ml)	14.3±0.4, 10	13.1±0.4, 10	14.0 ± 0.2, 8
Alveolar chord length (μm)	39.8±1.0, 10	37.8±1.2, 10	36.7 ± 0.7, 10
$\frac{\text{Chord length}}{\text{Fixed Volume}^{1/3}}$	16.4±0.4, 10	16.0±0.4, 10	15.4±0.3, 8
Septal wall intercept (μm)	4.83±0.22, 10	5.12±0.27, 10	5.35 ± 0.19, 10
1 MONTH POST-EXPOSURE			
Fixed lung volume (ml)	14.8±0.2, 9	13.9±0.2, 10[a]	14.0±0.2, 10[a]
Alveolar chord length (μm)	37.6±0.5, 10	37.2±1.1, 10	36.8±0.5, 10
$\frac{\text{Chord length}}{\text{Fixed Volume}^{1/3}}$	10.7±0.1, 9	11.8±0.7, 10	11.6±0.4, 10
Septal wall intercept (μm)	4.34±0.06, 10	4.92±0.30, 10	4.81±0.18, 10

[a] Mean significantly different from control, $t=3.5$, $p<0.01$.
[b] Mean significantly different from control, $t=3.3$, $p<0.01$.

suggested at end-exposure analysis, and statistically significant at the 1 month post exposure analysis.

Table 8. Lung tissue volume fractions for alveolar duct and bronchioles in the 1 month and 6 month exposure experiments. Data are mean ± SE.

1 MONTH EXPOSURE	Purified Air	Low	Medium	High
	N = 10	N = 9	N = 10	N = 9
Alveoli	0.444±0.022	0.450±0.011	0.398±0.018	0.486±0.024
Alveolar duct	0.301±0.015	0.310±0.011	0.286±0.013	0.263±0.016
Bronchioles	0.023±0.005	0.035±0.004	0.049±0.005[a]	0.037±0.007

[a] Mean significantly different from control, t=3.6, p<0.01.

6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
End Exposure			
Alveoli	0.491±0.018, 10	0.466±0.010, 10	0.455±0.013, 10
Alveolar duct	0.359±0.012, 10	0.399±0.018, 10	0.391±0.014, 10
Bronchioles	0.061±0.006, 10	0.050±0.011, 10	0.066±0.011, 10
1 Month Post-exposure			
Alveoli	0.484±0.013, 10	0.412±0.012, 10[b]	0.433±0.020, 10
Alveolar duct	0.382±0.007, 10	0.440±0.013, 10[c]	0.419±0.013, 10
Bronchioles	0.046±0.009, 10	0.030±0.007, 10	0.078±0.017, 10

[b] Mean significantly different from control, t=2.6, p<0.05
[c] Mean significantly different from control, t=3.6, p<0.01

2. Nasal Epithelial Composition.

Nasal epithelial composition was significantly altered in dose dependent fashion by the 1 month exposure (Table 9). Both nasal respiratory epithelium and transition epithelium

showed a decrease in representation of ciliated cells and in transition epithelium there was a significant increase in the proportion of secretory cells. This pattern was not evident in the 6 month exposure to the medium concentration mixture (Appendix Table 3), although there was one significant difference in proportion of ciliated cells at the analysis 1 month post-exposure.

3. Epithelial Secretory Density.

Histochemical staining for secretory products within nasal respiratory epithelium showed a significant increase in alcian blue-periodic acid schiff stain density in rats exposed 1 month to the medium concentration of the 5 component mixture. However, no significant changes were observed in the 6 month exposure (Appendix Table 4). Tracheal epithelium, however, showed decreased stain density in medium and high concentrations at 1 month exposure. Depression of secretory activity was also present following 6 months exposure to the medium concentration (Table 10). This effect was not present for exposure to O_3 and the response showed recovery at the 1 month post-exposure analysis.

4. Mast Cell Density in the Lobar Bronchus.

Mast cell numbers in the lobar bronchus were not affected by the 1 month exposure to 5 component mixtures, but 6 months exposure to the medium concentration of the mixture significantly elevated the proportion of mast cells in the epithelium (Table 11). 1 month post-exposure, mast cell numbers had recovered toward control purified air values.

5. Acid Phosphatase Stain Density in Pulmonary Macrophages.

1 month exposure to graded concentrations of the 5 component mixture induced a shift in distribution of pulmonary macrophages among acid phosphatase stain density classes (Figure 1). The 6 month exposure did not result in significant differences in acid phosphatase density at end exposure, but there was a significant shift from class 1 to class 2 density for both groups exposed to the 5 component mixture and to O_3 alone.

Table 9. cellular composition of the nasal epithelium in 1 month exposure to 3 concentrations of the multicomponent mixture. Data are mean ± SE, n.

	Purified Air	Low	Medium	High
Respiratory Epithelium	n = 10	n = 10	n = 10	n = 10
Cell Type				
Ciliated	0.313±0.023	0.338±0.017	0.290±0.017	0.248±0.017[a]
Secretory	0.443±0.016	0.384±0.017	0.417±0.022	0.463±0.020
Basal	0.244±0.020	0.278±0.014	0.288±0.015*	0.290±0.022
Transition Epithelium	n = 10	n = 9	n = 9	n = 9
Cell Type				
Ciliated	0.286±0.026	0.214±0.021	0.209±0.028	0.117±0.028[b]
Secretory	0.430±0.018	0.491±0.021	0.499±0.021	0.562±0.025[c]
Basal	0.285±0.026	0.294±0.014	0.292±0.011	0.321±0.022

[a] Significantly different from control, $t=2.5$, $p<0.05$.
[b] Significantly different from control, $t=4.6$, $p<0.001$.
[c] Significantly different from control, $t=4.5$, $p<0.001$.

Table 10. Tracheal epithelial secretory density in 1 and 6 month exposures. Data are relative density of alcian blue-periodic acid schiff stain per epithelial cell (mean ± SE, n).

	Purified Air	Low	Medium	High
1 MONTH EXPOSURE	10.3±0.5, 10	9.55±0.73, 7	5.91±0.42, 10[a]	7.18±0.53,9[b]

6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
End-exposure	9.971±0.69, 9	7.31±0.54, 10[c]	9.31±0.85, 10
1 Month Post-exposure	8.10±0.75, 8	8.39±0.79, 10	8.07±0.67, 10

[a] Mean significantly different from control, $t = 6.3$, $p < 0.001$.
[b] Mean significantly different from control, $t = 4.3$, $p < 0.001$.
[c] Mean significantly different from control, $t = 2.6$, $p < 0.05$.

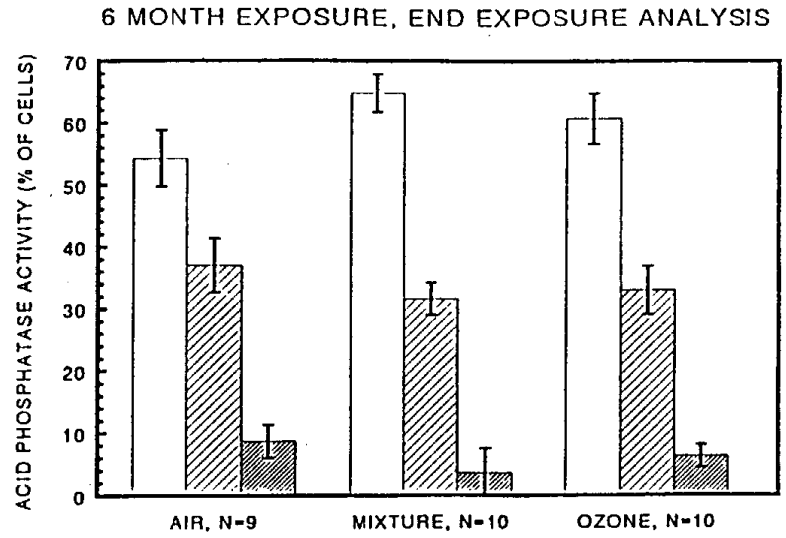
Table 11. Proportion of mast cells in epithelium of the lobar bronchus in 1 and 6 month exposures. Data are mast cells as a percent of epithelial cells (mean ± SE, n).

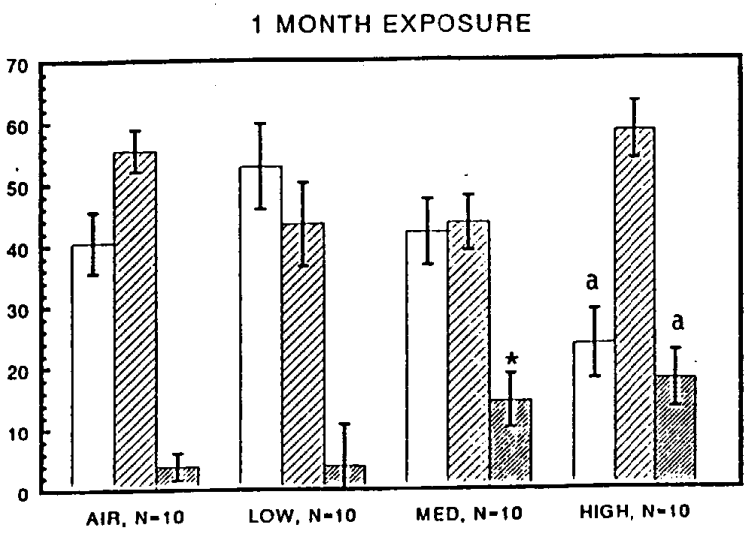
	Purified Air	Low	Medium	High
1 MONTH EXPOSURE	2.37±0.40, 8	2.02±0.35, 10	2.33±0.36, 8	2.29±0.41, 9

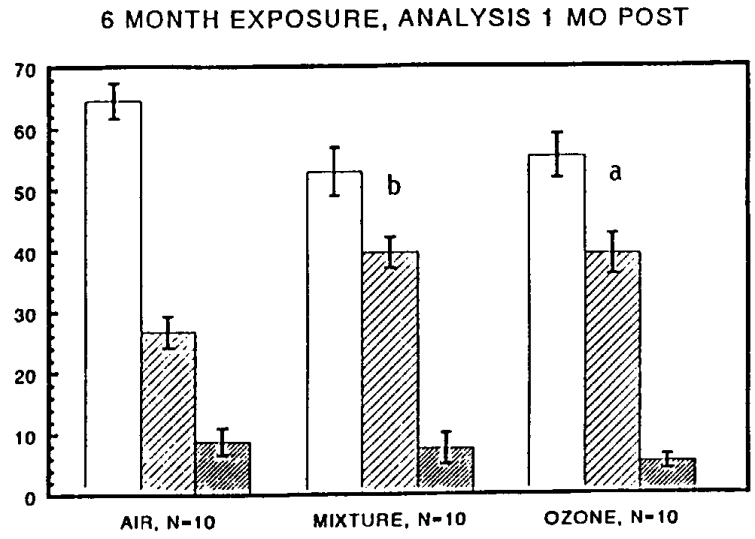
6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
End-exposure	2.66±0.29, 10	4.16±0.39, 10[a]	3.50±0.43, 9
1 Month post-exposure	2.16±0.25, 10	3.51±0.48, 10	3.12±0.37, 10

[a] Mean significantly different from control, $t = 2.8$, $p < 0.001$.

Figure 1. Acid phosphatase stain density in pulmonary macrophages from rats in 1 and 6 month exposures. Data are fraction of macrophages in each stain intensity class (mean ± SE, n = 10). Open panels are class 1, coarse cross hatched panels are class 2 and fine cross hatched panels are class 3 stain intensity. Footnote p values are for Bonferroni adjusted multiple comparisons.





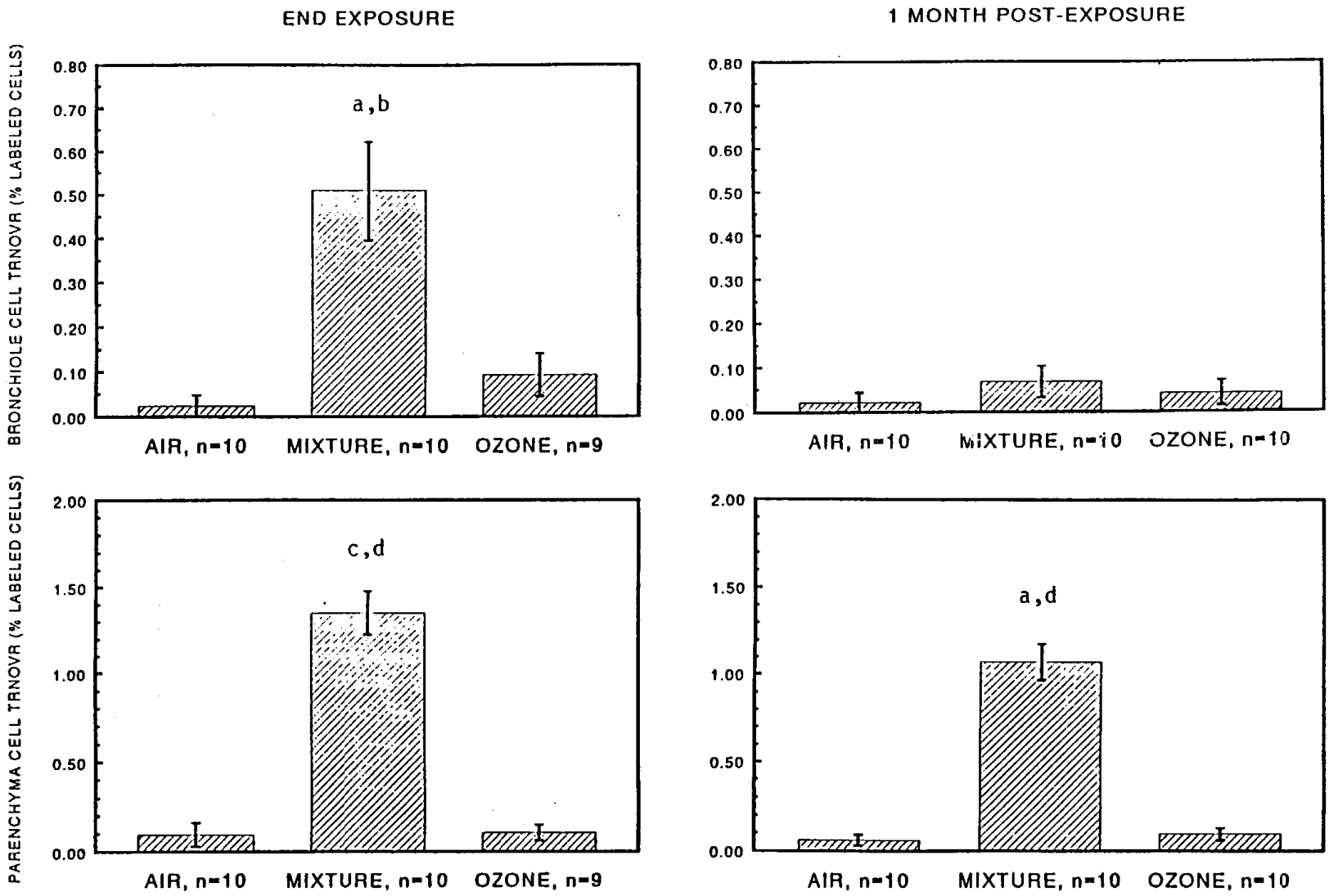


[a] Mean significantly different from control, $p < 0.05$.
[b] Mean significantly different from control, $p < 0.05$.

6. Epithelial Cell Proliferation.

Cell proliferation measured by incorporation of ^{3}H-thymidine into epithelial tissues is shown for all tissues samples in Appendix Tables 5 and 6, and for bronchiole and lung parenchymal tissues of rats in the 6 month exposure in Figure 2. The 1 month exposure resulted in significant elevation of epithelial cell proliferation in all measured tissues of the group exposed to the high concentration (Appendix Table 5). Tracheal epithelium and lung parenchyma had elevated proliferation rates in the medium and high concentrations. In the 6 month exposure to the medium level concentration,
significant elevation of cell proliferation was observed at end exposure in the terminal bronchiole epithelium and in the lung parenchyma (Figure 2 and Appendix Table 6). 1 month post-exposure, epithelial cell proliferation in these tissues showed recovery toward purified air control levels, however proliferation was still significantly elevated in the lung parenchyma.

Figure 2. Epithelial cell proliferation of rats in the 6 month exposure. Data are percent of epithelial cells labeled with ^{3}H-thymidine (mean ± SE, n). Footnotes are Bonferroni adjusted multiple comparisons.



[a] Mean significantly different from control, $p<0.001$.
[b] Mean significantly different from ozone, $p<0.01$.
[c] Mean significantly different from control, $p<0.01$.
[d] Mean significantly different from ozone, $p<0.001$.

C. Breathing Pattern and Minute Ventilation

In the 1 month exposure, breathing pattern (breath frequency and tidal volume) and minute ventilation were measured during the fourth hour of exposure on the first exposure day of week 1 and on the first and third exposure day of subsequent weeks. Exposure to the medium and high mixture concentrations induced a rapid-shallow breathing response on the first day of exposure (Table 12). Breathing pattern on the third exposure day of successive weeks is also shown in Table 12. Repeated measures analysis of variance was used to test for differences in the pattern of breathing pattern responses over the successive weeks of exposure. In high concentration, the rapid shallow breathing pattern response progressively increased as shown in significant exposure x time interactions for breath frequency and tidal volume. In the medium concentration, the low tidal volume response at hour 4 was successively diminished over the 4 weeks of episodic exposures, and by the end of the entire exposure, tidal volume in medium concentration had returned to near purified air control levels. These results demonstrate a pronounced distinction between responses to the high concentration and medium concentration mixtures; the medium concentration induced a small acute response that was diminished with repeated episodic exposure, while the high concentration induced a strong rapid-shallow breathing response that increased with repeated episodic exposure. Minute ventilation (Appendix Table 7) did not show consistent patterns of exposure effects.

Table 13 shows breathing pattern and minute ventilation of rats on the first exposure day of the 6 month exposure. Breath frequency and minute ventilation were significantly elevated during the fourth hour of exposure to the mixture, however tidal volume was not significantly changed, and as was the case in the 1 month exposure to the medium concentration, a clear rapid shallow breathing pattern response to this concentration of mixture was not present.

With repeated episodic exposure, data for hour 4 of the third exposure day at successive 4 week intervals showed significant main effects of exposure atmosphere for breath frequency of rats exposed to the mixture (Appendix Table 8). Breath frequency was significantly elevated in comparison to the purified air control group and to the O_3 group.

Table 12. Breath frequency, tidal volume, and minute ventilation in the 1 month exposure during hour 4 of day 1 and for the third day of successive weeks. Data are mean ± SE, n = 8.

	Purified Air	Low	Medium	High
Day 1				
Frequency (min-1)	157 ±7	149 ±5	178 ±12	231 ±6[a]
Tidal Volume (ml)	1.0 ±0.04	0.9 ±0.04	0.7 ±0.05[b]	0.5 ±0.04[c]
Minute Ventilation (ml min-1)	160 ±12	127 ±5	128 ±8	120 ±8

a significantly different from control, t = 6.8, p<0.001.
b significantly different from control, t = 4.8, p<0.001.
c significantly different from control, t = 8.2, p<0.001.

Frequency (min-1)	Purified Air	Low	Medium	High[d]
Day 3	165 ±7	146 ±6	175 ±7	191±12
10	159 ±10	159 ±7	175 ±12	227±17
17	140 ±4	152 ±8	161 ±9	275±22
24	163 ±7	157 ±6	145 ±6	304±11
Tidal Volume (ml)	**Purified Air**	**Low**	**Medium[e]**	**High[f]**
Day 3	0.8 ±0.04	0.9 ±0.1	0.8 ±0.03	0.6 ±0.1
10	0.7 ±0.1	0.9 ±0.1	1.0 ±0.1	0.6 ±0.1
17	1.1 ±0.03	1.0 ±0.04	1.0 ±0.04	0.6 ±0.04
24	1.0 ±0.04	0.8 ±0.1	1.0 ±0.1	0.4 ±0.03
Minute Ventilation (ml min-1)	**Purified Air**	**Low**	**Medium**	**High**
Day 3	135 ±5	126 ±9	133 ±7	114 ±9
10	113 ±6	142 ±7	164 ±13	132 ±12
17	149 ±6	146 ±9	157 ±5	155 ±11
24	159 ±9	129 ±7	132 ±9	127 ±7

d significantly different from control, exposure x time interaction F=10.8, p<0.0003.
e significantly different from control, exposure x time interaction F=6.1, p<0.002.
f significantly different from control, exposure x time interaction F=9.4, p<0.0003.

Table 13. Breath frequency, tidal volume, and minute ventilation in the 6 month exposure during hour 4 of day 1. Data are mean ± SE, n = 8.

	Purified Air	Mixture	Ozone
Frequency (min^{-1})	140 ±3	168 ±5[a,b]	130 ±4
Tidal Volume (ml)	1.1 ±0.06	1.2 ±0.11	1.2 ±0.04
Minute ventilation ($ml\ min^{-1}$)	148 ±9	199 ±16[c,d]	150 ±6

[a] Significantly different from control, $t=4.8$, $p<0.001$.
[b] Significantly different from O_3, $t= 6.4$, $p<0.001$.
[c] Significantly different from control, $t=3.1$, $p<0.05$.
[d] Significantly different from O_3, $t=3.0$, $p<0.05$.

One month post-exposure, breathing pattern and minute ventilation of rats tested in purified air did not differ among test exposures (Table 14).

Table 14. Breathing pattern of rats 1 month post-exposure measured in purified air. Data are mean ± SE, n.

	Exposure History		
	Purified Air	Mixture	Ozone
Breath Frequency (min^{-1})	108 ±6, 8	117 ±9, 8	129 ±8, 7
Tidal Volume (ml)	1.4 ±0.1, 8	1.3 ±0.2, 8	1.2 ±0.1, 7
Minute Ventilation ($ml\ min^{-1}$)	154 ±11, 8	150 ±15, 8	157 ±6, 7

D. Airway Epithelial Permeability

Epithelial permeability for nasal and bronchoalveolar regions are shown in Table 15. In the 1 month exposure, nasal epithelial permeability was not significantly altered by the exposure, although there was a trend for increased permeability in all exposure groups. Bronchoalveolar epithelial permeability was significantly increased in the group exposed to the high concentration of mixture. The 6 month exposure to the medium level concentration of the mixture induced a trend of increased nasal epithelial permeability which was not present when the analysis was performed at 1 month following the exposure.

Table 15. Epithelial permeability of rats in 1 month and 6 month exposures. Nasal epithelial permeability was 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 (mean ± SE, n). Tracheobronchial permeability is protein (mg/ml) in bronchoalveolar lavage fluid (mean ± SE, n).

1 MONTH EXPOSURE	Purified Air	Low	Medium	High
Nasal	0.18±0.04, 6	0.36±0.06, 6	0.35±0.07, 7	0.38±0.06, 7
Bronchoalveolar	0.224±0.011, 6	0.264±0.013, 6	0.262±0.021, 7	0.293±0.016, 7[a]

[a] Mean significantly different from control, t=3.0, $p < 0.05$.

6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
Nasal			
End-exposure	0.374±0.063, 9	0.693±0.124, 10	0.482±0.129, 9
1 Month post-exposure	0.345±0.080, 10	0.344±0.056, 10	0.459±0.107, 10
Bronchoalveolar			
End-exposure	0.235±0.013, 10	0.255±0.021, 10	0.237±0.023, 9
1 Month post-exposure	0.248±0.007, 11	0.250±0.009, 10	0.224±0.007, 11

E. Particle Clearance

Measures of respiratory tract clearance in the 1 and 6 month exposures are shown in Table 16. In the 1 month exposure, respiratory tract clearance was measured at the end of the exposure. Rats received ^{51}Cr labeled polystyrene latex particles by inhalation deposition, and early clearance was measured over the first 50 h post-deposition by counting activity of labeled particles cleared from the respiratory tract and appearing in the feces. This early clearance measurement primarily reflects muco-ciliary clearance of particles from the upper respiratory tract. Late clearance was measured by counting lungs for ^{51}Cr activity 30 days post exposure, and this measure primarily reflects clearance of particles from the

Table 16. Respiratory tract clearance in the 1 month and 6 month exposures. In the 1 month exposure early clearance, $T_{50\%}$, is time in hours required to excrete 50% of total activity excreted in first 50 h post deposition. Late clearance (A_{30d}) is an index of activity remaining in the lungs at 30 days post deposition normalized to activity present 2 days post-deposition. In the 6 month exposure, late clearance was measured by an index of activity remaining in the respiratory tract normalized to activity present 4 days post-deposition (A_{6mo}). Data are mean ± SE, n.

1 MONTH EXPOSURE	Purified Air	Low	Medium	High
Early Clearance				
$T_{50\%}$	14.2±0.5, 28	13.8±0.5, 29	14.3±0.4, 28	14.8±0.5, 29
Late Clearance				
A_{30d}	11.5±0.1, 30	11.5±0.1, 30	11.6±0.1, 30	11.4±0.1, 30

6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
Late Clearance			
A_{6mo}	30 ± 2, 33	36 ± 2, 32	33 ± 2, 24

deep lung by macrophages. For the longer 6 month exposure, particles were labeled with ^{54}Mn, and deposition was performed at the beginning of the 6 month exposure. The clearance measure is late clearance occurring over the course of the 6 month exposure and is based on activity remaining in lung tissues normalized to activity measured by chest counting following the early clearance phase. There were no significant differences in short or long term clearance in the 1 month exposure. In the 6 month exposure, there was a delay in late clearance of rats exposed to the mixture that was significant in a 2 sample comparison uncorrected for multiple comparisons, and the value for O_3 alone was intermediate between the purified air and mixture exposure values.

F. Bronchopulmonary Lavage Fluid Analysis and Macrophage Function

Analysis of bronchopulmonary lavage fluid showed trends toward increases but no significant effects of exposure atmospheres on lactate dehydrogenase, prostaglandin E_2, or Interleukin-1 in the 1 month exposure nor were there significant effects of the 6 month exposure on cell differential or lactate dehydrogenase (Appendix Table 9). Pulmonary macrophage functions analyzed in the 1 month and 6 month exposures are shown in Table 17. In the 1 month exposure, the high concentration mixture induced a significant reduction in both FcR binding capacity and in phagocytosis of latex polystyrene particles. In the 6 month exposure there was a significant depression of FcR binding capacity in rats exposed to the mixture and analyzed at end-exposure. This response showed recovery at 1 month post-exposure, however there was a trend for depressed FcR binding still present and this trend was also present for O_3 alone, but only at 1 month post-exposure.

Table 18 shows fractions of macrophages that contained carbon particles from animals in 1 month and 6 month exposures to 5 component mixtures. In the 1 month exposure, analysis of macrophages recovered from bronchoalveolar lavage and of macrophages present in histological sections of lung showed highly significant linear relationships to exposure concentration index: [carbon in BAL macrophages] = -0.009 + 0.068 C, $p<0.001$, $r^2=0.76$ and [carbon in lung macrophages] = 0.034 + 0.23 C, $p<0.001$,

Table 17. Pulmonary macrophage function assays of macrophages recovered from bronchoalveolar lavage in the 1 month and 6 month exposures. Data are mean ± SE, n. Viability is percent viable macrophages in BAL. Fc receptor binding capacity is percent rosette formation of macrophages with sheep red blood cells and IgA antibody. In vitro phagocytosis is percent macrophages containing 2 or more particles following challenge with a suspension of polystyrene latex microspheres.

1 MONTH EXPOSURE	Purified Air	Low	Medium	High
Fc receptor binding (%)	49.9±3.3, 10	44.3±3.1, 10	41.3±1.9, 10	38.7±3.7, 10[a]
Polystyrene latex phagocytosis (%)	51.5±5.8, 10	35.8±5.1, 10	35.7±7.7, 10	27.6±5.1, 10[b]

[a] Mean significantly different from control, t=2.6, p<0.05.
[b] Mean significantly different from control, t=2.8, p<0.05.

6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
Macrophage viability (%)			
End-exposure	93.7 ± 0.6, 15	94.0 ± 0.6, 13	94.8 ± 0.6, 12
1 Month post-exposure	87.6 ± 1.1, 12	86.2 ± 2.3, 12	86.7 ± 1.6, 12
Fc receptor binding (%)			
End-exposure	54.8 ± 2.0, 14	47.7 ± 1.3, 12[c,d]	58.3 ± 1.3, 12
1 Month post-exposure	56.2 ± 1.5, 12	52.0 ± 1.7, 12	51.0 ± 1.9, 12
Polystyrene latex phagocytosis (%)			
End-exposure	50.4 ± 1.2, 14	46.0 ± 2.5, 13	52.4 ± 2.2, 11
1 Month post-exposure	57.0 ± 1.9, 11	59.0 ± 3.6, 11	57.2 ± 3.0, 10

[c] Mean significantly different from control, t=3.1, p < 0.05.
[d] Mean significantly different from ozone, t=4.5, p < 0.001.

r^2=0.61. In the 6 month exposure, there was a greater proportion of macrophages containing carbon in the sample population recovered in bronchoalveolar lavage than there was in the sample from lung parenchymal section. Bronchoalveolar lavage macrophages also showed a large decrease in numbers containing carbon between the end-exposure and the 1 month post-exposure analyses, and while there was a decline in proportion containing carbon in the parenchyma population, the difference was not statistically significant.

Table 18. Carbon particles in macrophages recovered from bronchoalveolar lavage and in lung parenchyma for rats exposed to 5 component mixtures in the 1 and 6 month exposures. Data are percent of macrophages containing carbon particles (mean ± SE, n). Data for lung parenchyma are from histological sections from animals that were not lavaged.

1 MONTH EXPOSURE	Low	Medium	High
Bronchoalveolar lavage	6.4±2.1, 12	10.9±2.1, 12	24.8±2.3, 12
Lung parenchyma	0.22±0.07, 10	0.64±0.12, 10	0.90±0.12, 10

6 MONTH EXPOSURE	End-exposure	1 Month Post-exposure
Bronchoalveolar lavage	63.0 ± 4.3, 11	44.0 ± 2.6, 11[a]
Lung parenchyma	1.41 ± 0.14, 10	1.24 ± 0.16, 10

[a] Mean significantly different from end-exposure, t=4.4, p<0.001.

DISCUSSION

The 6 month exposure to the mixture at the medium level concentrations of the oxidant-acid-particle components (Table 5) induced significant alterations of respiratory tract structure and function. The O_3 component alone generally did not show significant changes, however for variables in which the mixture showed significant effects, there were usually trends of change in O_3 alone that were in the same direction. These patterns are described below for the broad categories of biological endpoints analyzed and are followed by a summary of the exposure effects.

Respiratory Tract Morphometry and Histopathology.

Morphometric analysis of lung tissues showed significant reductions in fixed lung volume in rats exposed 6 months to the 5 component mixture and to the O_3 component alone (Table 7). The most pronounced changes occurred 1 month post-exposure although trends of change were present at end-exposure, and these included changes in alveolar chord length and septal wall thickness. The 1 month exposure to the medium level concentration induced larger changes (Table 6) in chord length and septal wall intercept and indicates that the subchronic response at 1 month is attenuated with extended repeated exposure. The differences between the end 6 months exposure analysis and 1 month post-exposure analysis were not substantial; while crossing the critical values for statistical significance between end-exposure and 1 month post-exposure, the trends were present at end-exposure, and the patterns likely represent conditions that persisted rather than any dramatic change in the elastic properties of the lungs or major remodeling of alveoli over the 1 month post-exposure period. Volume fractions of lung tissue compartments in the 6 month exposure showed a shift of representation from alveoli to alveolar duct in the mixture group with a similar trend present in O_3 alone (Table 8), again most prominent in the analysis at 1 month post-exposure. The most likely explanation for the small changes in morphometric variables observed at the end and 1 month following the 6 month exposure is a small change in elasticity of peripheral lung tissues. The 1 month exposure likely involved a pulmonary inflammatory effect, because lesions were visible in lungs from the high concentration group,

fixed lung volume was not consistently affected and differences in volume did not remove the effect on chord length when expressed per unit cube root of volume (Table 6).

Nasal epithelial cell composition shifted to increased representation of secretory cells in 1 month exposure to the high concentration mixture (Table 9). Although this trend was present in the medium concentration group at 1 month exposure, in the 6 month exposure the trend appeared in the transition epithelium of the 1 month post-exposure group of animals but not in the end-exposure group (Appendix Table 3). While the 1 month exposure established a strong mixture dose-response relationship for nasal epithelial composition, the medium level concentration has a small effect that was not readily detected in the 6 months exposure.

Epithelial secretory products (glycoprotein compounds) in the epithelial tissues of the nose and trachea showed opposite responses to 1 month exposure to mixture concentrations (Table 10 and Appendix Table 4). Secretory density in the nasal respiratory epithelium increased while tracheal epithelial secretory density decreased with mixture concentration in the 1 month exposure. In the 6 month exposure, nasal secretory density did not show consistent responses, however tracheal secretory activity remained depressed in the mixture exposure group at end exposure. Clearly, the relationships of epithelial secretory density to exposure and respiratory tract position are complex, however Harkema et al. (1993) also found increased secretory density in the nasal epithelium and decreased secretory density in the tracheal epithelium of rats exposed to 1.0 ppm O_3 6 h/d, 5 d/wk, for 20 months. The tracheal response is apparently relatively labile, for in the present study, secretory density showed recovery 1 month post-exposure (Table 10).

The proportion of mast cells in epithelium of the lobar bronchus increased by 50% following the 6 month exposure to the mixture (Table 11). The fact that this response was not observed in the 1 month exposure to an even higher concentration as well as the medium level concentration indicates that this response requires a extended exposure to be elicited. 1 month post-exposure, mast cell numbers showed a recovery response, although a trend for elevated numbers was still present. Exposure to O_3 alone resulted in a trend, although non-significant, of increased mast cells numbers. Increased number of mast cells in bronchial airways could lead to increased sensitivity to airborne allergens resulting in

increased incidence or severity of asthma.

Pulmonary macrophages in lung tissues prepared with acid phosphatase stain showed significant changes in intracellular acid phosphatase density (Figure 1). In the 1 month exposure, there was a clear dose-related shift in proportions of cells toward higher density of acid phosphatase. In the 6 month exposure, this response was not as prominent; the shift appeared only at the 1 month post-exposure analysis point. The response was, however, present for O_3 exposed animals as well as those exposed to the mixture. The consequences of this response are uncertain. It clearly represents a response of the macrophages to the exposure, however, whether it represents increased synthesis and release rate or simply increased storage of this enzyme is not known at present.

One of the most significant findings of this investigation was the increased rates of epithelial cell proliferation and turnover rates observed in terminal bronchioles and lung parenchymal tissues in the 6 month exposure (Figure 2 and Appendix Tables 5 and 6). In the 1 month exposure to the high concentration of mixture, nearly all epithelial tissues sampled from the nose to the lung parenchyma had elevated proliferation rates. In the medium level concentration, trends of elevated rates were apparent for most tissues and significant differences were present in trachea and lung parenchyma. With longer term exposure in the 6 month exposure, elevated cell turnover persisted in the terminal bronchioles and lung parenchyma of mixture exposed animals (Figure 2), and 1 month following exposure, turnover was still elevated in the parenchyma. Exposure to the mixture resulted in an irritation of pulmonary tissues that leads to early cell death and high turnover rates. This pattern apparently continued throughout the exposure period and the observation that high turnover rate persisted through 1 month post-exposure suggests that elevated proliferation may be related to the continued presence of particles in the lung also observed at 1 month post-exposure (Table 18).

Breathing Pattern and Minute Ventilation.

In the 1 month exposure, the first day of exposure induced a strong rapid-shallow breathing pattern in the high concentration and a weak rapid-shallow breathing pattern in

the medium concentration (Table 12). These shifts in frequency and tidal volume that developed over the course of the 4 h exposure are indicative of pulmonary irritation and are the well known response to oxidant inhalation. With the successive days and weekly episodes of exposure, the medium concentration exposure group showed an attenuation of the rapid-shallow breathing response on the third days of successive episodes, whereas the high concentration group had a progressively exacerbated response. In the 6 month exposure, rapid-shallow breathing was not evident, however breath frequency was significantly elevated over the third days of episodes sampled at monthly intervals (Appendix Table 8). Over the 6 month exposure frequency during hour 4 on the third days of episodes averaged 143 min^{-1} in the mixture and 129 min^{-1} in purified air control. During hour 1 of these exposure days there was no difference (average mixture exposed frequency = 132 min^{-1} and average control frequency = 136 min^{-1}). Ozone alone did not induce significant main effects of exposure on breath frequency, and 1 month following the 6 month exposure, breathing patterns of rats measured in purified air were not significantly different (Table 14). Because the breath frequency response to mixture appeared only on the last days and not on the first days of the 3 day episodes, and then only in the last of 4 hours of exposure to the mixture, it likely represents an irritant response to the 3 successive days of exposure. If it were due to any progressive change in mechanical properties of the lungs developing over the 6 month period, it would be expected to develop over all days measured rather than only on the third days of episodes and it would be expected to persist through the 1 month post-exposure period. The frequency response to the mixture was not large and represents the continued pattern of pulmonary irritation also evidenced by increased epithelial cell proliferation rates present in the mixture exposure group.

Airway Epithelial Permeability.

In the 1 month exposure to the high concentration mixture, epithelial permeability in the bronchoalveolar zone was significantly increased (Table 15). However, in the 6 month exposure the medium level concentration did not significantly alter bronchoalveolar permeability. Nasal epithelial permeability showed a strong trend of increase at the end of

the 6 month exposure to the mixture. This trend was not present for the exposure to O_3 alone, nor for the exposure to the mixture analyzed 1 month post-exposure. Although the increased nasal epithelial permeability in the group exposed to the mixture did not quite reach the criterion for statistical significance with 3 multiple comparisons, this trend is noteworthy, because the nasal epithelium is the first line of respiratory tract defenses, and increased permeability can result in increased sensitivity to allergens and infectious agents.

Particle Clearance.

The only significant effect on clearance was an inhibition of long-term (presumably deep lung) clearance after 6 months of exposure to the mixture. Because effects were not seen in any group at 1 month of exposure, we conclude that cumulative alterations in the lung are responsible. Compared to controls, the lung burdens of tracer particles (6 months after deposition) were 20% greater in the mixture-exposed rats. This is a substantial increase in uncleared material. The implications to chronic lung disease are unclear. However, in the cases of the pneumoconioses (lung dust diseases) and pleural mesothelioma (asbestos cancer), uncleared toxic material is clearly causal. The perturbation in clearance observed here should not be forgotten in relation to the potential for air pollutant inhalation to cause or exacerbate chronic deep lung diseases.

Bronchoalveolar Lavage Analysis and Macrophage Function.

Fc receptor binding as measured in this study by the antibody-directed rosette formation of sheep red blood cells, is indicative of the alveolar macrophages ability to target and attack foreign antigenic material. this is an important part of the pulmonary defense against inhaled pathogens and impairment of this function would suggest increased susceptibility to respiratory infections.

Immediately after the conclusion of the 6 month exposure, rats exposed to the mixture showed a significant decrease in macrophage Fc receptor binding compared to

controls, while rats exposed to O_3 alone did not. There are several possible mechanisms by which FcR binding might be reduced. One possible mechanism might be a reduction in the number of Fc receptors on the macrophage surface or a decrease in the affinity of the receptor for the Fc fraction of immunoglobulins. This has been reported for macrophages from rats exposed for 8 weeks to components of PM10 (Ziegler et al., in press). Another possible mechanism might be a change in the characteristics of the macrophage membrane, such as a change in fluidity. Such changes have been seen after oxidative stress induced by inhaled toxicants such as environmental tobacco smoke (Hannon et al., 1989) and other compounds found to alter phagocytic activity (Ross et al., 1992; Illinger et al., 1990; Buescher et al., 1990). While membrane surface characteristics such as fluidity were not measured in this study, we did measure non-specific phagocytic activity, which is in part dependent upon membrane surface characteristics. Exposure to the mixture for 6 months caused a small, but not significant, reduction in phagocytic activity.

Taken together, these findings suggest that exposure to the mixture resulted in impairment of phagocytic activity in general. This suggests that lung clearance might also be impaired, and is consistent with the clearance results shown in Table 16. It is interesting to note, however, that while one month post-exposure, phagocytosis of PSL particles returned to control values, FcR binding was still depressed relative to control although the level of significance was poorer. Continued exposure to residual particles in the lung (Table 18), as demonstrated by carbon measurements in the lung parenchyma, could be responsible for reduced expression or affinity of macrophage surface Fc receptors.

Summary of Effects of 6 Months Exposure to the 5 Component Mixture and O_3 Alone.

The 6 month chronic exposure to episodes of the medium level concentration of the oxidant-acid-particle mixture (Table 5) resulted in significant alterations of respiratory tract structure and function. This medium or intermediate level concentration was selected for study based on the results of the 1 month exposure to 3 sets of mixture concentrations. The medium level mixture had showed trends or significant effects among several of the

biological endpoint variables analyzed following 1 month of exposure. It was hypothesized that with 6 months of exposure to this mixture, trends of response would show an emergent pattern of interrelated effects indicative of increased risk of pulmonary disease. Furthermore, if such a set of responses were present with long-term exposure to this intermediate level mixture, the results would form a strong basis to test the hypothesis that the effects of the mixture differed from the effects of the O_3 component of this mixture. Finally, the medium concentration level represented pollutant concentrations observed in the urban environment of Azusa and adjacent parts of the San Gabriel Valley in the South Coast Air Basin, and was therefore a relevant mixture level for toxicological evaluation.

The principal changes observed following the 6 month exposure to this mixture included elevated cell proliferation rates in the epithelium of terminal bronchioles and alveoli (Figure 2), increased numbers of mast cells in the lobar bronchi (Table 11), a depression of tracheal epithelial glycoprotein density (Table 10), depression of pulmonary macrophage Fc receptor binding capacity (Table 17), elevation of breath frequency on the final days of episodes (Table 13 and Appendix Table 8), trends of increased permeability of the nasal epithelium (Table 15), and decrease in long term particle clearance rate (Table 16). These results are summarized in Table 19 which catalogs significant changes in comparison to purified air controls observed in biological endpoint variables. In nearly all cases, significant differences among the 3 groups were differences between a pollutant exposure (O_3 or mixture) and purified air control. It is striking that while exposure to the mixture significantly affected a substantial number of these variables, exposure to O_3 alone showed very few significant effects. It is apparent that at the concentrations used in this study, the mixture is considerably more toxic than O_3 alone. This does not necessarily mean that O_3 had no effect and that the toxic effects of the mixture are due to compounds other than O_3. There is, in fact, evidence in the emergent pattern of significance testing that O_3 alone had effects that paralleled the effects of the mixture but were not large enough to be significant for individual variables with the sample sizes used in this study. In nearly all cases in which the mixture was significantly different from purified air control, the mixture effect was <u>not</u> significantly different from O_3 alone. The reason for this is that when the mixture effects were significantly different from control, the mean value

Table 19. Summary of biological responses of 6 months chronic exposure to the medium concentration 5 component oxidant, acid, and fine particle pollutant mixture and to the ozone component alone. Plus symbols indicate presence of statistically significant effects in 1 or more variables in the variable category and minus symbols indicate no significant effects observed. Data are shown for pollutant exposure *vs.* purified air control with Bonferroni correction for 3 multiple comparisons ($p < 0.05$).

	End-exposure		1 Month Post Exposure	
	Mixture	O_3	Mixture	O_3
Lung morphometry	-	-	+	+
Epithelial cell composition				
Nose	-	-	+	-
Mast cells in lobar bronchus	+	-	-	-
Epithelial secretory density				
Nose	-	-	-	-
Trachea	+	-	-	-
Pulmonary macrophage acid phosphatase activity	-	-	+	+
Epithelial cell proliferation				
Upper respiratory tract	-	-	-	-
Lower respiratory tract	+	-	+	-
Breathing pattern	+	-	-	-
Epithelial permeability				
Upper respiratory tract	-	-	-	-
Lower respiratory tract	-	-	-	-
BAL macrophage functions				
Fcr binding	+	-	-	-
Phagocytosis	-	-	-	-
Lavage fluid analysis				
Cell differential	-	-	-	-
LDH	-	-	-	-
Long term particle clearance	-	-	-	-

for O_3 exposure was usually intermediate between that of the mixture and purified air control. If O_3 alone had no effect on the variables, the mean value for the purified air control group should be intermediate to O_3 and mixture as often as O_3 is intermediate to control and mixture, and significance testing of mixture *vs* O_3 would show many significant differences. The only cases in which an O_3 mean was not intermediate when the mixture was significant (resulting in significant differences between mixture and O_3) were for breath frequency in hour 4 of day 1 (Table 13) and end-exposure FcR binding capacity (Table 17), and the only additional case in which the test of mixture *vs* O_3 was significant was for epithelial cell proliferation in the terminal bronchioles and parenchyma (Figure 2). The consistent pattern of O_3 appearing as the intermediate group argues for the presence of a small O_3 effect that was too small to be statistically significant in tests of individual variables with the sample sizes designed for this study. Therefore, the salient feature of the effects of the mixed pollutant atmosphere may be an interaction of O_3 with the acid, nitrogen oxides, and particulate components of the mixture.

The changes observed in the 6 month exposure suggest that continued episodic exposure to the mixture can result in continued irritation of lung tissues and can compromise pulmonary defenses. This is consistent with hypotheses relating pollutant exposures to increased risks of lung infections, stiffening of the lung tissues (which could be an early indication of development of lung fibrosis), and release into the lung of inflammatory mediators and other serum factors. These events could aggravate asthma and could, in turn, promote chronic lung inflammation which, in humans, is strongly related to the development of chronic bronchitis and emphysema. In previous studies we have demonstrated acute effects of ozone alone on respiratory structure and function. The results of the present study suggest that longer term exposure to ozone alone does not produce large changes in the biological endpoints measured, but a multicomponent mixture of oxidants, acids, and particles can produce a set of adverse biological effects on the respiratory system.

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APPENDIX

Supplemental Tables of Results.

Table 1. Lung tissue volume fractions in the 1 month exposure experiment.

Table 2. Lung tissue volume fractions in the 6 month exposure experiment.

Table 3. Cellular composition of the nasal epithelium in 6 month exposure to the 5 component mixture and O_3 alone.

Table 4. Nasal respiratory epithelial secretory density in 1 and 6 month exposures.

Table 5. Epithelial cell proliferation of rats in 1 month exposure to 3 concentrations of the multicomponent mixture.

Table 6. Epithelial cell proliferation of rats in 6 month exposure to the multicomponent mixture and to O_3 alone.

Table 7. Minute ventilation (ml/min) of rats during hour 4 of exposures in the 1 month exposure experiment.

Table 8. Breathing pattern and minute ventilation during hour 4 of the third exposure days of weeks 1, 4, 8, 12, 16, 20, 24, and 26 in the 6 month exposure experiment.

Table 9. Bronchoalveolar lavage fluid analyses in the 1 month and 6 month exposures.

Table 1. Lung tissue volume fractions in the 1 month exposure experiment. Data are mean ± SE.

	Purified air N = 10	Low N = 9	Medium N = 10	High N = 9
Alveoli	0.444±0.022	0.450±0.011	0.398±0.018	0.486±0.024
Alveolar duct	0.301±0.015	0.310±0.011	0.286±0.013	0.263±0.016
Bronchioles	0.023±0.005	0.035±0.004	0.049±0.005[a]	0.037±0.007
Lobar and secondary bronchi	0.152±0.030	0.133±0.014	0.189±0.021	0.159±0.026
Vascular tissue	0.074±0.007	0.070±0.012	0.073±0.013	0.041±0.007
BALT	0.005±0.002	0.003±0.002	0.004±0.002	0.006±0.004

[a] Mean significantly different from control, $t=3.6$, $p<0.01$.

Table 2. Lung tissue volume fractions in the 6 month exposure experiment. Data are mean ± SE.

END-EXPOSURE	Purified air	Mixture	Ozone
Alveoli	0.491±0.018, 10	0.466±0.010, 10	0.455±0.013, 10
Alveolar duct	0.359±0.012, 10	0.399±0.018, 10	0.391±0.014, 10
Bronchioles	0.061±0.006, 10	0.050±0.011, 10	0.066±0.011, 10
Lobar and secondary bronchi	0.062±0.014, 10	0.045±0.019, 10	0.054±0.016, 10
Vascular tissue	0.026±0.006, 10	0.038±0.009, 10	0.028±0.007, 10
BALT	0.000±0.000, 10	0.002±0.002, 10	0.006±0.003, 10
1 Month post-exposure			
Alveoli	0.484±0.013, 10	0.412±0.012, 10[a]	0.433±0.020, 10
Alveolar duct	0.382±0.007, 10	0.440±0.013, 10[b]	0.419±0.013, 10
Bronchioles	0.046±0.009, 10	0.030±0.007, 10	0.078±0.017, 10
Lobar and secondary bronchi	0.061±0.014, 10	0.058±0.014, 10	0.047±0.014, 10
Vascular tissue	0.026±0.008, 10	0.032±0.007, 10	0.026±0.006, 10
BALT	0.002±0.001, 10	0 001±0.001, 10	0.003±0.003, 10

[a] Mean significantly different from control, $t=2.6$, $p<0.05$
[b] Mean significantly different from control, $t=3.6$, $p<0.01$

Table 3. Cellular composition of the nasal epithelium in 6 month exposure to the 5 component mixture and O_3 alone. Data are mean ± SE, n.

RESPIRATORY EPITHELIUM	Purified Air	Mixture	Ozone
End-exposure	n = 9	n = 7	n = 9
ciliated cells	0.393 ± 0.011	0.378 ± 0.009	0.381 ± 0.009
Secretory cells	0.310 ± 0.013	0.334 ± 0.017	0.302 ± 0.008
Basal cells	0.297 ± 0.017	0.288 ± 0.009	0.317 ± 0.010
1 Month post-exposure	n = 7	n = 10	n = 9
ciliated cells	0.370 ± 0.014	0.376 ± 0.009	0.368 ± 0.008
Secretory cells	0.303 ± 0.010	0.301 ± 0.006	0.309 ± 0.012
Basal cells	0.326 ± 0.009	0.323 ± 0.010	0.324 ± 0.012
TRANSITION EPITHELIUM			
End-exposure	n = 8	n = 7	n = 9
ciliated cells	0.489 ± 0.022	0.515 ± 0.018	0.469 ± 0.012
Secretory cells	0.289 ± 0.013	0.286 ± 0.014	0.303 ± 0.013
Basal cells	0.222 ± 0.022	0.199 ± 0.01	0.227 ± 0.006
1 Month post-exposure	n = 7	n = 10	n = 8
ciliated cells	0.525 ± 0.009	0.476 ± 0.014	0.510 ± 0.015
Secretory cells	0.245 ± 0.010	0.290 ± 0.015	0.284 ± 0.011
Basal cells	0.230 ± 0.010	0.234 ± 0.013	0.206 ± 0.010

Table 4. Nasal respiratory epithelial secretory density in 1 and 6 month exposures. Data are relative density of alcian blue-periodic acid schiff stain per epithelial cell (mean ± SE, n).mean ± SE, n.

	Purified Air	Low	Medium	High
1 MONTH EXPOSURE	9.83±1.27, 10	8.98±0.90, 10	14.9±1.5, 10[a]	13.6±1.4, 10

6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
End-exposure	10.78±0.55, 10	8.72±0.81, 10	11.40±2.08, 9
1 Month post-exposure	8.48±0.78, 7	9.40±0.70, 10	11.22±1.24, 9

[a] Mean significantly different from control, $t = 2.8$, $p < 0.05$.

Table 5. Epithelial cell proliferation of rats in 1 month exposure to 3 concentrations of the multicomponent mixture. Data are percent of epithelial cells labeled with ^{3}H-thymidine (mean ± SE, n).

	Purified Air	Low	Medium	High
Nasal respiratory epithelium	0.10±0.03, 9	0.11±0.06, 10	0.19±0.07, 10	0.64±0.13, 10[a]
Nasal transition epithelium	0.10±0.05, 9	0.07±0.06, 10	0.29±0.06, 10	2.03±0.59, 10[b]
Trachea	0.12±0.06, 10	0.58±0.17, 8	0.60±0.15, 10[c]	0.74±0.15, 10[d]
Lobar bronchus	0.04±0.04, 8	0.03±0.03, 10	0.38±0.29, 8	0.33±0.15, 9
Terminal bronchiole	0.17±0.10, 10	0.26±0.17, 10	0.84±0.17, 10	7.35±7.24, 10[e]
Lung parenchyma	0.67±0.14, 10	1.01±0.12, 10	1.40±0.09, 10[f]	2.08±0.22, 10[g]

[a] Mean significantly different from control, $t=4.8$, $p<0.001$.
[b] Mean significantly different from control, $t=4.4$, $p<0.001$.
[c] Mean significantly different from control, $t=2.6$, $p<0.05$.
[d] Mean significantly different from control, $t=3,3$, $p<0.01$.
[e] Mean significantly different from control, $t=4.4$, $p<0.001$.
[f] Mean significantly different from control, $t=3.4$, $p<0.01$.
[g] Mean significantly different from control, $t=6.6$, $p<0.001$.

Table 6. Epithelial cell proliferation of rats in 6 month exposure to the multicomponent mixture and to O_3 alone. Data are percent of epithelial cells labeled with ^{3}H-thymidine (mean ± SE, n).

END-EXPOSURE	Purified Air	Mixture	Ozone
Nasal respiratory epithelium	0.30±0.11, 10	0.07±0.05, 9	0.23±0.07, 10
Nasal transition epithelium	0.08±0.06, 10	0.04±0.04, 9	0.11±0.08, 10
Trachea	0.37±0.13, 9	0.63±0.21, 9	0.70±0.15, 10
Lobar bronchus	0.03±0.03, 10	0.09±0.05, 10	0.03±0.03, 9
Terminal bronchiole	0.02±0.02, 10	0.51±0.11, 10[ab]	0.09±0.05, 9
Lung parenchyma	0.10±0.07, 10	1.35±0.13, 10[cd]	0.11±0.05, 9
1 MONTH POST-EXPOSURE			
Nasal respiratory epithelium	0.10±0.05, 6	0.53±0.34, 10	0.10±0.05, 8
Nasal transition epithelium	0.15±0.07, 6	0.60±0.49, 10	0.06±0.06, 8
Trachea	0.41±0.12, 9	0.74±0.18, 10	0.58±0.17, 10
Lobar bronchus	0.04±0.04, 10	0.07±0.04, 10	0.03±0.03, 10
Terminal bronchiole	0.02±0.02, 10	0.07±0.03, 10	0.04±0.03, 10
Lung parenchyma	0.06±0.03, 10	1.07±0.11, 10[ef]	0.09±0.04, 10

[a] Mean significantly different from control, $t=4.6$, $p<0.001$.
[b] Mean significantly different from ozone, $t=3.9$, $p<0.01$.
[c] Mean significantly different from control, $t=4.0$, $p<0.01$.
[d] Mean significantly different from ozone, $t=5.0$, $p<0.001$.
[e] Mean significantly different from control, $t=10.8$, $p<0.001$.
[f] Mean significantly different from ozone, $t=10.5$, $p<0.001$.

Table 7. Minute ventilation (ml/min) of rats during hour 4 of exposures in the 1 month exposure experiment. Data are mean ± SE, n = 8.

Day	Purified Air	Low	Medium	High
1	159.9±12.0	126.7±4.6	127.6±8.2	120.4±8.2
3	135.2±4.6	126.2±9.4	132.8±7.1	113.5±9.2
10	112.9±5.5	141.6±6.8	164.0±12.8	131.5±11.9
17	148.6±5.7	146.1±8.6	157.3±4.8	154.9±10.9
24	159.3±9.2	128.7±7.0	131.6±9.3	127.1±6.5

Table 8. Breathing pattern and minute ventilation during hour 4 of the third exposure days of weeks 1, 4, 8, 12, 16, 20, 24, and 26 in the 6 month exposure experiment. Data are mean ± SE, n.

Breath Frequency (min^{-1})			
Exposure Day	Purified Air	Mixture[a]	Ozone
3	154 ±9, 8	160 ±5, 8	157 ±6, 8
24	148 ±6, 7	146 ±4, 8	143 ±7, 8
52	144 ±7, 7	139 ±5, 8	130 ±9, 8
80	141 ±9, 8	132 ±5, 8	138 ±6, 8
108	116 ±5, 8	136 ±4, 8	120 ±6, 7
136	121 ±5, 8	170 ±16, 8	114 ±9, 8
164	94 ±5, 8	102 ±6, 8	123 ±9, 8
178	102 ±8, 8	155 ±17, 8	112 ±10, 8

Main effect of exposure: mixture significantly different from control, $F = 6.6$, $p<0.05$.

Tidal Volume (ml)			
Exposure Day	Purified Air	Mixture	Ozone
3	1.1 ±0.07, 8	1.1 ±0.07, 8	1.2 ±0.05, 8
24	1.3 ±0.07, 7	1.2 ±0.04, 8	1.2 ±0.04, 8
52	1.3 ±0.03, 7	1.3 ±0.09, 8	1.3 ±0.09, 8
80	1.2 ±0.06, 8	1.3 ±0.07, 8	1.2 ±0.06, 8
108	1.4 ±0.04, 8	1.4 ±0.10, 8	1.6 ±0.13, 7
136	1.4 ±0.09, 8	1.1 ±0.15, 8	1.3 ±0.15, 8
164	1.4 ±0.08, 8	1.5 ±0.14, 8	1.1 ±0.12, 8
178	1.3 ±0.13, 8	1.0 ±0.13, 8	1.4 ±0.07, 8

Minute ventilation (ml min^{-1})			
Exposure Day	Purified Air	Mixture	Ozone
3	169 ±11, 8	177±15, 8	176 ±9, 8
24	190 ±14, 7	169±7, 8	175 ±11, 8
52	187 ±13, 7	178±11, 8	180 ±26, 8
80	165 ±11, 8	174±10, 8	167 ±11, 8
108	161 ±8, 8	186±16, 8	178 ±16, 7
136	162 ±11, 8	190±30, 8	169 ±17, 8
164	131 ±6, 8	156±24, 8	140 ±13, 8
178	129 ±6, 8	135±14, 8	139 ±13, 8

Table 9. Bronchoalveolar lavage fluid analyses in the 1 month and 6 month exposures. Data are mean ± SE, n.

1 MONTH EXPOSURE	Purified Air	Low	Medium	High
Lactate dehydrogenase (units/ml)	30.9±2.9, 10	35.8±4.4, 10	34.2±4.6, 10	36.5±3.7, 10
Prostaglandin E_2 (ng/10^6 cells, samples pooled from 4 rats)	1.9±0.7, 2	2.4, 1	2.3±0.9, 2	3.1±0.7, 2
Interleukin-1 (counts/min/10^3 cells, 4 rats pooled)	2.47±0.86, 2	3.68±0.63, 2	3.07±0.04, 2	3.71±0.82, 2

6 MONTH EXPOSURE	Purified Air	Mixture	Ozone
Cell differential (%)			
End exposure	n = 10	n = 9	n = 11
Monocyte	95.9±0.2	95.8±0.8	96.5±0.8
Lymphocyte	1.0±0.2	1.2±0.9	0.6±0.2
Epithelia	1.8±0.2	1.9±0.5	1.9±0.5
PMN	0.9±0.1	0.9±0.2	0.7±0.2
Multinuclear	0.3±0.1	0.1±0.1	0.2±0.1
Other	0.0±0.0	0.0±0.0	0.0±0.0
1 Month post-exposure	n = 12	n = 11	n = 11
Monocyte	94.9±0.8	96.2±0.8	94.3±0.9
Lymphocyte	1.3±0.2	1.4±0.4	2.2±0.6
Epithelia	2.7±0.6	1.5±0.4	1.8±0.4
PMN	0.7±0.3	0.5±0.2	0.9±0.3
Multinuclear	0.4±0.1	0.3±0.1	0.6±0.2
Other	0.0±0.0	0.0±0.0	0.1±0.1
Lactate dehydrogenase (units/ml)			
End-exposure	30.4 ± 1.8, 15	30.6 ± 2.2, 13	33.3 ± 3.6, 13
1 Month post-exposure	41.3 ± 2.5, 12	44.3 ± 4.5, 12	43.8 ± 3.1, 12