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## Toxic Effects of Short-Term Exposures to Acids and Diesel Exhaust

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### ABSTRACT

The objective of this study was to evaluate the toxicity of inhaled diesel engine-generated fine carbon particles, when an acid mixture of the type found in California's air was also present. Rats were exposed nose-only for 5 hours per day for 5 consecutive days. The target concentrations of the pollutants in the three atmospheres studied were: (a) 0.5 mg/m<sup>3</sup> diesel exhaust soot, (b) 0.35 mg/m<sup>3</sup> nitric acid + 0.15 mg/m<sup>3</sup> sulfuric acid, and (c) 0.5 mg/m<sup>3</sup> diesel exhaust soot + 0.35 mg/m<sup>3</sup> nitric acid + 0.15 mg/m<sup>3</sup> sulfuric acid. Control rats were exposed using the same schedule to purified air. The evaluation of potential toxic effects in exposed rats included: (a) measurement of respiratory tract clearance of insoluble tracer particles; (b) histopathologic examination of respiratory tract tissues, including autoradiographic measures of cell turnover as an index of cell killing; and (c) measurement of functional effects on pulmonary alveolar macrophages.

Biological effects were observed for each of the atmospheres studied. None of the atmospheres produced significant lung cell damage, but retained soot deposits were seen in the lung after exposure to atmospheres that contained soot. Although the diesel soot + acids atmosphere did not alter early or late clearance of tracer particles, this atmosphere - and the other two atmospheres studied - depressed pulmonary macrophage functions. This interference with macrophage functions may have significant implications to human health.

### INTRODUCTION

#### Atmosphere Selection

Persons living in the South Coast Air Basin of southern California - an area heavily impacted by vehicular and stationary source air emissions - are exposed to a large variety of airborne particles and gases. Included in the particulate pollutants are

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KEY WORDS: acid, diesel exhaust, toxicity, macrophage

various metals, minerals, water-insoluble inorganic compounds, water-soluble inorganic compounds including weak and strong acids, a great variety of organic compounds, elemental carbon, and water. This study focused on a particular combination - fine carbon particles plus acid - which has been associated with excess deaths and morbidity in past air pollution disasters (Goldsmith and Friberg, 1977), and which may represent a significant present and future airborne health hazard.

Elemental (graphitic) carbon in the fine particle size range (aerodynamic diameters below 10 micrometers) is emitted into the South Coast Air Basin at a rate of about 15 metric tons per day (Cass et al., 1982). Although elemental carbon represents only about one-third of the total particulate emission in the air basin, it is not only a substantial portion of the total fine particle inventory, but also is known to have significant retention times in the lung due to its resistance to normal particle clearance mechanisms (Nau et al., 1962; Wiester et al., 1980; Griffis et al., 1983; McClellan, 1987).

Acid mists and fogs in the South Coast Air Basin have been collected and found to have pH values as low as 2.2 (Brewer et al., 1983). Ion analyses indicated that nitrate and sulfate ions were present in ratios that were consistent with the relative emission rates of nitrogen oxides and sulfur dioxide into the air (Waldman et al., 1982; Brewer et al., 1983). The acid mist or acid fog droplets contained a variety of anionic and cationic species, but hydrogen, ammonium, nitrate and sulfate ions accounted for about 90% of the total ionic species on a chemical equivalency basis (Brewer et al., 1983).

Since it isn't possible to precisely simulate complex ambient atmospheres in the laboratory, a surrogate pollutant mixture has therefore been developed for exposure of rats in an effort to assess the potential toxic effects on the respiratory system. The atmosphere studied was a mixture of sulfuric acid-coated diesel soot particles formed from diesel exhaust in the presence of sulfuric acid ( $H_2SO_4$ , which is formed when sulfur dioxide is present in a photochemically pollutant atmosphere) and nitric acid ( $HNO_3$ ) vapor (which is formed by ozone and nitrogen dioxide in ambient air). Separate groups of rats were exposed to (a) diesel exhaust and (b) the acids in order to determine the effects of the component parts of the combined atmosphere. A 5 day period of daily exposures of rats was chosen to simulate an environmental exposure to a complex carbon-acid mixture atmosphere during an extended air pollution episode, or to simulate a visit to a polluted area from a cleaner environment.

### Previous Studies

Studies of the effects of diesel exhaust using laboratory animals show accumulation of particles in the deep lung, production of "nonspecific" cellular damage, and deterioration of lung defenses (Health Effects Panel of the Diesel Impacts Study Committee, 1981; McClellan, 1987). Repeated exposure appears to prolong retention times for carbon in lung tissue over those seen after a single exposure (Griffis et al., 1983). The cellular and mucus changes, except for those observed following long-term exposures or high concentration studies, appear to reverse over time. In the lungs of rats exposed to  $3.5 \text{ mg/m}^3$  or above, clusters of macrophages filled with black pigment were observed in areas that did not yet show fibrotic or cancerous changes (McClellan, 1986). In rats inhaling  $0.35 \text{ mg/m}^3$ , the carbon particle load did not continue to increase, even with extended times of exposure; hence, the rate of particle clearance probably matched the rate of particle deposition. Pigment (carbon) storage in macrophages was dose-related. McClellan (1987)

subsequently estimated that the cancer risk to the U.S. human population from diesel exhaust inhalation was "fewer than 200 deaths per year."

Most of the available literature regarding the health effects of acid is concerned with sulfuric acid. Sulfuric acid droplets, when inhaled, have been associated with several effects. These effects include bronchoconstriction, alterations in ciliary beating, loss of cilia, sloughing of epithelial cells and alterations in clearance of particles from the lung (Amdur, 1958; Lippmann et al., 1987; Schlesinger et al., 1978; Schiff et al., 1979; Phalen et al., 1980; Wolff et al., 1981, 1986; Chen and Schlesinger, 1983). Very similar effects on particle clearance have been seen in rats, rabbits, dogs, donkeys and humans. At low concentrations of inhaled  $H_2SO_4$ , a stimulation of clearance is observed, but clearance is depressed at higher levels (Chen and Schlesinger, 1983). A single one hour exposure to  $1 \text{ mg/m}^3$  of  $H_2SO_4$  aerosol can cause effects on clearance that persist to 1 week post-exposure (Wolff et al., 1981). Exposures (up to 120 hours, cumulatively) of laboratory animals to  $H_2SO_4$  aerosols at concentrations ranging from 0.3 to  $5 \text{ mg/m}^3$ , and sizes in the range of 0.3 to 1.0 micrometer mass median aerodynamic diameter (MMAD), have resulted in changes in respiratory tract morphology including concentration-dependent increases in the thickness of the tracheal mucus layer in guinea pigs (Wolff et al., 1986), edema and cellular infiltration around bronchi and parenchymal blood vessels in guinea pigs (Bushtueva, 1957), and increased epithelial thickness and increased numbers of small airways and secretory cells in rabbits (Schlesinger et al., 1983). Exposures to  $H_2SO_4$  for longer durations (cumulative exposure times between 120 and 700 hours) resulted in hypertrophy (excessive growth) of epithelial cells, mainly in the alveolar duct region in rats (Juhos et al., 1978), and increased density of secretory cells throughout the bronchial tree accompanied by decreased lumen sizes of small airways in rabbits (Gearhart and Schlesinger, 1988).

Despite the fact that nitric acid is widely used in industry, the toxicology data base is very limited. Abraham et al. (1982) exposed sheep to 1.6 ppm ( $5.5 \text{ mg/m}^3$ ) nitric acid for 4 hours and found that airway reactivity to aerosolized carbachol was increased. Studies with an ozone + nitrogen dioxide atmosphere at high relative humidity, in which nitric acid was readily formed, showed that both airway permeability and lung pathology were altered (Bhalla et al., 1987; Mautz et al., 1988). In these studies effects were seen when the ozone and nitrogen dioxide concentrations were as low as 0.35 ppm and 0.6 ppm, respectively, and the associated nitric acid formed was about  $0.05 \text{ mg/m}^3$ .

Schiff et al. (1979) exposed hamsters for 3 hours to carbon alone ( $1.5 \text{ mg/m}^3$ , 0.3 micrometer diameter), sulfuric acid alone ( $1.1 \text{ mg/m}^3$ , 0.12 micrometer diameter), or to the mixture. Carbon alone did not change the tracheal cilia beating frequency, but it produced occasional rounded, swollen epithelial cells and focal loss of ciliated cells. Sulfuric acid alone was associated with statistically significant lowering of ciliary beating frequency, patchy uneven swollen epithelium, some loss of cilia, rounding and sloughing of epithelial cells, and alterations in the mucus which covers the trachea. The carbon plus sulfuric acid had effects that were of the type seen with acid alone, but in general were more severe and of longer duration. In a study of the effects of pollution on susceptibility to infection, mice exposed to acid-coated carbon particles (300 hrs cumulative at  $2.9 \text{ mg/m}^3$ ) showed higher mortality and pulmonary consolidation following a challenge exposure to viruses than did mice exposed to equivalent amounts of either carbon alone or acid alone (Fenters et al., 1979).

## Biological Endpoints Used

Without effective clearance the respiratory tract would quickly overload with contaminants and disease would be inevitable. In mammals the airways of the head, neck and bronchial tree are coated with mucus which is steadily driven by small hair-like cilia toward the throat for swallowing. The proper functioning of this clearance mechanism requires mucus-producing cells and glands and a large healthy population of mature ciliated cells. Deeper in the lung the clearance of insoluble foreign materials apparently depends largely upon a population of self-mobile macrophages. Macrophages, which can engulf foreign materials, may transport that material to the mucus, whereby it is subsequently swallowed, or to lymph nodes for long-term storage. In some cases macrophages with ingested debris remain in the deep lung for long periods. Our study included assessments of these clearance phenomena during the early (mucociliary) and late (macrophage) clearance phases. The tracer particles used to follow clearance were radiolabeled so that they could be easily monitored by external detectors after inhalation. The monodisperse tracer particles were about 1.8 micrometers in aerodynamic diameter, and thus deposited throughout the entire respiratory tract.

Changes in the structure of respiratory tract tissues were investigated using histopathologic (light microscopy) techniques. Histopathologic evaluations were made of the respiratory tract from the nasal cavity to the lung alveolar region in order to identify and measure any cellular injury or derangement induced by inhalation of the pollutants. Measurements of stimulated cell division at different sites of injury in nasal, tracheal, and bronchial tissues were performed using an autoradiographic technique after labeling the whole animal with a radioactive DNA precursor. In addition, a quantitation of the number of lung cells containing soot particles was also performed. We were interested in the effects of acidity on the degree of accumulation of soot particles in the lung, since excessive accumulation of soot has been implicated as a possible contributor to lung cancer (McClellan, 1987).

Pulmonary macrophages were evaluated to determine if this element of pulmonary defense was compromised. In addition to their role as phagocytes, the pulmonary macrophages also (a) function as potent secretory cells which release a wide variety of biologically active products, among which are compounds that can attract and activate other cells to initiate an inflammatory response, and (b) carry out a recently discovered immunoregulatory function, in that they have been shown to modulate lymphocyte response. Macrophages play an integral role in the host lung defense and their role has been reviewed extensively (Brain, 1986; Herscovitz, 1985; Bienenstock, 1984). Macrophages possess receptors on their surfaces for the Fc portion of immunoglobulin (IgG) which help in the recognition of foreign particles (Gaafar et al., 1971) and facilitate phagocytosis and destruction of foreign material 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), and mediates antibody-dependent cellular cytotoxicity (Johnston et al., 1985). Inhalation exposure to nickel (Reichrtova et al., 1986), and in vitro exposure to lead (Jian et al., 1985) have been shown to cause an inhibitory effect on FcR activity (ability to fix cytophillic antibody on the surface of macrophages). In addition, we (Prasad et al., 1988) have shown the inhibitory effects of three different atmospheres on FcR activity, and have established the importance of this endpoint as a marker of lung injury. The ability of macrophages to engulf foreign particles (polystyrene latex microspheres) by phagocytosis was also measured using macrophages recovered from lungs of experimental and control rats.

## MATERIALS AND METHODS

### Experimental Design

Rats were exposed by inhalation for 5 hours per day for 5 successive days to atmospheres containing diesel exhaust soot and/or a mixture of nitric and sulfuric acids. A matched group of control rats was exposed to purified air using the same exposure regimen. The biological evaluations performed using the exposed animals were chosen to allow us to observe both structural (S) and functional (F) alterations in the upper respiratory tract (URT) and deep lung (DL) regions. These evaluations included: (a) measurement of early (F, URT) and late (F, DL) respiratory tract clearance of insoluble tracer particles; (b) histopathological examination of respiratory tract tissues (S, DL), including autoradiography of cell turnover (S, URT) and soot particle accumulation (F, DL); and effects on pulmonary alveolar macrophages (F, DL). The details of the methods involved in exposing the rats and performing the biological evaluations are provided below.

### Animals and Exposures

Barrier-reared Sprague-Dawley rats used in this study were purchased from Hilltop Lab Animals, Inc. Male rats (200 - 225 g, about 6 weeks of age) were delivered to the laboratory in filter-equipped shipping boxes to minimize prior exposure of the animals to particulate pollutants. The rats were then housed in a laminar air-barrier caging system (with high-efficiency gas and particle filtration) for about one week before the start of the exposure. Microbiological assays, supplied by Hilltop, indicated that the rats were free of respiratory infection. This fact was confirmed by quality control histopathologic examinations on 10% of the animals at our laboratory.

Groups of rats were exposed 5 hours per day for 5 consecutive days to either purified air or the pollutant atmospheres using unique 1 m<sup>3</sup> stainless-steel Rochester chambers modified for nose-only exposure. This exposure system has been described in detail previously (Prasad et al., 1988). Each rat was placed into a nose-only exposure tube, which was then inserted and sealed into a nose-only port in a wall of an exposure chamber. This exposure system was specifically designed to prevent the neutralization of acids in the pollutant atmospheres by ammonia generated from the rats' excreta. The chambers were supplied with purified air (prior to the injection of pollutants) at a temperature of about 23°C, and a relative humidity of about 83%. The chamber air contained about 5 ppb of ammonia, which would neutralize less than 5% of the sulfuric acid present.

Diesel exhaust aerosols were obtained from an 11 horsepower, single-cylinder diesel engine operating at constant speed (1800 r.p.m.) and load (80% of maximum). The engine exhaust was rapidly diluted with purified air to prevent particle agglomeration before inhalation by the rats. (A schematic diagram of the diesel soot + acids generation system is presented in Figure 1.) The fuel used was Phillips #2 Diesel Control Fuel. The sulfuric acid coating on the diesel soot aerosol was produced by bubbling a stream of dry air through fuming sulfuric acid. The resultant sulfur trioxide vapor was mixed with the carbon aerosol in the presence of water vapor to produce the sulfuric acid coating on the surface of the particles (Walters et al., 1988). The coating of sulfuric acid on the soot particles was investigated using electron microscopic techniques. Approximately 95% of the soot particles were coated with acid. Nitric acid vapor was generated by bubbling purified air through concentrated nitric acid immersed in a constant temperature bath.

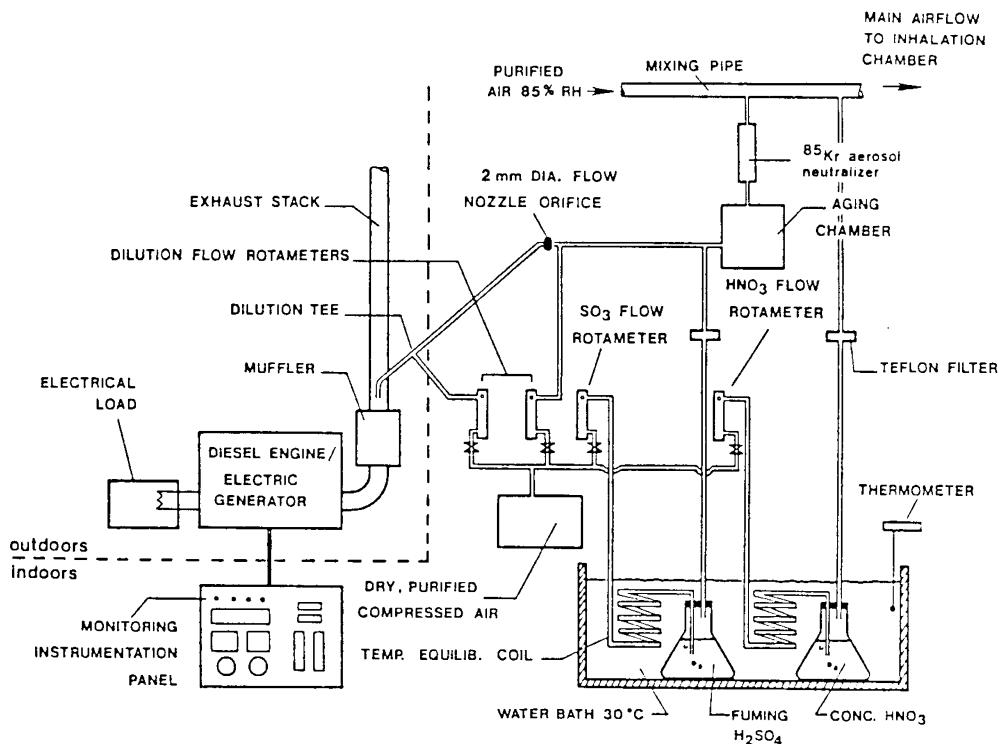


FIGURE 1. Diagram of the Diesel Soot + Acids Generation System.

Cascade impactor samples were used to size-classify particles for chemical and gravimetric analyses. The size (mass median aerodynamic diameter) of the acid-coated diesel soot aerosol was about 0.15 micrometers. The geometric standard deviation was approximately 2. Aerosols and vapors were collected on filters for gravimetric and chemical analyses (nylon filters (Shaw et al., 1982) were used for nitric acid sampling). The analysis of filter samples collected during the study indicated that the diesel soot aerosol particles were about 92% elemental carbon and 8% extractable organic matter. The aerosol concentration stability during exposures was determined using a real-time aerosol mass monitor. In the case of diesel exhaust-containing atmospheres, nitrogen oxides and carbon monoxide were monitored using calibrated gas monitors. All sampling (for both aerosols and gases) was from the rat's breathing zone.

The atmosphere characterization data obtained during the exposures indicated that the observed mean pollutant concentrations for the three atmospheres studied were: (a) 0.56 mg/m<sup>3</sup> diesel exhaust soot, (b) 0.40 mg/m<sup>3</sup> nitric acid + 0.17 mg/m<sup>3</sup> sulfuric acid, and (c) 0.55 mg/m<sup>3</sup> diesel exhaust soot + 0.39 mg/m<sup>3</sup> nitric acid + 0.19 mg/m<sup>3</sup> sulfuric acid. The mean nitric oxide (NO), nitrogen dioxide (NO<sub>2</sub>) and carbon monoxide (CO) concentrations in the diesel exhaust-containing atmospheres were about 6 ppm, 1 ppm and 10 ppm, respectively.

### Biologic Endpoints

#### 1. Particle Clearance

The radioactive tracer particles were labeled at this laboratory with tightly bound <sup>51</sup>Cr (Hinrichs et al., 1978). Aerosols were

generated using a Lovelace-type compressed air nebulizer (Mercer et al., 1968). The aerosolized particles were dried, diluted with filtered air, and passed through a  $^{85}\text{Kr}$  discharger; rats were exposed for 20 minutes. The aerosol, sampled from the breathing zone of the rats using a calibrated seven-stage impactor, had an activity median aerodynamic diameter of 1.8 micrometers and a geometric standard deviation of about 1.2. The animals were placed in individual plastic counting tubes and inserted into a collimated 3-in NaI(Tl) gamma ray detector apparatus for an initial count, and then they were given their final 5 hour exposure (on the fifth exposure day). After the rats were removed from the exposure chambers their feces were collected at 10 fixed times during the first 50 hours after the deposition of the tracer particles. Early clearance was characterized by the resulting fecal activity excretion curves. Fecal output rates and water consumption rates were monitored for each rat so that an effect of the pollutant exposure on the gastrointestinal system could be detected. During the first 400 hours post-deposition of the tracer particles, 5 thoracic counts were performed on each animal in order to characterize late clearance. The rats were sacrificed at 30 days post-deposition and their lungs counted in order to provide another measure of late clearance (termed the  $A_{30}$  for activity remaining at 30 days). Early clearance 50% clearance times ( $T_{50\%}$ ), late clearance biological half-times ( $T_L$ ), and  $A_{30}$  values were determined for each rat individually. Means and standard deviations were calculated for the purified air and pollutant exposed groups of rats, and the group  $T_{50\%}$ ,  $T_L$ , and  $A_{30}$  values were compared statistically using two-tailed t-tests.

## 2. Histopathology

A detailed description of the histological techniques used in the quantification of lung lesions has been given elsewhere (Mautz et al., 1985). A brief summary is provided here. Rats were deeply anesthetized and then humanely killed by exsanguination about 4 hours after their last 5 hour exposure. The thoracic cavity was opened, the lungs and trachea carefully exposed, and the lung surfaces grossly examined. The distal portion of the trachea was cannulated and this segment with attached lungs was fixed by airway perfusion with 10% neutral-buffered formalin at 30 cm fluid pressure for 72 hours. The remaining trachea with attached larynx was removed and immersed in formalin fixative. In addition, the rat was decapitated, and that portion of the head containing the intact nasal cavity was immersed in formalin fixative. After the lungs were removed from the perfusion apparatus, the lobes of the right lung were separated. Appropriate portions of the caudal lobe were prepared for embedding in an automatic tissue processor. Lung and tracheal tissues were embedded in paraffin and sectioned at 6 micrometers on a rotary microtome. Complete lobar sections of lung were cut close to the midline of the main bronchus, and were used for microscopic examination and grid area determinations. The trachea was split longitudinally and cell turnover rates were examined. After fixation the heads were decalcified and specimens of nasal cavity were prepared by free hand cutting a 2-3 mm slice through the hard palate at the incisive papillae. This slice was sectioned at 4 micrometers and stained. Lung grid area determinations were made using an ocular grid calibrated with a stage micrometer. Lung parenchymal area determinations were similarly made at a lower magnification. Only lung parenchyma was counted. The magnification factor for the two grid counting systems was 1:8. The total lung parenchymal area was computed and the percent lung lesion obtained. The linear grid scans of lung parenchyma gave a count of total lesion area per alveolar zone of lung section.



Autoradiographic techniques (Mautz et al., 1988) were used to identify sites of cell proliferation (associated with cell death) in various regions in the respiratory tract. Rats were injected intraperitoneally with tritiated thymidine, a DNA precursor ( $H^3T$ , 1 uCi/gm body wt.), one hour before killing. Slides containing the respiratory tissues were subsequently dipped into sensitive photographic emulsion. After radiation exposure and photographic development, slides were stained and the percentage of labeled cells per epithelial cell population in the trachea and nasal cavity was determined by cell counts for each animal.

As an additional measure of the effects of the soot-containing atmospheres on the deep lung, a quantitation of the lung cell populations containing soot particles was also performed. The percentage of cells containing soot was compared statistically with control values using two-tailed t-tests and analyses of variance.

### 3. Macrophage Studies

Prasad et al., (1988) described in detail the procedures for determining the phagocytic activity and FcR activity of pulmonary macrophages. In brief, 0.1 ml of cell suspension in HBSS (Hank's balanced salt solution, viable cell concentration  $1 \times 10^6$ /ml), obtained from mincing the lungs, was added to wells of Lab-Tek chambers containing 0.4 ml HBSS. The Lab-Tek chambers were incubated for 1 hr at  $37^\circ C$  and washed with HBSS to remove non-adherent cells. Then 0.1 ml of a suspension of spherical polystyrene latex particles (diameter 1.1 micrometers) was added to each chamber; the chambers were then incubated for 60 minutes. The wells were then washed with calcium-free and magnesium-free PBS (Phosphate Buffered Saline, pH 7.2) to remove any non-engulfed latex particles, and the slides were observed under an inverted phase contrast microscope. Macrophages that had engulfed the latex particles were counted. A minimum of 300 macrophages from each rat was counted. The percentage of latex positive cells was then determined.

Pulmonary macrophage FcR activity was determined by a rosette assay method. Briefly, 0.2 ml of anti-SRBC (Sheep Red Blood Cell) serum at a predetermined concentration was added to wells of Lab-Tek chambers pre-incubated with cell suspension as before, and incubated at  $37^\circ C$  for 30 minutes. Then the serum was removed and 0.1 ml of SRBC ( $1 \times 10^7$  cells/ml) was added to each of the wells, along with 0.2 ml of HBSS, followed by incubation for 30 minutes at  $37^\circ C$ . Unbound SRBCs were then washed away gently with HBSS and each chamber was examined with an inverted phase contrast microscope to determine the number of macrophages forming rosettes. After counting a minimum of 300 cells, the percentage of macrophages that formed rosettes was calculated. Data for each rat, in the form of latex-positive macrophages or net rosettes, were assembled and analyzed using two-tailed t-tests, analyses of variance, and the Newman-Keuls multiple comparison test. A level of significance of 0.05 or less was considered statistically significant.

## RESULTS

### Particle Clearance Study

The results of the particle clearance experiment in which rats were exposed to the diesel soot + acids atmosphere are shown in Table 1. The data indicate that this combined pollutant atmosphere did not significantly affect the early or late clearance of the tracer particles. The results of both the thoracic counting ( $T_1$ ) and lung

TABLE 1

Effects of the Diesel Soot + Acids Atmosphere on Early and Late Clearance of Radiolabeled Tracer Microspheres				
<b>Early Clearance</b>	N	$\bar{T}_{50x} \pm SD(\text{hr})$	$\Delta T_{50x} \pm SE(\text{hr})$	$p^b$
Purified Air	63	$9.9 \pm 2.1$	----	--
Diesel Soot + Acids	56 <sup>a</sup>	$10.0 \pm 2.2$	$0.1 \pm 0.4$	0.80
<b>Late Clearance</b>	N	$\bar{T}_L \pm SD(\text{hr})$	$\Delta T_L \pm SE(\text{hr})$	$p^b$
Purified Air	63	$624 \pm 217$	----	--
Diesel Soot + Acids	57	$646 \pm 205$	$22 \pm 39$	0.57
<b>A<sub>30</sub></b>	N	$\bar{A}_{30} \pm SD$	$\Delta A_{30} \pm SE$	$p^b$
Purified Air	63	$20.7 \pm 3.9$	----	--
Diesel Soot + Acids	57	$20.9 \pm 3.9$	$0.2 \pm 0.7$	0.78

$T_{50x}$  = time required to excrete 50% of the total activity excreted through 50 hours postdeposition.

$T_L$  = late clearance biological half-time.

$A_{30}$  = index of late clearance.

a - one rat was excluded from the early clearance data analysis for failure to meet established defecation criteria.

b - Two-tailed t-test.

sacrifice ( $A_{30}$ ) analyses are in agreement that the clearance of the radiolabeled tracer particles from the deep lung region of the rats was not affected by the 25 hour diesel soot + acids exposure. (Rats were not exposed to diesel soot alone or the acids alone for the clearance endpoint.)

### Histopathology Studies

Despite the intense histopathological coverage and large numbers of careful measurements performed, no significant injury was observed. This is a clear negative with respect to tissue disruption at the light-microscopic level. None of the atmospheres studied (diesel soot + acids, or the component atmospheres) produced statistically significant quantities of deep lung focal lesions in pollutant-exposed rats. The autoradiographic analyses of respiratory tissues indicated that none of these atmospheres produced a significant acceleration in cell turnover in the nasal, tracheal or deep lung epithelia. (While only epithelial cells were counted, no attempt was made to distinguish the among the various cell types within the epithelium.

The data from the endpoint involving the quantitation of lung cell populations containing soot particles are shown in Table 2. The cells containing soot were mostly free cells (i.e., macrophages) which were found in alveolar spaces or against the alveolar walls (Figure 2). The distribution of these cells appeared to be random throughout the lung - although an occasional aggregate of soot-containing cells (between 3 and 8 cells) was found in proximal ducts. The results of the study demonstrate that a significantly lower percent of cells containing soot was found in the rats exposed to the diesel soot +

TABLE 2

Comparison of Carbon Retention in the Deep Lung for Groups of Rats Exposed to Diesel Soot-containing Atmospheres

	NO. OF RATS N	CELLS WITH SOOT PER FIELD(SE)	% OF TOTAL CELLS WITH SOOT(SE)
Diesel Soot + Acids	7	0.75(0.06)	0.51(0.04)
Diesel Soot	6	1.15(0.03) <sup>a</sup>	1.07(0.05) <sup>a</sup>

a = p < 0.01 vs. Diesel Soot + Acids group (two-tailed t-test).

acids atmosphere than were seen in the animals exposed to diesel soot alone. Although the diesel soot + acids and the diesel soot alone studies were performed using different batches of rats, an analysis of variance indicated that there was not a statistically significant (p<0.05) difference between the two studies in the total number of cells observed per microscope field. Therefore, the experiment was not complicated by the occurrence of hyperplasia, and the statistical comparison of the results obtained for the two groups was valid.

#### Macrophage Studies

The results of the experiment performed to examine the effects of the diesel soot + acids atmosphere on the Fc receptors and on the phagocytic capability of rat alveolar macrophages are listed in Table 3. The data indicate that there was a significant reduction (p<0.05) in the binding of sheep red blood cells to the macrophages obtained from the diesel soot + acids group of rats on days 0 (the last exposure day) and 2. This is evidenced by the reduction in the percentage of rosettes formed as compared to the purified air controls. Consistent with these results, it was observed that there was a significant reduction in the percentage of macrophages from the diesel soot + acids group which engulfed polystyrene latex particles. This effect was also present immediately after exposure and persisted for at least 3 days. The significance of the data was also tested using an analysis of variance. The results indicate that both the Fc receptors and the phagocytic capability were strongly and persistently affected by the atmosphere.

The results from the study which involved exposures to diesel soot alone and the acid combination alone are also presented in Table 3. The acid combination produced a statistically significant reduction in the binding of the sheep red blood cells to the macrophages obtained on days 0, 2 and 4. A significant decrease in rosette formation was also noted on days 0 and 2 with rats exposed to diesel soot. By day 4 the percentage of rosettes had returned to normal. Regarding the effects of the atmospheres on phagocytosis, the acid combination significantly depressed phagocytic activity immediately after the exposure, and the effects persisted through day 4. A similar effect was observed with rats exposed to diesel soot, but in this case the effect only existed in rats sacrificed on day 2. The phagocytosis and Fc receptor data were also compared using an analysis of variance test. Both the Fc receptor formation and the

TABLE 3

Effect of Pollutant Atmospheres on Pulmonary Macrophage Functions				
Percent of rosettes formed on day Di after pollutant exposure (Mean $\pm$ standard deviation)				
Exposure	Purified Air Controls <sup>a</sup>	D0	D2	D4
Diesel Soot + Acids	28.6 $\pm$ 5.9 (n=8)	16.8 $\pm$ 4.2 <sup>b</sup> (n=5)	16.1 $\pm$ 1.8 <sup>b</sup> (n=5)	19.1 $\pm$ 1.3 <sup>c</sup> (n=5)
Diesel Soot	44.0 $\pm$ 3.6 (n=12)	18.7 $\pm$ 2.8 <sup>b</sup> (n=6)	35.8 $\pm$ 3.7 <sup>b</sup> (n=4)	42.0 $\pm$ 3.2 (n=4)
Acids	42.1 $\pm$ 3.7 (n=12)	12.8 $\pm$ 1.7 <sup>b</sup> (n=6)	23.0 $\pm$ 2.2 <sup>b</sup> (n=4)	35.5 $\pm$ 3.4 <sup>b</sup> (n=4)
Percent of macrophages with polystyrene latex particles on day Di after pollutant exposure				
Diesel Soot + Acids	66.1 $\pm$ 8.8 (n=8)	41.1 $\pm$ 9.4 <sup>b</sup> (n=5)	53.3 $\pm$ 3.6 <sup>b</sup> (n=5)	50.7 $\pm$ 2.5 <sup>b,c</sup> (n=5)
Diesel Soot	83.2 $\pm$ 2.9 (n=12)	79.8 $\pm$ 4.7 (n=6)	77.5 $\pm$ 3.1 <sup>b</sup> (n=4)	80.5 $\pm$ 2.4 (n=4)
Acids	77.0 $\pm$ 4.2 (n=12)	65.0 $\pm$ 2.4 <sup>b</sup> (n=6)	71.5 $\pm$ 3.9 <sup>b</sup> (n=4)	71.5 $\pm$ 2.1 <sup>b</sup> (n=4)

a - Control rats were sacrificed on Days 0, 2 and 4, and the data were averaged.

b -  $p < 0.05$  vs. control (two-tailed t-test, and Newman-Keuls multiple comparison test).

c - Data from rats sacrificed on day 3.

phagocytic capability were found to be significantly depressed from the control values by the diesel soot and the acids atmospheres. While the Fc receptors exhibited a pronounced effect on day 0 with a trend towards baseline over the next 3 days, such a progression was less evident for the phagocytosis endpoint.

An analysis of variance was also used to compare the results (Fc receptors and phagocytosis) of the diesel soot + acids study to the results of diesel soot alone and acids alone studies. A statistically significant difference in the effects of the three atmospheres was noted. However, the analysis revealed that there was a significant "study" effect - i.e., the control values varied greatly between the diesel soot + acids study and the other two studies. A Newman-Keuls multiple comparison test was then used, and the results of this test indicated that any differences in the effects of the diesel soot + acids atmosphere and the other two atmospheres were marginal. In other words, both the diesel soot and the acids atmospheres produced effects which were not statistically different from the effects of the diesel soot + acids atmosphere.

#### DISCUSSION AND CONCLUSIONS

The findings are intriguing from a toxicological point of view. They indicate that the deep lung regions were the most affected by the exposures. The diesel soot particles were able to penetrate into the

deep lung because of their low deposition efficiency in the nose. Diesel soot, the acid combination and the diesel soot + acids atmosphere all affected the macrophages of the deep lung. The macrophage changes warn of the potential of the atmospheres for producing immunologic perturbations in human populations which could lead to increased susceptibility to infection.

From a review of the literature on the toxicological effects of diesel exhaust particles, it is obvious that the majority of the studies were conducted to discover the mutagenic and carcinogenic effects following long-term exposures (greater than 6 months) with high soot levels. However, a few other effects reported include: (a) exposure concentration-related dark discoloration of lungs and thoracic lymph nodes; (b) an increase in the number and size of alveolar macrophages; (c) diesel particle accumulation in macrophages, alveoli, interstitium, lymphatic channels and eosinophils; (d) a restrictive disorder of pulmonary function; (e) an increase in the total number of cells in thoracic lymph nodes, and a trend towards an increase in the number of IgM antibody forming cells; and (f) an alteration in biochemical and cellular constituents of lung lavages, which was consistent with an inflammatory response. Each of these effects have been observed with moderate ( $3.5 \text{ mg/m}^3$ ) to high ( $7.0 \text{ mg/m}^3$ ) levels of exposure for periods exceeding 6 months (McClellan, 1986).

Wolff et al. (1987) exposed groups of rats to low ( $0.35 \text{ mg/m}^3$ ), medium ( $3.5 \text{ mg/m}^3$ ) and high ( $7.0 \text{ mg/m}^3$ ) levels of diesel exhaust soot for 7 h/day, 5 days per week, for up to 2 years. None of the exposures produced significant effects on tracheal mucociliary (early) clearance. While statistically significant delays in pulmonary (late) clearance were noted following exposures to the medium and high soot levels, no significant changes were seen in the group exposed to the low level (a concentration close to the one used in our study). Lung burdens of diesel soot in the rats at all exposure levels showed a progressive increase with time—an effect which was also observed by Chan et al. (1984) following exposures of rats to  $0.25 \text{ mg/m}^3$  or  $6.0 \text{ mg/m}^3$  of diesel exhaust soot for 20 h/day, 7 days per week, for up to 112 days. In that study the group exposed to the low soot level did not exhibit a significant effect on pulmonary clearance, while the high level did produce a statistically significant delay. In our study, we did not observe any statistically significant changes in the clearance patterns of rats exposed to diesel soot + acids. Although our study was of considerably shorter duration, and in spite of the co-presence of the acids in the exposure atmosphere, our results are similar to those observed in the prior studies. (Our particle clearance measurement was designed to detect (with 90% certainty) a group change of  $\pm 10\%$  in the early and late clearance rates.) In view of this observation, and since the lung burden increases with dose and exposure duration, it is reasonable to hypothesize that the altered clearance in the high level studies may be because of an overload of particulate material both in the lung parenchyma and macrophages. However, it should be noted that ambient exposures do not involve such high doses. Hence, it is possible that clearance mechanisms may not be affected on exposure to diesel exhaust particles at low levels. But clearance effects may be observed following chronic exposures, resulting from an accumulation of the particulate material.

Figure 2 and Table 2 show qualitative and quantitative assessments of soot accumulation in cells, which is an important observation of this study. Even in the absence of an effect on the clearance mechanism, for a short-term exposure (5 hours per day for 5 days) at relatively low pollutant concentrations ( $0.50 \text{ mg/m}^3$ ) the soot accumulation is evident. This observation supports the hypothesis that progressive soot accumulation is possible in the absence of an effect on the clearance mechanism. As was previously

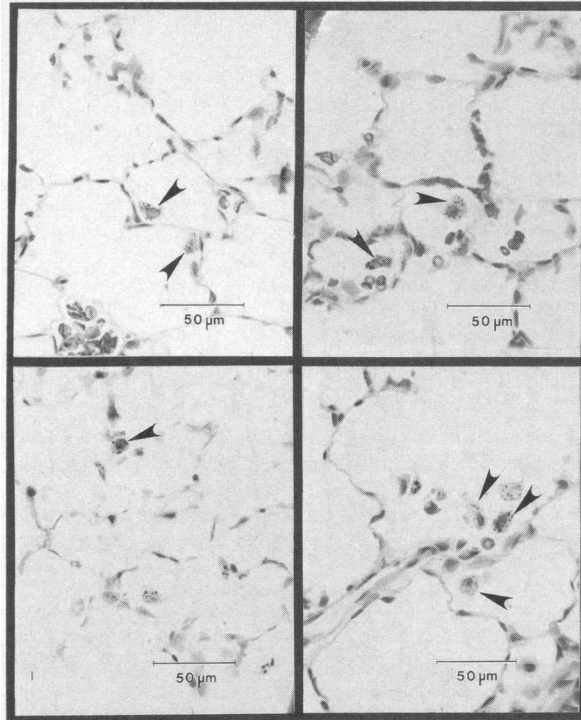


FIGURE 2. Macrophages Containing Soot Particles (arrows).

mentioned, a significant portion of the accumulated soot was observed in macrophages. From Table 3 it is evident that another macrophage function - Fc receptor-mediated binding of opsonized material - was also compromised by the pollutant atmospheres, in absence of any observable effect on late clearance of inhaled particles, another functional parameter measured in the study.

Bice et al. (1985) have reported that diesel particle accumulation is seen not only in macrophages, but also in the interstitium, alveoli, lymphatic channels, etc., of the lung. This leads to the speculation that an alteration in the permeability of the alveolar region may be contributing to the soot accumulation in different compartments of the lung. From preliminary data provided by Michael Kleinman and Deepak Bhalla of our laboratory (unpublished), it appears that permeability of the alveolar region is altered in rats exposed to atmospheres containing comparable levels of diesel exhaust particles.

Exposures of rats to atmospheres containing diesel exhaust particles alone and in combination with the acids have resulted in the inactivation of the Fc receptors of pulmonary macrophages (Table 3). We have previously reported similar toxic effects produced by ozone and ozone-containing multicomponent atmospheres (Prasad et al., 1988). Although the mechanism(s) that induces this phenomenon is not clearly understood, it has been hypothesized that soluble immune complexes and suppressor T cells may play significant roles (Rao et al., 1979a; Rao et al., 1979b; Rao et al., 1980). Therefore, it seems clear that the immune competence of the macrophages was compromised by exposure to the test atmospheres at lower pollutant concentrations than have been used in other studies in which toxic effects were observed.

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