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## Respiratory Tract Responses to Repeated Inhalation of an Oxidant and Acid Gas-Particle Air Pollutant Mixture

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The purpose of this study was to examine a broad range of toxicologic responses in rats exposed to a multi-component pollutant atmosphere. Cumulative and adaptive respiratory tract responses to 3 concentrations of an inhaled particle-oxidant mixture were examined in Fisher 344 N rats exposed 4 h/day, 3 days/week for 4 weeks. The mixtures contained O<sub>3</sub>, NO<sub>2</sub>, NH<sub>4</sub>HSO<sub>4</sub>, carbon particles, and HNO<sub>3</sub> vapor. Irritant-induced, rapid-shallow breathing responses were present during the first 4-h exposure to medium and high concentrations. Successive exposures showed diminished responses in medium concentrations and exacerbated responses in high concentrations. At the end of 4 weeks, rats exposed to high concentrations exhibited lung lesions. Lavaged pulmonary macrophages showed dose-dependent depressions of Fc-receptor binding and phagocytosis. Lung tissue macrophages showed dose-dependent increases in acid phosphatase staining density and carbon particles. Respiratory tract clearance of tracer particles was not significantly affected by the exposures. Bronchoalveolar epithelial permeability was increased by the high concentration. Epithelial cell-proliferation labeling showed a dose-dependent increase at all levels of the respiratory tract. Progressively exacerbated breathing-pattern responses at high concentrations were associated with lung lesions and high cell-proliferation labeling in the nose transitional epithelium and terminal bronchioles. Attenuating or adaptive breathing-pattern responses occurred in the presence of smaller, but in many cases still significant, compromise of respiratory functions. Either attenuating or exacerbated breathing-pattern responses can occur in the presence of a significant dose-dependent compromise of other respiratory functions and lung tissue injury.

**Key Words:** air pollution; particles; ozone; nitrogen dioxide; nitric acid; ammonium bisulfate; oxidants; acids; complex mixtures; inhalation toxicology.

Understanding the adverse health effects of urban air pollution is complicated by recent epidemiologic studies implicating fine particles as being of major toxicologic importance (Abbey *et al.*, 1991; Lebowitz, 1996; National Research Council, 1998; Pope *et al.*, 1991, 1995). In addition, laboratory exposure

studies show that repeated daily ozone exposures result in pulmonary function responses that peak on the first or second day of exposure and are subsequently attenuated, in some cases returning to control levels (Foxcroft and Adams, 1986; Horvath *et al.*, 1981; Linn *et al.*, 1988; Wiester *et al.*, 1995). Changes in breathing patterns are sensitive indications of respiratory tract irritation from inhaled pollutants (Alarie, 1973), and breath frequency and tidal volume responses often attenuate with repeated daily exposure to oxidant pollutants in both humans and laboratory animals. Attenuated or adaptive responses to repeated ozone exposure observed for spirometric pulmonary-function measures may not occur for other oxidant-injury responses of the lung (McKinney *et al.*, 1998; Sun and Chung, 1997; Tepper *et al.*, 1989). Furthermore, repeated exposures to ozone in combination with an acidic carbon particle aerosol showed significant modification and disruption of the attenuated responses to repeated exposures to ozone alone (Kleinman *et al.*, 1999, 2000). The mechanism by which particle exposure can blunt ozone-response attenuation is not known, but such ozone-particle interactions raise the possibility of an important role for particles in toxicity of urban air pollution.

The purpose of the present study was to examine, in laboratory rats, a diverse range of respiratory effects of inhaling a fine particle and oxidant gas mixture representative of photochemical air pollution. By studying a variety of endpoints, a more complete picture of the responses could be obtained, and effects on related lung functions could be compared. Nose-only inhalation exposures were conducted for 4 h per day, 3 days per week for 4 weeks to a mixture of oxidant, acid and particulate pollutants. Elevated levels of urban air pollution tend to occur in repeated daily excursions driven by meteorological patterns, with more severe episodes lasting for several h per day for several consecutive days (Blumenthal *et al.*, 1978). Breathing patterns were recorded during the course of exposures to monitor the development and possible attenuation of pulmonary responses. At the end of the 4-week exposure, respiratory tract injury was evaluated with a variety of measures historically affected by ozone exposure including respiratory tract clearance, epithelial permeability, epithelial cell

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TABLE 1  
Component Concentrations of the Oxidant Gas-particle Pollutant Mixtures

Exposure group	Component pollutant	Target concentration	Exposure concentrations (mean $\pm$ SD)
Low	O <sub>3</sub> (ppm)	0.15	0.16 $\pm$ 0.02
	NO <sub>2</sub> (ppm)	0.10	0.11 $\pm$ 0.01
	ABS (mg/m <sup>3</sup> )	0.05	0.05 $\pm$ 0.02
	C (mg/m <sup>3</sup> )	0.03	0.03 $\pm$ 0.01
	HNO <sub>3</sub> (mg/m <sup>3</sup> )	0.025	0.020 $\pm$ 0.005
	Aerosol size ( $\mu$ m MMAD)	0.3	0.29 $\pm$ 1.6 GSD
Medium	O <sub>3</sub> (ppm)	0.30	0.30 $\pm$ 0.03
	NO <sub>2</sub> (ppm)	0.20	0.21 $\pm$ 0.02
	ABS (mg/m <sup>3</sup> )	0.10	0.10 $\pm$ 0.02
	C (mg/m <sup>3</sup> )	0.06	0.06 $\pm$ 0.02
	HNO <sub>3</sub> (mg/m <sup>3</sup> )	0.05	0.04 $\pm$ 0.01
	Aerosol size ( $\mu$ m MMAD)	0.3	0.31 $\pm$ 1.4 GSD
High	O <sub>3</sub> (ppm)	0.60	0.59 $\pm$ 0.04
	NO <sub>2</sub> (ppm)	0.40	0.39 $\pm$ 0.01
	ABS (mg/m <sup>3</sup> )	0.20	0.22 $\pm$ 0.05
	C (mg/m <sup>3</sup> )	0.12	0.10 $\pm$ 0.02
	HNO <sub>3</sub> (mg/m <sup>3</sup> )	0.10	0.11 $\pm$ 0.03
	Aerosol size ( $\mu$ m MMAD)	0.3	0.31 $\pm$ 2.1 GSD
All groups	Relative humidity 60.5 $\pm$ 0.3%; temperature 23.5 $\pm$ 0.8°C		

Note. Data are means  $\pm$  standard deviation (SD) of daily average values. ABS is ammonium bisulfate, NH<sub>4</sub>HSO<sub>4</sub>. Aerosol size was measured from the mixture sampled at the breathing zone of the exposed animals and includes both carbon and sulfate components.

proliferation labeling, pulmonary macrophage functions, and inflammatory injury. The exposure mixture, which contained oxidants (ozone and nitrogen dioxide), acids (ammonium bisulfate and nitric acid), and fine particulate carbon (Table 1) was based upon ambient measurements made in the San Gabriel Valley of the South Coast Air Basin in California (Solomon *et al.*, 1996). This is an area with intense levels of photochemically derived oxidants and particles. This region was of interest because prior epidemiological studies (Tashkin *et al.*, 1994), and a current multi-community children's health study (McConnell *et al.*, 1999) has demonstrated adverse effects in sensitive children in these communities. Many urban communities in the United States and other countries have significant levels of ozone, nitrogen oxides, sulfates and carbonaceous particles (Kley *et al.*, 1999).

## MATERIALS AND METHODS

**Animals and experimental design.** This study was conducted under an Institutional Animal Care and Use Committee-approved protocol and in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Male Fischer 344/N rats, barrier reared and specific-pathogen free (Simonsen Laboratories, Inc., Gilroy, CA), were tattooed for identification. Rats were housed in wire-bottomed cages (Hoeltge, Inc., Cincinnati, OH) over rock salt to absorb moisture from excreta and prevent ammonia formation (Phalen, 1997). Laminar flow isolators supplied each cage with filtered air (Mautz and Kleinman, 1997). The animals were provided with dry laboratory chow (Wayne Lab Blox, Western Research Products, Orange, CA) and water *ad libitum*. Upon arrival, 10 animals were sacrificed for quality control analysis. Lungs were examined grossly and microscopically for any signs of respiratory infection. Serum from 3 animals was collected and tested

in a viral and mycoplasma exposure panel by the University of Southern California Animal Diagnostic and Disease Surveillance Laboratory (University of Southern California, Los Angeles, CA). During exposures, sentinel animals were maintained, and 3 were sacrificed for quality control every 2 weeks. The rats were held in the laboratory for 1 week prior to exposures, which began when they were 11 weeks old. Nose-only exposures were 4 h per day on 3 consecutive days per week for 4 weeks. The animals were handled by trained personnel wearing clean lab coats, surgical masks, head covers, shoe covers, and gloves in order to prevent infections. Exposure tubes were washed in hot soapy water and disinfected with bleach daily. Animal quarters were cleaned twice weekly, and prior to delivery of rats, housing and exposure areas were fumigated with formaldehyde vapor (Buckle *et al.*, 1981; Lach, 1990). There were no signs of pulmonary infections during the study.

Rats were randomly assigned to experimental groups. Within each of the 4 pollutant exposure groups, subgroups of animals were assigned to different analyses of pollutant effects. Sample sizes for these subgroups were as follows: respiratory tract clearance,  $n = 30$ ; nasal epithelial permeability,  $n = 8$ ; bronchoalveolar lavage and pulmonary macrophage function,  $n = 10$ ; and lung histopathology,  $n = 10$ . Breathing pattern during exposure was monitored in 8 of the 10 animals assigned for lung histopathology. An additional  $n = 5$  animals in each pollutant exposure group were available to replace any other individuals that did not complete the exposure and effects analysis procedure.

**Generation and monitoring of exposure atmospheres.** Air pollutant exposure mixtures were generated at 3 different concentration levels that differed by successive factors of 2 (Table 1). Each mixture and purified air stream was delivered to 1-m<sup>3</sup> volume, Rochester-type chambers modified to accept nose-only exposure tubes in ports that placed each rat's nose into an individual stream of a test atmosphere (Mautz, 1997; Phalen, 1997). Air supplying the chambers was passed through coarse-particle filters and gas scrubbers, humidified, and then passed through high-efficiency particle filters (Phalen, 1997). Carbon aerosol was generated with a modified MRE-type 3-jet collision nebulizer model CN-24 (BGI, Inc., Waltham, MA) containing ultrasonically agitated fresh suspensions of carbon black (Monarch 120, Cabot Chemical). Ammonium bisulfate aerosol was nebulized from a dilute solution in a second

collision nebulizer. Each aerosol was dried and diluted with air, passed through a  $^{85}\text{Kr}$  aerosol discharger, equilibrated with purified air at 60% relative humidity, and then introduced into the exposure chamber air stream (Kleinman *et al.*, 1999, 2000). Ozone was generated by passing medical-grade oxygen through a corona-discharge ozonizer (Sander type III, Osterberg, Germany). Nitrogen dioxide (1% in nitrogen; Matheson) was metered into purified air and mixed with diluted ozone in a fluorocarbon delay line, to allow for a dynamic equilibrium to be reached between the ozone and nitrogen dioxide precursors of nitric-acid vapor. Vapor-phase nitric acid was formed by reaction of  $\text{O}_3$  and  $\text{NO}_2$  in the absence of UV light. The resulting oxidant gas and nitric-acid vapor mixture was equilibrated with purified, humidified air and introduced into the chamber air stream to yield the appropriate mixture concentrations at 60% relative humidity at the rat's breathing zone. The mixture of  $\text{O}_3$  and  $\text{NO}_2$  was expected to also yield nitrate radical and  $\text{N}_2\text{O}_5$  (Mautz *et al.*, 1988; Mustafa *et al.*, 1984; Pitts, 1983), but these reactive compounds were not measured. Each chamber air stream, containing mixed component pollutants, entered the modified Rochester-type chamber by a tangential inlet to an expanding plenum for swirl mixing, followed by distribution to individual rat exposure-tube ports. Samples of the mixture for component pollutant analyses were continuously drawn from unoccupied exposure-tube ports during the exposure.

Ozone was measured with a calibrated ultraviolet-absorption continuous monitor (Dasibi Environmental Corp., Glendale, CA, Model 1003-AH). Nitrogen dioxide was measured by chemiluminescence using a Monitor Labs Model 8440 detector. Size distribution of mixed sulfate and carbon particles was measured using an 8-stage cascade impactor (Andersen Model 210, Graseby, Atlanta, GA). Total aerosol samples were collected on acid-washed and distilled-water-rinsed quartz fiber filters and weighed with a sensitive electrobalance. Elemental carbon was measured by combustion of the filter in oxygen and measurement of evolved  $\text{CO}_2$  by non-dispersive infra-red absorption (Dasibi Model 3003 modified with a  $\text{CO}_2$  absorption cell). Samples were also collected using a fluorocarbon-coated quartz fiber prefilter (for  $\text{SO}_4^{2-}$ ,  $\text{NO}_3^-$  and  $\text{H}^+$ ) and a nylon backup filter (for nitric acid vapor). Filter and impactor samples were extracted in aqueous media and analyzed by ion chromatography. The mass concentration of aerosol was adjusted during exposures using a nephelometer (RAM-1, GCA Corporation, Bedford, MA).

**Breathing pattern during exposure.** Breathing patterns of rats were monitored using nose-only exposure tubes modified to function as plethysmographs during exposures. A latex membrane at the neck separated respiratory orifices from the trunk. Breathing movements displaced air from the body tube through a pneumotachograph, which measured tidal volume and frequency (Mautz, 1997; Mautz and Bufalino, 1989). The system was calibrated with a rodent ventilator (Harvard Apparatus, Holliston, MA) over the range of breath frequencies measured. Measurements were made at 20-min intervals during 4-h exposures on the first day of exposure and thereafter on day 3 of each week. Frequency, tidal volume, and minute ventilation were measured and analyzed by repeated-measures analysis of variance (ANOVA) to test for effects developing during the 4-h-exposure periods and for changes in these responses over successive exposures. Body mass was monitored over the course of the experiment to ensure that pollutant mixtures did not alter growth rates and indirectly affect breathing patterns.

**Respiratory tract clearance.** Respiratory tract clearance of insoluble radioactive tracer particles was measured as 2 phases: a short-term early clearance of particles, largely mucociliary clearance from the upper airways, and a longer-term phase of particle clearance, largely from the deep lung (Mannix *et al.*, 1983, 1996). Monodisperse  $1.2\ \mu\text{m}$  diameter polystyrene latex tracer particles were labeled with tightly bound  $^{51}\text{Cr}$  (Hinrichs *et al.*, 1978). Aerosols were generated from a 0.1% (by volume) aqueous suspension of the particles using a Lovelace-type compressed air nebulizer (Mercer *et al.*, 1968). The aerosolized particles were dried by heating, diluted with filtered air, and passed through a  $^{85}\text{Kr}$  discharger before entering a nose-only exposure chamber (Raabe *et al.*, 1973). The aerosol, sampled from the breathing zone of the rats using a calibrated 7-stage impactor (Mercer *et al.*, 1970), had an activity median aerodynamic diameter of  $1.8\ \mu\text{m}$  and a geometric standard deviation of  $1.2\ \mu\text{m}$ . The rats were exposed to the tracer aerosol for 20 min on the day

following the final pollutant exposure of the study. After the particle deposition, the rats were removed from the nose-only chamber and their noses washed of externally deposited tracer. Feces were collected 8 times during the first 50-h period after deposition, to define individual excretion curves. Early clearance was characterized from these excretion curves by fitting them to a logarithmic function, and by then determining  $T_{50\%}$ , the time at which 50% of the radioactivity was excreted for each rat. For late clearance, the rats were sacrificed at 30 days post-deposition, and the residual lung radioactivity was determined by counting excised lungs in a collimated and shielded cylindrical  $3 \times 3$ -inch NaI(Tl) gamma-ray detector. The lung activity for each rat was corrected for decay of the  $^{51}\text{Cr}$  back to 50 h post-deposition and then normalized by dividing by the value obtained from a thoracic count of the same animal at 50 h post-deposition. The resulting values for uncleared radioactivity at 30 days constitute an index of late clearance termed the  $A_{30}$  (Mannix *et al.*, 1996).

**Epithelial permeability.** Permeability of the nasal epithelium was measured at 1 h following the last exposure (Bhalla *et al.*, 1986; Bhalla and Young, 1992). Animals were anesthetized with 73 mg/kg sodium pentobarbital, polyethylene tubing (PE-90) was placed in the trachea, and a polyethylene catheter (P-10) was placed in the femoral artery. The oropharynx of tracheostomized rats was filled with dental impression cream to block the posterior nares. A radiolabeled tracer inoculum containing  $^{99m}\text{Tc}$  labeled diethylenetriaminepentaacetate ( $^{99m}\text{Tc}$ -DTPA, molecular weight 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 min after the start of instillation. Blood samples were counted for  $^{99m}\text{Tc}$  radioactivity in a gamma counter. Isotope counts for  $^{99m}\text{Tc}$  were expressed as the percent of the inoculum transferred from the site to the blood of each rat. An index of epithelial permeability was obtained by interpolation of a regression of the fraction of label transferred at the mid-time point for the 5 samples.

**Broncho-alveolar lavage and pulmonary macrophage function.** Broncho-alveolar lavage (BAL) was performed at 1 and 18 h after the last exposure. The animals were anesthetized, prepared with tracheal cannulas, and lavaged with  $\text{Ca}^{+2}$ - and  $\text{Mg}^{+2}$ -free HEPES-buffered Hanks balanced salt solution (HBSS) as described previously (Kleinman *et al.*, 2000). Cells were separated from the recovered lavage fluid by centrifugation at  $300 \times g$  for 10 min. Assay of lactate dehydrogenase was performed on a sample of the supernate, and the remainder was frozen at  $-70^\circ\text{C}$  for later analyses. Total protein in the supernate fluid was determined by a bicinchoninic acid (BCA) procedure (Smith *et al.*, 1985) using a bovine serum albumin (BSA) standard and Pierce BCA Protein Assay Reagents (Pierce Chemical CO, Rockford, IL). For the group sampled at 18-h post-exposure, total protein, albumin, and lactate dehydrogenase were measured. Lactate dehydrogenase was analyzed spectrophotometrically by enzymatic conversion of pyruvate to lactate in the presence of NADH (Sigma Chemical, St. Louis, MO). Albumin concentration was evaluated with an enzyme-linked immunosorbent assay (ELISA) as described by Bhalla and Young (1992).

The cell pellet from the 18-h post-exposure BAL animals was resuspended in HBSS containing  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  for analysis of pulmonary macrophage function. The number of viable cells recovered was determined by trypan blue exclusion using a bright line hemocytometer. Between 0 and 5% of the cells from any sample were non-viable, independent of pollutant exposure. Cells were diluted to  $10^6$  per ml, and a 0.1-ml aliquot of cells was mounted onto a glass microscope slide using a cytocentrifuge, and was stained with Wright-Giemsa stain for cell differential counts. The recovered cells were  $>95\%$  macrophages. Assays of pulmonary macrophage function included measures of Fc-receptor binding, phagocytosis of polystyrene latex (PSL) particles, and carbon particle inclusions. Phagocytic activity of pulmonary macrophages was measured in suspension, as previously described (Kleinman *et al.*, 2000). Cell suspensions containing  $2 \times 10^5$  pulmonary macrophages per ml and  $2 \times 10^8$  fluorescent polystyrene latex microspheres ( $1\ \mu\text{m}$  in diameter, Duke Scientific, Palo Alto, CA) per ml were incubated with gentle agitation for 1 h. Cells were

then pelleted onto a slide using a cytocentrifuge, then the slides were fixed with methanol, immersed in xylene for 10 min to remove extracellular PSL microspheres, and stained with light green stain (Diff-Quick). Engulfed spheres in the cell cytoplasm were counted, and the percentage of a sample of 100 cells containing >2 microspheres was the phagocytic activity index.

Pulmonary macrophage Fc-receptor (FcR) binding activity was assayed as macrophage capacity to bind sheep red blood cells following activation of FcR with anti-sheep red blood cell antibody, as previously described (Kleinman *et al.*, 1993, 2000; Prasad *et al.*, 1988, 1990; Rao *et al.*, 1980). Activated cell preparations coating well slide chambers were incubated 30 min at 38°C in 5% CO<sub>2</sub> with sheep red blood cells (10<sup>7</sup> cells suspended in HBSS). The unbound red blood cells were washed away gently, and the number of cells that formed rosettes with 3 or more blood cells out of a total sample of 300 macrophages was recorded.

**Lung histopathology.** Histopathological analyses were performed 2 days following the last exposure. At 24-h post-exposure, rats were given a subcutaneous administration of tritiated thymidine ([<sup>3</sup>H]-dThd) in sterile 0.9% NaCl (2 μCi/g body mass), and the animals sacrificed 24 h later to allow for complete metabolism of the tracer (Cleaver, 1967). They were anesthetized with sodium pentobarbital and exsanguinated via the dorsal aorta. The cranial portion of the trachea was removed and fixed in 10% buffered formalin, and the lungs were removed and inflation-fixed by airway perfusion for 72 h at 30 cm H<sub>2</sub>O with buffered 10% formaldehyde. The left lobe provided sections for morphometric, autoradiographic, and histochemical analyses. The left 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 an image analysis system (American Innovision, San Diego, CA). After embedding in paraffin, 5 μm sections were cut, mounted, and digitized to determine shrinkage. The head was skinned, external tissue and muscle removed, and the nasal portion of the head was fixed by immersion with vacuum degassing in 10% buffered formalin. Decalcification was performed in 6% EDTA followed by embedment in paraffin. Cross-sections cut approximately midway between the nares and the eye provided sections containing squamous, transitional, respiratory, and olfactory epithelia (Level 1 of Young, 1981). Paraffin 5-μm sections were stained with hematoxylin and eosin, neutral red-fast green, or for acid phosphatase.

Focal sites of inflammation in the lung were quantified after the method of Elias and Hyde (1980, 1983). Volume fractions of parenchyma and non-parenchyma (including large airways, large vessels, and other tissues) were estimated following Weibel (1966, 1979) using a 10 × 10 grid laid over a video image. A stratified scan of 100 randomly selected fields was performed at ×400, and points in the parenchyma falling on lesions scored. Focal lesions were readily identified by increased cellularity, thickened alveolar walls, infiltration of inflammatory cells and large numbers of free cells and cellular debris in alveolar spaces. Separate scans were made of sections stained with neutral red-fast green to score the percentage of pulmonary macrophages containing carbon particle inclusions from samples of 350–450 macrophage cells (Kleinman *et al.*, 1999). In sections stained for acid phosphatase, pulmonary macrophages were classified by relative differences in stain density. Color thresholds on the image analysis system were first set to encompass the hue and saturation range of macrophages in the stained sections, and then to classify macrophages into 3 divisions of this range of color thresholds: weakly stained (class I), orange stained (class II), or bright red stained (class III). A random sample of 10–15 cells was analyzed from each section, and proportions of each staining class were recorded.

Autoradiographic (ARG) analyses were performed on 5 μm paraffin sections of the nasal transitional epithelium, trachea, left lung lobar bronchus, terminal bronchioles, and parenchyma, 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 autoradiographs at 400 × 20 randomly selected lung fields, which were scored, reader-blind, for each section and tissue type including lung parenchyma and terminal bronchiolar and lobar bronchus epithelia. Separate sections of nose and trachea were used for ARG analysis of epithelia. Prepa-

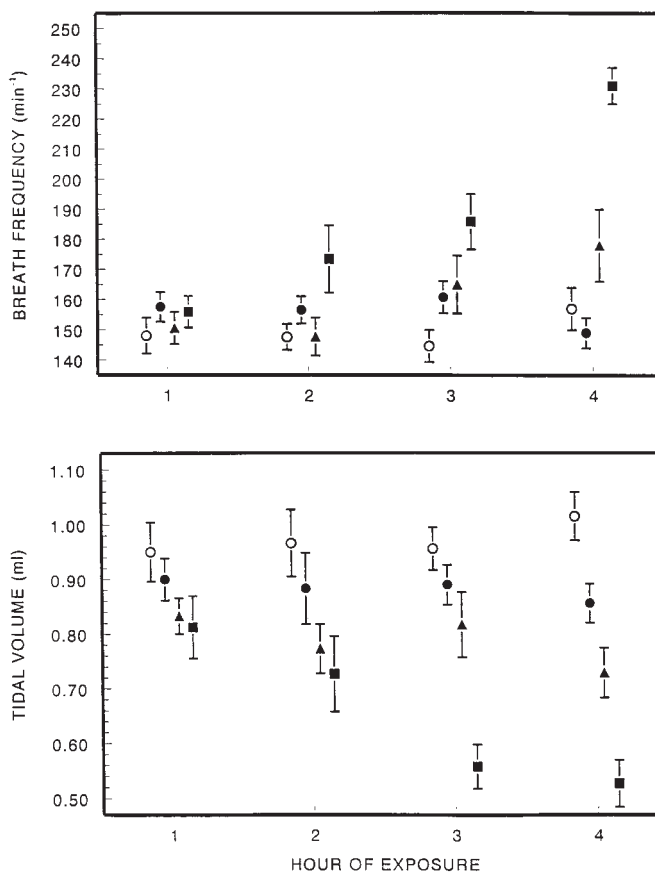


FIG. 1. Effects of oxidant gas-particle mixtures on breathing patterns of rats during the first day of exposure. Data are hourly means  $\pm$  SE,  $n = 8$ . Open circles are purified air, closed circles are low-, triangles, medium-, and squares, high-concentration mixtures.

rations with tissue processing problems, which could contribute to analysis errors (such as uneven lung inflation or imprecise nasal section plane), were excluded.

Data were analyzed using analysis of variance (ANOVA), and tested with multiple comparisons of group means using Bonferroni adjustment of critical values (Dixon, 1988).

## RESULTS

Table 1 shows concentrations of air pollutants measured during the exposures. There was good agreement between the target concentrations and concentrations measured from nose-only exposure ports at the breathing zone of rats during exposures. The low, medium, and high concentration groups were effectively factors of 2 increases.

Breathing pattern is influenced by body mass, and rat mean mass during exposure increased from 247 to 264 g. All groups increased in parallel and there was no significant interaction between change in mass over time and exposure group. Figure 1 shows breath frequency and tidal volume during each h of the first exposure day. The high concentration induced a rapid-shallow breathing pattern developing during hours 3 and 4 of

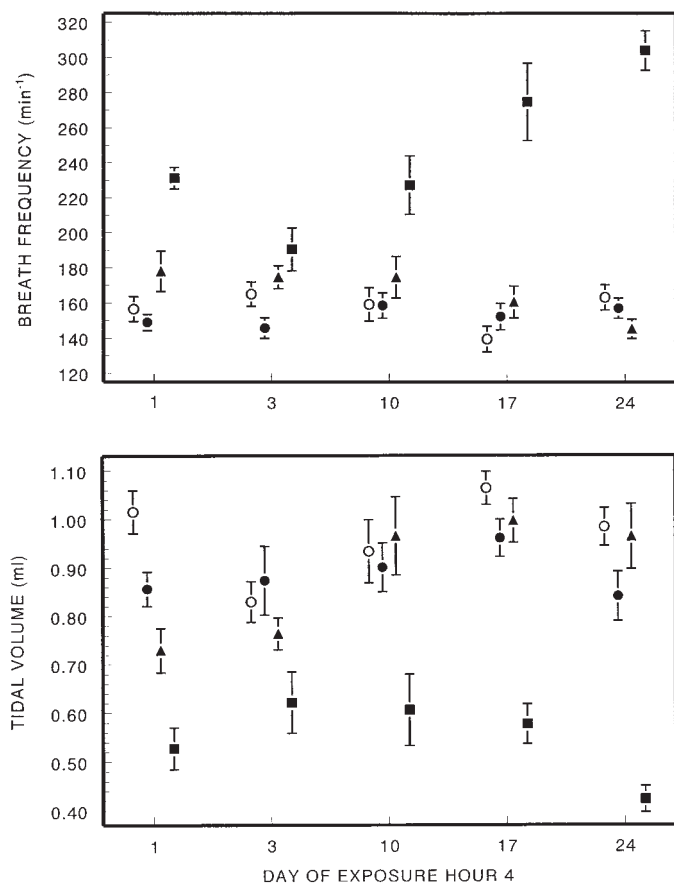


FIG. 2. Effects of oxidant gas-particle mixtures, at 3 concentration levels, on breathing patterns of rats during the 4th hour of exposure on day 1 and on day 3 of weeks 1, 2, 3, and 4. Data are hourly means  $\pm$  SE,  $n = 8$ . Open circles are purified air, closed circles are low-, triangles are medium-, and squares are high-concentration mixtures.

the exposure (high vs. control exposure-time interactions for breath frequency and for tidal volume ( $p < 0.0001$ )). The medium concentration induced a smaller degree of rapid-shallow breathing (medium vs. control exposure-time interactions for breath frequency  $p < 0.03$  and for tidal volume  $p < 0.03$ ). The rapid-shallow breathing pattern was modified with successive daily and weekly exposures. Figure 2 shows the breathing pattern during hour 4 of the first day and then on the third day of each weekly 3-day episode. Over the successive days and weeks of exposure, the high concentration rapid-shallow breathing pattern progressively increased (breath frequency high vs. control exposure-time interaction,  $p < 0.0001$ ; tidal volume exposure  $\times$  time interaction,  $p < 0.0001$ ). In the medium concentration, the rapid-shallow breathing observed at hour 4 on day 1 was successively diminished over the 4 weeks of episodic exposures (tidal volume medium vs. control exposure-time interaction,  $p < 0.0004$ ; and breath frequency medium vs. control exposure-time interaction,  $p < 0.06$ ). By the end of the entire exposure, tidal volume in medium concentration had returned to near-purified air control levels. The me-

TABLE 2  
Effects of Oxidant Gas-Particle Mixture Exposures on Early and Late Clearance of Radiolabeled Tracer Microspheres

	Purified air	Mixture Concentration		
		Low	Medium	High
Early clearance	14.2 $\pm$ 0.5	13.8 $\pm$ 0.5	14.3 $\pm$ 0.4	14.8 $\pm$ 0.5
T <sub>50%</sub> (h) <sup>a</sup>	28	29	28	29
Late clearance	11.5 $\pm$ 0.1	11.5 $\pm$ 0.1	11.6 $\pm$ 0.1	11.4 $\pm$ 0.1
A <sub>30%</sub> <sup>b</sup>	28	29	28	29

Note. Data are mean  $\pm$  SE,  $n$ .

<sup>a</sup>Early clearance T<sub>50%</sub> is the time required to excrete 50% of the total activity excreted through 50 h post-deposition.

<sup>b</sup>A<sub>30%</sub> index of late clearance is residual lung radioactivity remaining at 30 days post-deposition.

dium concentration induced an acute response that was attenuated with repeated episodic exposure, while the high concentration induced a strong, rapid-shallow-breathing response that was exacerbated with repeated episodic exposure.

Neither early- nor late-term particle clearance was significantly affected by the 4 weeks of exposures (Table 2). Because the tracer particle deposition occurred after the end of the exposures, an additional analysis was performed to verify that all groups had roughly equivalent total deposition, as measured by radioactivity excreted in feces up to 50 h post-deposition. The lung count activities after 30 days (A<sub>30</sub> values) indicated that the pollutant exposures had no significant effect on the deep lung clearance rates of the rats.

Although nasal epithelial permeability, measured with DTPA tracer 1 h after the end of the last exposure (Fig. 3), was elevated in all groups relative to control, the changes were not dose-dependent and individual group differences from control were not statistically significant. Protein concentrations in

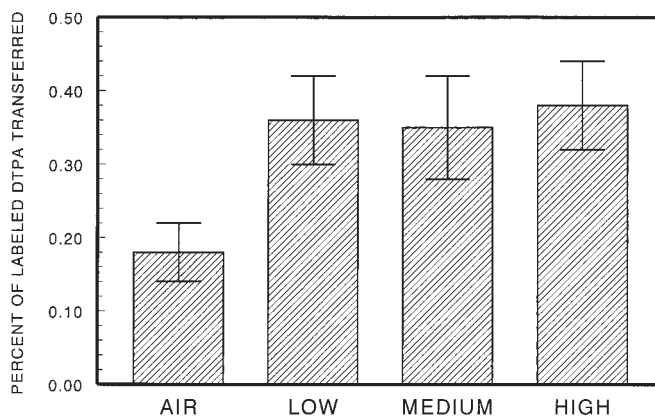


FIG. 3. Effects of oxidant gas-particle mixtures, at 3 concentration levels, on nasal epithelial permeability of rats measured 1 h following the last exposure. Permeability is the percent of <sup>99</sup>Tc labeled DTPA inoculum transferred from the nasal cavity to the blood at 8-min following instillation. Data are mean  $\pm$  SE,  $n = 6-7$  per group.

TABLE 3  
Effects of Oxidant Gas-Particle Mixtures on Bronchoalveolar Lavage Compounds at 1 or 18 Hours Following the Last Exposure

	Mixture concentration			
	Purified air	Low	Medium	High
Total protein (mg/ml)				
1 h post-exposure	0.22 ± 0.01	0.26 ± 0.01	0.26 ± 0.02	0.29 ± 0.02 <sup>a</sup>
18 h post-exposure	0.20 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
Albumin (mg/ml)				
18 h post-exposure	0.059 ± 0.005	0.057 ± 0.004	0.055 ± 0.003	0.057 ± 0.004
Lactate dehydrogenase activity (units/ml)				
18 h post-exposure	30.9 ± 2.9	35.8 ± 4.4	34.2 ± 4.6	36.5 ± 3.7

Note. Data are mean ± SE,  $n = 6-7$  for 1 h post exposure groups and  $n = 10$  for 18 h post-exposure groups.

<sup>a</sup>Significantly different from control ( $p < 0.05$ ).

bronchoalveolar lavage fluid (BALF) are shown in Table 3. Samples collected 1 h following the last exposure exhibited elevated levels of total protein in the exposure groups, and the difference between purified air control levels and the high-concentration group was significant ( $p < 0.05$ ). This change was apparently rapidly reversible. Protein and albumin analyses performed on lavage samples collected 18 h after the last exposure showed no statistically significant differences; total protein and lactate dehydrogenase were slightly elevated in the exposed groups compared to control.

Pulmonary macrophages recovered from BALF had a viability of  $94 \pm 2$  SD%, with no significant differences between exposure groups. Figure 4 shows significant dose-dependent depression in Fc-receptor binding, polystyrene latex (PSL) particle phagocytosis, and significant dose-dependent increases in carbon particle content of lavaged macrophages.

Histopathologic measures of lung tissue and pulmonary macrophages in the lung are shown in Table 4. Focal inflammatory lesions were observed only at the high concentration. Pulmonary macrophages in sections showed increasing proportions of cells with carbon particles with increasing exposure concentration, paralleling the results observed with macrophages collected in BALF (Fig. 4). Pulmonary macrophages showed an increased acid phosphatase stain density in pollutant-exposed groups. The high and medium exposures shifted the proportions of macrophages in the 3 separate classes from lower- to higher-density classes.

Epithelial cell proliferation measured following the last exposure is shown for tissues at several levels of the respiratory tract in Figure 5. Increases in the labeling index above control levels likely represent cell proliferation in areas where epithelial cells were killed or damaged by the exposures. The high-concentration group showed increased cell labeling in all tissues examined, the medium concentration group had significantly elevated labeling in trachea and lung parenchyma, and tracheal epithelium showed significantly increased labeling in all pollutant exposures. At the high concentration, nasal

transitional epithelium and terminal bronchiolar epithelium showed large elevations of proliferation labeling.

## DISCUSSION

Toxic interactions among pollutant compounds need to be considered because of the potential for antagonism, additivity, and synergism in comparison to single components (Last *et al.*, 1986; Mauderly, 1993; Mautz *et al.*, 1988; Schlesinger *et al.*, 1992; Warren and Last, 1987). In particular, if mixtures of oxidant or acid-forming gases with particles exhibit synergism, the importance of particulate air pollution, as revealed in epidemiologic investigations, might be better understood (Abbey *et al.*, 1991; Lebowitz, 1996; National Research Council, 1998; Pope *et al.*, 1991, 1995). Acute symptoms that disappear upon repeated exposure may be only a subset of a larger array of injury processes, others of which may be persistent or progressive. We selected the 5-component oxidant, acid, and particle mixture and an episodic exposure pattern to represent an environmentally relevant challenge, while recognizing that real ambient air pollution is yet more complex than our model mixture, both in diversity of chemical species and in aerosol size distribution. We examined a proven battery of inhalation-injury variables in order to explore possible relationships among variables at different dose levels.

Measures of pulmonary function show strong sensitivity to one of our components, ozone (Alarie, 1973; Lippmann, 1989; Mautz and Bufalino, 1989; Menzel, 1984), and pulmonary function variables are readily measured markers of respiratory irritant exposure in humans. However, as these responses may attenuate on repeated daily exposure, it is important to understand what health effects they are indicating. In rats exposed daily to 0.5 ppm O<sub>3</sub>, Tepper *et al.* (1989) observed an attenuation of the rapid-shallow breathing pattern response in the presence of progressive lung epithelial damage, inflammation, and increased concentrations of lavagable protein. The rapid-shallow breathing pattern response to ozone and other so-called

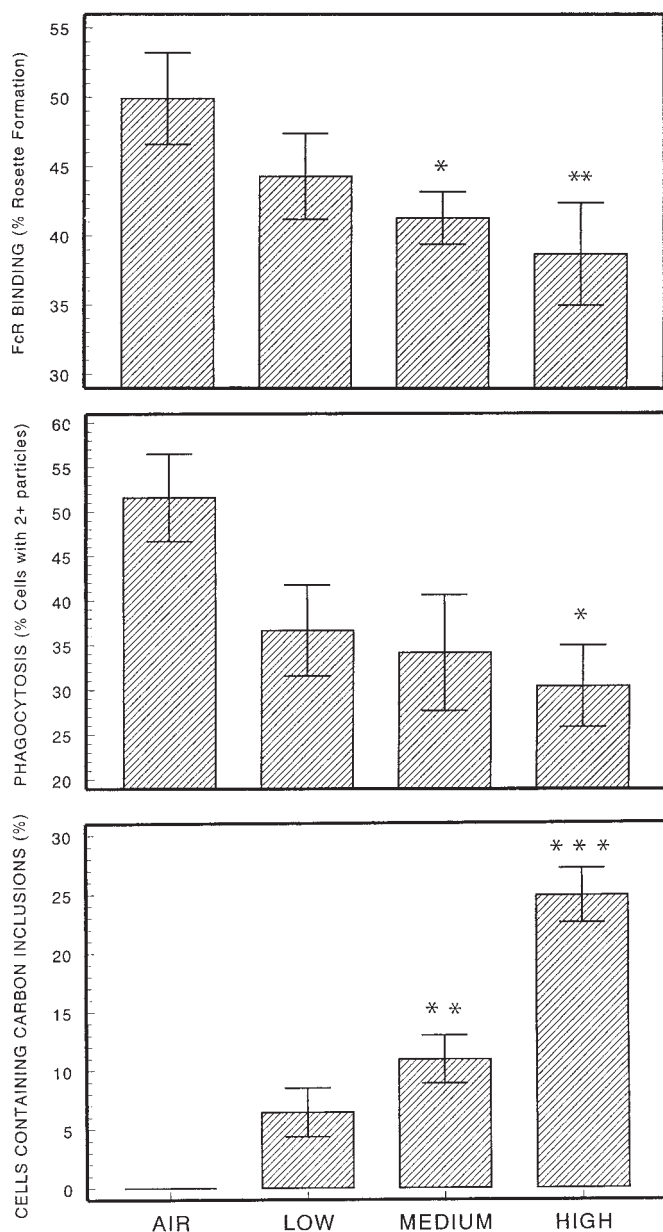


FIG. 4. Pulmonary macrophage function assays of macrophages recovered from bronchio-alveolar lavage following exposure to 3 concentration levels of oxidant gas-particle mixtures. Data are mean  $\pm$  SE. Fc receptor-binding capacity ( $n = 10$  per group) is percent rosette formation of macrophages with sheep red blood cells and IgA antibody. *In vitro* phagocytosis ( $n = 12$  per group) is percent macrophages containing 2 or more polystyrene microspheres following challenge with a microsphere suspension. Cells containing carbon ( $n = 12$  per group) is the percent of macrophages containing carbon particle inclusions from the exposure mixtures. Asterisks indicate means significantly different from control: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

deep lung or respiratory irritants is a vagally mediated reflex stemming from stimulation of lung C nerve fibers (Coleridge and Coleridge, 1984; Coleridge *et al.*, 1993; Schelegle *et al.* 1993). The mechanism of response attenuation to repeated ozone exposure is not well understood, but has been suggested

to involve replacement of oxidant-damaged epithelial cells with more resistant cells (Moffatt *et al.*, 1987; Nikula *et al.*, 1998), or with increases of anti-oxidant compounds in the lung (Duan *et al.*, 1996; Tepper *et al.*, 1989; Wiester *et al.*, 1995, 2000). Response attenuation to repeated oxidant exposure could represent a beneficial process, but the attenuation does not appear to include all tissue oxidant injury processes.

In the rats exposed here to the 5-component oxidant, acid, and particle mixture, there were distinct differences in breathing-pattern responses among groups. The low-concentration group did not show significant breathing pattern responses, the medium group showed an initial day-1 exposure response that attenuated over successive exposures, and the high-concentration group showed progressively exacerbated rapid-shallow breathing patterns over the 4-week exposure (Fig. 2). The fact that the exposures were to a mixture and were episodic may have modified the expected response to the oxidant gases. Single exposures to combinations of  $O_3$  and  $NO_2$ , which also contained formed  $HNO_3$  vapor, strongly enhanced oxidant lung injury compared to  $O_3$  exposure alone (Mautz *et al.*, 1988). Five successive daily 4-h exposures to mixtures of 0.4 ppm  $O_3$  with sulfuric acid-coated carbon particles blunted the breathing pattern-attenuation response observed in similar exposures to  $O_3$  alone (Kleinman *et al.*, 1999). Tepper *et al.* (1989) observed breathing-pattern attenuation in daily 2.25-h exposures of rats to 0.5 ppm  $O_3$  and no attenuation in rats exposed to 1.0 ppm  $O_3$ . In the present study, the mixture with  $O_3$  at 0.3 ppm induced attenuation with repeated exposure, but the higher concentration mixture with  $O_3$  at 0.6 ppm induced an exacerbated breathing pattern response with each of the 4 3-day episodes of exposure (Fig. 2). Because of the complexity of our mixture, comparisons to other studies are difficult, but, within the study, responses can be compared.

Respiratory tract clearance, both short-term and long-term, did not show significant alterations from purified-air control animals at the end of any of the mixture-exposure groups (Table 2). Whether this resulted from absence of response to all 3 concentrations or adaptive responses is uncertain, because measurements were not made following single exposures. Our laboratory has conducted more than 20 studies on the effects of inhaled air pollutants on the short- and long-term clearance of radiolabeled tracer particles (Phalen *et al.*, 1994). In these studies, 4-h exposures of resting rats to  $O_3$  above 0.6 ppm delayed short-term clearance and accelerated long-term clearance. In the short-term exposures,  $O_3$ , if present, typically dominated the effects on clearance. In multi-day exposures (4 h/day, 21 days)  $O_3$  did not dominate the effects of mixtures on clearance, suggesting an adaptation of clearance mechanisms to the effects of  $O_3$ . Furthermore, when focal lesions were extensive in the lung parenchyma, they were correlated with accelerated long-term clearance. The present study is consistent with these prior findings in that the clearance of tracer was unaffected in repeated resting exposures to  $O_3$  containing atmospheres.



TABLE 4  
Effects of Oxidant Gas-Particle Mixture Exposures on Lung Parenchymal Inflammatory Foci and *in Situ* Pulmonary Macrophages

	Mixture concentration			
	Purified air	Low	Medium	High
Lung parenchyma				
Inflammatory foci (area %)	0.0	0.0	0.0	2.15 ± 0.59 <sup>d</sup>
Pulmonary macrophages				
Containing carbon particles (%)	0.0	0.22 ± 0.07 <sup>b</sup>	0.64 ± 0.12 <sup>d</sup>	0.90 ± 0.12 <sup>d</sup>
Acid phosphatase stain				
Density (% of cells sampled)				
Class I	41 ± 5	53 ± 7	42 ± 5	24 ± 6 <sup>b</sup>
Class II	56 ± 3	43 ± 8	44 ± 5	59 ± 5
Class III	4 ± 2	4 ± 7	15 ± 4 <sup>b</sup>	18 ± 5 <sup>c</sup>

Note. Data are mean ± SE, *n* = 10 for all groups.

<sup>a</sup>Five of 10 rats showed focal lesions.

<sup>b</sup>Significantly different from control (*p* < 0.05).

<sup>c</sup>Significantly different from control (*p* < 0.01).

<sup>d</sup>Significantly different from control (*p* < 0.001).

Lavagable protein was significantly elevated by 1 h after the last high-concentration-mixture exposure (Table 3). In repeated O<sub>3</sub> exposures, elevated lavage protein levels have been observed to attenuate in one study (Canning *et al.*, 1991), but Tepper *et al.* (1989) observed sustained significant elevation of lavaged protein levels in rats that demonstrated response attenuation in breathing pattern. While BAL protein may show attenuated responses under some repeated exposure conditions, it does not appear to be tightly coupled to other attenuation responses. Increases in BAL protein generally means that pulmonary epithelia have increased permeability (Bhalla, 1999). When broncho-alveolar epithelial permeability is increased, inhaled oxidants and reactive oxidation products in the fluid lining of the lung have greater access to lower levels of epithelia. This increase might also support greater capacity to achieve higher concentrations at pulmonary C fibers and to override breathing pattern-response attenuation. Nevertheless, the difference in permeability, as indexed by lavagable protein between the high- and lower-concentration groups, was modest at 1 h following the last exposure (Fig. 3) and was not evident 18 h post-exposure (Table 3). The differences in breathing-pattern responses among the mixture exposure groups, however, were dramatic (Fig. 2). BAL levels of lactate, dehydrogenase as an indicator of tissue injury, showed only a weak trend of increase in the mixture-exposure groups, but no significant difference among the groups (Table 3). Tissue injury and inflammation was evident in histological examination of the lung parenchyma exposed to the high concentration (Table 4). This exposure-induced parenchymal injury is apparently not sufficient to substantially elevate BAL indicators in total protein, albumin, or lactate dehydrogenase.

While BAL proteins were weakly, if at all, affected by the exposures, pulmonary macrophages collected by BAL or analyzed histologically *in situ* did show strong concentration-

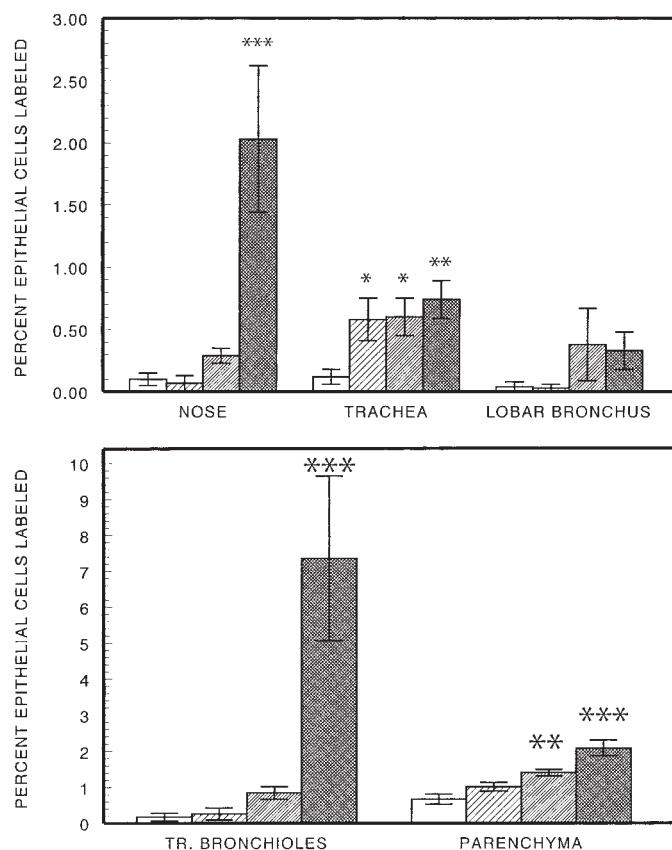


FIG. 5. Effects of oxidant gas-particle mixture exposures at 3 concentration levels on epithelial cell proliferation in the respiratory tract. Open bars are purified air control, and bars with cross-hatching at increasing density represent respectively low-, medium-, and high-concentration mixture exposures. Data for the nose are from transitional epithelium. Data are mean ± SE, *n* = 10 of the percent of labeled cells in epithelia. Asterisks indicate means significantly different from control: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

response relations in all measures examined, including the functional capacities, Fc receptor binding, and phagocytosis, and in their carbon particle-inclusion load and acid phosphatase-staining density (Fig. 4, Table 4). The high-concentration group progressed through the latter weeks of exposure with extreme rapid-shallow breathing patterns (Fig. 2), and these might be expected to substantially alter dose-deposition of gases and particles in the lung compared to other groups. However, the proportions of macrophages with carbon loads and the depression of their FcR-receptor binding and phagocytotic capacities was proportionate to airborne pollutant concentrations. Despite the impairment of macrophage immune recognition and phagocytotic capacity and the carbon-particle loads in pulmonary macrophages, the late-term clearance index, which is expected to be determined primarily by macrophages, was not significantly modified by the exposures (Table 2).

The sets of effects that most closely parallel the disparate breathing pattern differences among exposure groups were the patterns of epithelial-cell proliferation in the terminal bronchioles and nasal transitional epithelium (Fig. 5) and the presence of inflammatory lesions in the lung (Table 4). For these analyses, the high-concentration exposure group showed marked increases compared to medium- and lower-concentration exposure groups. The nasal transitional epithelium and the centri-acinar portions of the lung (including terminal bronchioles and proximal alveoli) were the foci of inflammatory responses in single acute exposures of rats to 0.8 and 1.2 ppm O<sub>3</sub> (Hotchkiss *et al.*, 1989). This pattern of injury, as revealed by high epithelial-cell labeling, was also apparent in our high-concentration group (Fig. 5). Increased epithelial cell labeling is observed over a period of days following single acute exposures to O<sub>3</sub> at 0.35–0.6 ppm and the cell-proliferation response exhibits attenuation with repeated 0.5 ppm O<sub>3</sub> exposure (Evans *et al.*, 1985; Mautz *et al.*, 1988). The results of the episodic exposure to the mixtures (Fig. 5) indicate that an injury and cell-proliferation response to the exposure persisted through the 4-week duration of the exposures. Lung parenchymal lesions were also visible in the high concentration group (Table 4), but only in half of the sample of 10 rats. No such lesions were observed in any of the other groups, including the medium concentration-exposure group, which showed breathing pattern-response attenuation (Fig. 2) and a more modest elevation of epithelial labeling in the lung parenchyma. This suggests that events in the terminal bronchiolar epithelium mediate the breathing-pattern attenuation response to repeated exposure. C-fiber endings are present in the bronchiolar epithelium as well as alveolar epithelia (Coleridge and Coleridge, 1984).

Oxidant breathing pattern response attenuation or adaptation to repeated exposure may involve induction of defense against high concentrations of oxidants penetrating to epithelial and sub-epithelial tissues in the terminal bronchioles, a defense that can be overridden by exposure conditions that further damage this epithelium. With sufficient injury to the epithelial barrier

in the terminal bronchioles, oxidant gases like O<sub>3</sub> still penetrate at high enough concentrations to stimulate C fibers and induce vagal reflex breathing-pattern changes. Tepper *et al.* (1989) found detectable centri-acinar lesions in rats that had demonstrated attenuated breathing pattern responses to repeated 0.5 ppm O<sub>3</sub>. Breathing pattern-response attenuation did not imply an absence of tissue inflammatory signs. In our exposure to the high-concentration mixture (including 0.6 ppm O<sub>3</sub>), breathing-pattern response was not attenuated, but rather exacerbated; over 2% of the lung parenchyma was involved in centri-acinar lesions, and epithelial cell proliferation was strongly elevated (Table 4, Figs. 2 and 5). When breathing-pattern responses to repeated exposures are persistent or increasing rather than attenuating, they may reflect underlying persistent or increasing patterns of lung-tissue inflammation. Attenuating responses in general may show dose-response relations where low doses induce a response that then attenuates on repeated exposure, but higher doses delivered in repetition result in an exacerbated response. Furthermore, the pattern of response development with repeated exposures may be altered by factors other than concentration-related dose: episodic exposure (Wiester *et al.*, 1995; and the present study), exposures to mixtures (Kleinman *et al.*, 1999, 2000; and the present study), or exercise during exposure (Horstman *et al.*, 1990). This study showed that various measures of response in the respiratory tract can respond in diverse ways to a relatively realistic air pollutant mixture exposure. This complexity of response was clearly revealed by the use of a battery of endpoints performed by different investigators that had shown previous utility in studying pollutants singly. Further investigation of the pattern of exposure to realistic concentrations of mixed air pollutants would clearly benefit from the collaboration of independent investigators using complementary endpoints.

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