21-DAY EXPOSURE TO MIXED AIR POLLUTANTS:
EFFECTS ON LUNG AIRWAYS AND MACROPHAGES

Research Contract No. A6-126-33
Final Report

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The California Air Resources Board

by

Robert F. Phalen, Ph.D. and Michael T. Kleinman, Ph.D.
Air Pollution Health Effects Laboratory
Department of Community and Environmental Medicine
University of California
Irvine, CA 92717

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I. ABSTRACT

The effects of exposure to a 7-component pollutant atmosphere on epithelial permeability and macrophage functions in rats have been studied. The components of this atmosphere (0.30 ppm ozone + 1.2 ppm nitrogen dioxide + 2.5 ppm sulfur dioxide + 0.27 mg/m³ ammonium sulfate + 0.22 mg/m³ ferric sulfate + 0.004 mg/m³ manganese sulfate + 0.15 mg/m³ ferric oxide) were selected in order to model the oxidant and sulfate-containing air pollutant mixtures which are common in the South Coast Air Basin of southern California. Separate groups of rats were exposed 4 hours per day to either purified air or the 7-component atmosphere for either 7 or 21 consecutive days. Results indicate that statistically significant (p < 0.05) effects on macrophage functions (Fc receptor activity and phagocytic activity) were observed in rats exposed to the 7-component mixture for both 7 and 21 days, with more pronounced effects seen in the group exposed for 21 days. In both cases these effects persisted up to 96 hours post-exposure. No statistically significant effects on nasal or bronchoalveolar epithelial permeability were found in either the 7 day or 21 day exposure groups. These findings indicate that a mechanism by which the deep lung is cleansed of particulate debris, macrophage activity, is compromised throughout the period of repeated exposure to the 7-component atmosphere, while no significant effects on the integrity of the airway epithelia are produced. Although the changes seen in macrophage function are not a disease per se, they indicate damage to an important defense mechanism that must function efficiently in order to maintain health.

II. ACKNOWLEDGEMENTS

Key technical personnel on the project included Dr. D. Bhalla, Dr. S. Prasad, Dr. S. Rao, R. Mannix, T. McClure, M. Azizian, T. Nguyen, S. Lavan, S. Bucher, B. Nordenstam, and D. Daniels. J. Sweaza, L. Romey, J. Cantonwine and S. Usdansky provided administrative, clerical and secretarial support. D. Westerdahl of the California Air Resources Board and Dr. T. Crocker made important technical suggestions. This report was submitted in fulfillment of ARB Research Contract No. A6-126-33, "21-Day Exposure to Mixed Air Pollutants: Effects on Lung Airways and Macrophages," by the Department of Community and Environmental Medicine, California College of Medicine, University of California, Irvine, under the sponsorship of the California Air Resources Board. Work was completed on July 31, 1987.
III. SUMMARY AND CONCLUSIONS

Rats were exposed by inhalation 4 hours per day for either 7 or 21 successive days to a mixture of air pollutants (Table 1). Biologic evaluations performed were epithelial permeability to tracers and lung macrophage cell functions. These evaluations were selected because they are sensitive and relevant to the ability of the respiratory tract to maintain a state of health.

Small elevations in permeability were seen in 7 of the 8 groups evaluated, but the changes were not statistically significant (at the 0.05 level) and were not large enough to cause concern from a health standpoint. On the other hand, lung macrophage cells were significantly depressed in function. We conclude the following:

1. Lung macrophage cells are sensitive to repeated exposure to the mixture atmosphere studied.
2. The changes seen in macrophage function imply that the immune defenses of the lungs of some people will be depressed throughout prolonged air pollution episodes.
3. The results of this 21-day study indicate a need for long-term animal exposures (over a significant portion of the animal's life span) to California-type air pollutant mixtures.

IV. RECOMMENDATIONS

There are two recommendations: first, that long-term animal toxicology studies be performed to evaluate the effects of oxidant and particle-containing atmospheres on lung defenses; second, that future human clinical and epidemiological studies of air-pollutant effects include measures of immune function.

V. INTRODUCTION

A. Atmosphere Selection

The selection of atmospheric components is crucial to the relevance of inhalation toxicology research to public health. With power plant and industrial emissions in mind, epidemiological, human clinical, and animal toxicologic information was searched in order to select pertinent experimental atmospheres. Toxicological studies by ourselves and others under controlled laboratory conditions indicated that in laboratory animals and human volunteers single compounds were not
toxic enough to account for the morbidity and mortality associated with notorious air pollution events. It appeared that some mixture of components present during such severe episodes were responsible for the observed effects on human health. Attempts to identify the likely etiological factor(s) have provided several candidate species. Goldsmith and Friberg (1977) implied that sulfur oxides and particles or oxidants might be causally related to health effects. Kagawa et al. (1980) attributed significant changes in human lung function to the combined influences of temperature, sulfur dioxide, nitrogen dioxide, oxidant and particles.

One hypothesis which can explain differences between results in the laboratory and those in the field is that the suspected compounds are not themselves causal, but that they are associated with other actual etiologic agents (Mazumdar et al., 1982). These etiological agents have perhaps not yet been identified because they have not been adequately monitored in ambient air. Therefore, inhalation toxicology studies with multicomponent atmospheres which represent polluted ambient air are needed. It is important to recognize that stationary source emissions in polluted urban atmospheres will interact with emissions from mobile sources and the products of photochemical reactions. We have therefore selected a multicomponent atmosphere that largely simulates one common type of polluted ambient air - that which has high proportions of both mobile and stationary source emissions and reaction products. We have based our selection of the relative concentrations of pollutants in our 7-component mixture on ambient air sampling data for Lennox, California. This is a site in the South Coast Air Basin for which extensive multiyear, multicomponent data are available. Airborne pollutants at this site are predominantly from primary source emissions including power plants, refineries and automobiles, with modest impact from photochemical oxidants (Cass, 1978).

The atmosphere which has been developed (through a research contract with the Electric Power Research Institute (EPRI)) for use in our studies is a laboratory surrogate mixture of gases and particles. The 7-component atmosphere contains ozone ($O_3$), sulfur dioxide ($SO_2$), nitrogen dioxide ($NO_2$), soluble sulfate aerosols, trace metal ions ($Fe^{+3}$ and $Mn^{+2}$) and acidic reaction products (such as bisulfate ion ($HSO_4^-$) and nitric acid vapor ($HNO_3$)) formed by interactions of the gases and particles which comprise the atmosphere. In order to project an episode in which conditions were such that the total ozone concentration (presumably the most toxic individual component of the mixture) approached 0.6 ppm, we proportionately increased the measured ambient concentrations from Lennox to compute the EPRI test atmosphere composition shown in Table 1. The concentrations for the present study were reduced to one-half of the EPRI concentrations in order to provide information on the effects of these lower concentrations and to allow an estimation of a dose-
response curve relationship. The exposure duration (21 days) was chosen in order to help bridge the gap between single, acute laboratory effects and prolonged, episodic human exposures.

### Table 1. 7-Component Atmosphere

<table>
<thead>
<tr>
<th>Component</th>
<th>EPRI Target Concentration</th>
<th>ARB Target Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO(_2) (ppm)</td>
<td>5.0</td>
<td>2.5 (2.5)*</td>
</tr>
<tr>
<td>O(_3) (ppm)</td>
<td>0.6</td>
<td>0.3 (0.3)*</td>
</tr>
<tr>
<td>NO(_2) (ppm)</td>
<td>2.5</td>
<td>1.2 (1.2)*</td>
</tr>
<tr>
<td>SO(_4)(^{-2}) (mg/m(^3))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(^{+3}) (mg/m(^3))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn(^{+2}) (mg/m(^3))</td>
<td>1.0 (combined)</td>
<td>0.5 (0.49)*</td>
</tr>
<tr>
<td>NH(_4)(^{+2}) (mg/m(^3))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(_2)O(_3) (mg/m(^3))</td>
<td>0.3</td>
<td>0.15 (0.15)*</td>
</tr>
<tr>
<td>H(_2)NO(_3) (mg/m(^3))**</td>
<td>(0.48)*</td>
<td>(0.13)*</td>
</tr>
<tr>
<td>H(^+) (µm/m(^3))++</td>
<td>(3.0)*</td>
<td>(2.0)*</td>
</tr>
</tbody>
</table>

* Actual mean concentration observed during the 21-day study.
** Formed in the exposure chamber by the reactions of generated atmospheric components.
++ Particulate phase acidity attributed to adsorption of H\(_2\)NO\(_3\) (g) by particles.

### B. Biologic Endpoints

The biologic endpoints employed in the study—nasal and bronchoalveolar permeability, Fc receptor activity, and phagocytic activity of macrophages—were chosen due to their coverage of many levels of the respiratory tract. A description of these endpoints is presented below.

1. **Epithelial Permeability**
Permeability characteristics of the airway mucosa (surface) provide a useful tool for identifying the sites of pollutant effects and the magnitude of injury following exposure to air pollutants (Bhalla et al., 1986a). The effect of ozone is maximal in the bronchoalveolar region (deep lung) and minimal in the nose. Formaldehyde, an upper respiratory irritant, produces substantial permeability changes in the nose (Bhalla et al., 1986b).

Airway epithelial transport functions have been studied in recent years by morphologic techniques, by isotope tracer techniques, and by measurements of transmucosal ionic flows. Under normal conditions the airway epithelium selectively restricts the transfer of exogenous antigens, macromolecules and particles from the airway lumen to blood. Structural integrity and organization of the sealing fibrils of tight junctions between adjacent cells constitute the major components responsible for the epithelial barrier function (Claude and Goodenough, 1973; Friend et al., 1972; Richardson et al., 1976).

Increased airway to blood transfer of macromolecules has been noticed following epithelial perturbations resulting from mechanical insult (Gordon and Lane, 1976), antigenic challenge (Hogg et al., 1979), drug treatment (Boucher et al., 1978; Ranga and Kleinerman, 1982; Gatzy and Stutts, 1980) or inhalation exposure to pollutants such as cigarette smoke (Boucher et al., 1980; Hulbert et al., 1981), nitrogen dioxide (Ranga et al., 1980) and ozone (Davis et al., 1980; Bhalla and Crocker, 1986a). Changes in permeability may occur in the absence of obvious epithelial desquamation, metaplasia or neoplasia, suggesting that altered permeability may be a more sensitive indicator of epithelial cell perturbation and injury than common histologic and cytologic changes (Simani et al., 1974; Jones et al., 1980).

In our previous studies involving single exposures to acid atmospheres we found that acidity plus ozone produced a greater and longer-lasting elevation in permeability than ozone alone. A prolonged elevation of nasal and bronchoalveolar permeability was also seen in a previous 21-day study of the 7-component atmosphere with high (EPRI) concentrations. Increased permeability, or "leakiness," of nasal and tracheobronchial epithelia was found using radiolabeled tracers, but the concentrations of pollutant components were high. The experiment was critical because it provided 21 exposures of 4 hr/d and thus served as a bridge between the effects of acute exposure and very long-term effects that require exposure for months to years. "Leakiness" of the nasal or bronchoalveolar epithelia, detected by transport of DTPA into the blood, was greater in pollutant exposed rats than in controls. The positive findings at 7 and 21 days were evidence that injurious
effects of the atmospheres were detectable even when pathologic lung changes were absent. These findings needed to be tested at lower atmospheric concentrations since increased permeability is not only a useful laboratory index of injury but is relevant to potential health effects of exposure to air pollutants. We regard such data as needed to fill an important gap in knowledge about air pollutants as primary causes of lung disease. The gap exists between effects of acute exposure in man and animals and the inconclusive epidemiologic evidence that chronic lung disease can be caused solely by photochemical air pollution. Effects of long-term animal exposures have supported this causal hypothesis but have required continuous breathing of the test atmosphere during most of the life-span of the test animals. The best animal data are, therefore, difficult to extrapolate to human exposure. An indicator of injury during more realistic exposure was needed to determine whether some less-than-continuous exposure can be shown to produce a meaningful biologic effect even when pathologic changes are not yet detectable.

2. Macrophage Studies

Macrophages (mobile cells that engulf foreign material) are one of the important immunological elements that protect the host from infection and other environmental insults. The residential macrophages that inhabit the lung are crucial for the lung to defend against the onslaught of environmental pollutants and pathogenic organisms. Two notable functions of macrophages that play a role in the host resistance include (1) removal of dead and damaged cell debris and foreign substances by endocytosis and phagocytosis, and (2) interaction of macrophages with other lymphoid cells to present the antigen and generate and/or stimulate the cytotoxic cells that are an important part of immunity. A breakdown in these functions will predispose the host to disease.

The phagocytic activity of macrophages is influenced by the presence of surface attachment sites called receptors (C and Fc) on the cell membrane. Immune activation of macrophages influences the density of the receptors per cell and also the ability of C and Fc receptors to permit engulfment of foreign particles. It has been demonstrated that Fc receptors in normal macrophages can mediate both the attachment and endocytosis of immunoglobulin G (IgG) coated particles and that C receptors mediate only attachment of complement (C) coated particles (Bianco et al., 1975). However, in the activated macrophages C receptors mediate both the attachment and endocytosis in the same way as Fc receptors of normal macrophages. Bactericidal and tumoricidal activities can be induced by immunostimulants (Miller et al., 1973; Rao et al., 1981, 1983). Macrophage phenomena have been examined both in-vitro and in prolonged diesel exhaust inhalation exposures (Strom, 1984;
Chen et al., 1980; Green et al., 1983; etc.) indicating increases in number of alveolar macrophages, increases in macrophage sizes, differences in macrophage surface adhering ability, and defective macrophage phagocytosis ability. It has been shown that lead ions possess the ability to inhibit immune phagocytosis and Fc receptor activity of pulmonary macrophages, which may partially account for the host immune impairment induced by lead (Jian et al., 1985). Pilot studies performed in our laboratory with ozone have shown that there is decreased binding of foreign red blood cells to the surface of macrophages as evidenced by decreased number of rosettes formed, indicating damage to the Fc receptors. The previous 21-day study of the 7-component atmosphere at the high (EPRI) concentrations did not involve these macrophage-related endpoints.

This study was intended to identify any alteration in macrophage phagocytic activity and any damage to Fc receptors during or after repeated exposures. A disruption of Fc receptors implies that the lung defenses against microorganisms, and carcinogenic events, are compromised.

C. Importance of the Study

As has been previously mentioned, changes in airway permeability or in the immunological function of macrophage cells may be considered to be adverse health effects. The sensitivity of these endpoints for detecting effects at low exposure concentrations appears to exceed that of histopathology.

Long-term repeated laboratory exposures to controlled multicomponent atmospheres are essentially unprecedented. The 7-component atmosphere has been developed over the past 10 years. It is designed for relevance to exposures of people in California who are living in areas heavily impacted by multiple sources of air pollutants. A daily repeated 21-day exposure represents an exposure which is intermediate between acute and chronic. The concentrations are selected to maintain an environmentally realistic level of ozone and to keep the components in ratios that have been observed in the environment.

The results of the study will be of relevance for several reasons: the mixture is chemically environmentally relevant from a health point of view; the multiple repeated exposures are more relevant than either single acute exposures or continuous (24 hour/day) exposures; and the endpoints have been selected due to their link to health and due to the possibility of eventually confirming our laboratory results in humans. The issue of development of tolerance (or lack
thereof) over a 21 day repeated exposure has important implications to public health and was a central issue in this study.

VI. MATERIALS AND METHODS

A flow diagram of the general procedures used in performing the study is shown in Figure 1.

Figure 1. Project flow diagram

ORDER AND QUARANTINE RATS

PERFORM 21 DAY NOSE-ONLY EXPOSURE

IMMUNOLOGY STUDY

A. EVALUATE RAT GROUPS AFTER 7 DAYS OF EXPOSURE
1. IMMED. SAC (n=8)
2. 48 H. SAC (n=8)
3. 96 H. SAC (n=8)
4. CONTROLS FOR ABOVE (n=8)

CALCULATE MEANS, SE & SD FOR EXPOSURE DATA IN ORDER TO CHARACTERIZE THE EXPOSURE

PERMEABILITY STUDY

A. EVALUATE RAT GROUPS AFTER 7 DAYS OF EXPOSURE
1. IMMED. SAC (n=8)
2. 24 H. SAC (n=8)
3. CONTROLS FOR 1 (n=8)
4. CONTROLS FOR 2 (n=8)

B. EVALUATE RAT GROUPS AFTER 21 DAYS OF EXPOSURE
1. IMMED. SAC (n=8)
2. 48 H. SAC (n=8)
3. 96 H. SAC (n=8)
4. CONTROLS FOR ABOVE (n=8)

C. EVALUATE RAT GROUPS HELD FOR 7 DAYS POST 21-D EXPOSURE
1. IMMED. SAC (n=8)
2. CONTROLS FOR ABOVE (n=8)

FINAL BIOLOGICAL DATA ANAL. ABSTRACT REPORT & PUBLICATION

n = Number of exposed rats
A. Animals

Barrier-reared Sprague-Dawley rats used in this study were purchased from Hilltop Lab Animals, Inc. Male rats weighing about 200-225 grams were delivered to the laboratory in filtered shipping boxes in order 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) in wire-bottom stainless steel cages over a relatively dust-free sodium chloride litter for about one week before the start of the exposure. The rats exposed to the 7-component atmosphere were distributed pseudo-randomly throughout the cage racks among the purified air rats so that the results of the experiment were not biased by rat placement. Microbiological assays, supplied by Hilltop, indicated that the rats were free of respiratory infection. This fact was confirmed by quality control histopathologic examinations at our laboratory. Five rats were autopsied upon arrival and five each at two additional times during the study to monitor the health of the rat colony. The rats’ lungs remained free of significant complicating infection throughout this study.

B. Exposures

Groups of rats were exposed to either purified air or the 7-component atmosphere using unique 1 m³ stainless steel Rochester chambers modified for nose-only exposure (Figure 2). Exposures were 4 hours per day for either 7 or 21 consecutive days. Each rat was placed into a cylindrical plastic tube with an aluminum nosepiece with a conical interior cavity tapered toward the forward end so that only the tip of the rat’s nose protruded into the atmosphere. The exterior of the nosepiece had a cylindrical fitting by which it was inserted and sealed into one of the nose-only ports in the wall of the chamber. Each port was supplied by a channel on the interior wall of the chamber which directed the atmosphere downward past the nose of the rat to prevent rebreathing of that atmosphere by the exposed rat and to prevent sharing of that sample of atmosphere with another rat. This exposure system was designed in order to provide for the comfort of the rats (to minimize stress) and to prevent the neutralization of acids in the pollutant atmospheres by ammonia generated from the rats’ excreta. The chambers were
Figure 2. Nose-only exposure system for exposure of rats

NOSE-ONLY MODIFICATION TO ROCHESTER CHAMBERS

TYPICAL ORIENTATION OF NOSE-ONLY PORTS

SECTIONAL VIEW

EXPOSURE CHAMBER INTERIOR WALL

AIRFLOW

BAFFLE

SQUARE CROSS SECTION CHANNEL ONE FOR EACH SLEEVE

RAT TUBE

TAILGATE

NOSE-PIECE

O-RING SEAL
supplied with air that had passed through coarse particulate filters, gas
scrubbers, a humidifier and a high efficiency particulate air (HEPA) filter.
Pollutants were injected into the airstream just prior to the exposure chamber.
The temperature and humidity were controlled; an exposure temperature of 72 ± 2°F
was selected to insure the comfort of the rats, and the relative humidity was 85 ± 2%.

Sulfate aerosols containing catalytic metal ions were generated by nebulizing
a solution of ammonium sulfate, ferric sulfate and manganese (II) sulfate into
highly purified dilution air, using a Collison nebulizer. Aerosols were passed
through a ^85 Kr discharger to reduce static charges to the Boltzmann equilibrium
level. Nitrogen dioxide and sulfur dioxide were metered into purified air from
compressed gas cylinders using calibrated rotameters equipped with needle valves.
Ozone was generated by metering purified oxygen into a commercial ozonizer (Sander
Ozonizer, Type III). Ferric oxide particles were generated by nebulization of
dilute colloidal suspensions of Fe$_2$O$_3$, yielding spherical aggregate particles
having a density of 2.5 g/cm$^3$, a mass median aerodynamic diameter of 0.3 μm, and a
geometric standard deviation of about 2. The aerosols are diluted with warm air,
discharged with a ^85 Kr discharger and introduced into purified air.

Nitrogen dioxide and nitric oxide were monitored using a chemiluminescent
detector (Monitor Labs 8080). Sulfur dioxide was monitored using a Teco (Series
43) pulsed fluorescence detector. Ozone was measured by U.V. absorption (Dasibi
Model 1003-AH). During exposures the stability of particulate components was
monitored using a real-time aerosol monitor (RAM-1, GCA Environ. Instr.). Cascade
impactor samples (Sierra Model 212 8-stage impactor) were collected to provide size-
classified particles for gravimetric and chemical analyses. Aerosols were
collected on pre-cleaned quartz fiber or teflon filters for gravimetric
determinations and for detailed analyses of atmospheric components and reaction
products. Nylon backup filters were used to collect nitric acid vapor (from the
reaction of NO$_2$ and O$_3$). Ion chromatography was used to analyze sulfate, sulfite,
nitrate and nitrite ions in filter extracts. Sample aliquots were analyzed for
manganese, "soluble iron" (ferric sulfate) and "insoluble iron" (ferric oxide)
using a differential extraction technique. Iron and manganese were determined by
atomic absorption spectrophotometry.
C. Biologic Endpoints

1. Epithelial Permeability

Permeability studies were performed in two localized regions of the respiratory tract: the nose and the bronchoalveolar zone. Animals were anesthetized with sodium pentobarbital. A polyethylene tube was placed in the trachea, and a polyethylene catheter was placed in the femoral artery. The radiolabeled tracer inocula contained $^{99\text{m}}$Tc or $^{111}$In labeled diethylenetriaminepentaacetate ($^{99\text{m}}$Tc DTPA, or $^{111}$In DTPA; MW 492).

For measuring permeability in the nose, the oropharynx was filled with dental impression cream to block the posterior nares. Tracer solution containing $^{111}$In DTPA was instilled to fill the nasal cavity. The total volume of the tracers needed to fill the entire nasal cavity ranged from 0.19 to 0.28 ml. For bronchoalveolar instillation, rats were held in a semivertical position. Inoculum containing $^{99\text{m}}$Tc DTPA (0.1 ml) was delivered over a 5-min period directly to a main stem bronchus using a tube inserted through a tracheostomy tube. Heparin solution (20 units in 0.2 ml saline) was injected into the rats through femoral artery catheters prior to blood sampling. Blood samples, 0.10 ml each, were drawn at 6, 7, 8, 9, and 10 min after the start of instillation. $^{99\text{m}}$Tc radioactivity in the blood samples was measured immediately after collection of the samples in a LKB Model 1275 Minigamma Counter. The radioactivity measurements were corrected for isotope decay. Samples were held for two days to allow decay of $^{99\text{m}}$Tc, and then counted again for $^{111}$In radioactivity.

The amount of label in blood samples was analyzed as a function of time by linear regression, and the concentration in blood at the mid-time point ($T_{\frac{1}{2}}$) for the 5 samples - i.e., at 8 min after the start of instillation - was determined. The results from individual rats were pooled and differences in the fractions of each labeled tracer transferred to blood in the control and the 7-component atmosphere-exposed groups were contrasted using an analysis of variance; differences were considered significant at the 0.05 level.

2. Macrophage Studies

After exposure to pollutants each group of rats was sacrificed by sodium pentobarbital injection at pre-planned time intervals (immediately, 2 days and 4 days post exposure). The lungs were removed under sterile conditions, cut into small fragments and residential cells were obtained by thoroughly shaking the
fragments in sterile tissue culture medium (Hanks' medium). The contaminating red cells were lysed using Boyle's solution. The intact cells were washed with medium, centrifuged at 300 g for 10 minutes, and the concentration of the cells was determined and adjusted to $1 \times 10^5$ cells in 0.1 ml of RPMI (Roosevelt Park Memorial Institute) medium, supplemented with 15% FCS (Fetal Calf Serum). The phagocytic activity was measured in Lab-Tek chambers (Rao et al., 1980). In brief, 0.1 ml of each cell suspension was placed into Lab-Tek chambers containing 0.5 ml of the medium and incubated for 1 hr at 37° C. The chambers were then washed with the medium to remove the non-adherent cells. Then 0.1 ml of a suspension of spherical latex micro-particles (diameter 1.1 µm) was then added to each chamber and they were incubated for 60-90 minutes. The cells were then washed with calcium and magnesium-free PBS (Phosphate Buffered Saline, pH 7.2) to remove the free latex particles. After dismantling the cell chambers from the Lab-Tek slides, the adherent cells (macrophages) were observed under an inverted-stage phase contrast microscope. The percentage of latex positive cells was determined. The experiment was conducted at 0, 48, and 96 hour intervals after the exposure to the pollutant atmosphere. The group of rats exposed to purified air was used as a control. The percent of the total macrophage population that has engulfed latex particles was calculated.

A rosette assay was used to determine the effect on surface Fc receptors which bind antigen to macrophages. Lab-Tek chambers, each containing $1 \times 10^5$ cells in 0.1 ml of RPMI medium supplemented with FCS and 0.59 ml of Hanks medium, were prepared as described earlier, for the individual rat lungs of each group. The Lab-Tek chambers were incubated for 1 hr at 37° C and later the non-adherent cells were removed by washing with the medium. The Fc receptors on macrophages were measured by rosette assay (Rao et al., 1980). In brief, 0.1 ml of anti-SRBC (Sheep Red Blood Cells) at a concentration determined by trials prior to the experiment in RPMI medium was added to each of the chambers and incubated for 30 minutes at 37°C.

After the incubation 0.1 ml of SRBC ($1 \times 10^7$ cells) was added to each of the chambers containing macrophages and incubated for 30 minutes at room temperature. The unbound SRBCs were washed away gently using the medium. The number of cells forming rosette's with SRBCs were counted using a microscope. Cells attached with three or more SRBCs were counted as positive rosettes. One to two hundred counts were accumulated. The percentage of rosette-forming macrophages was calculated as follows:
No. of macrophages forming rosettes (with antibody) \( \times 100 = (A) \)

Total No. of macrophages counted

No. of macrophages forming rosettes (without antibody) \( \times 100 = (B) \)

Total No. of macrophages counted

net rosettes = A - B

Preparation of anti-SRBC serum: SRBCs obtained in Alsever's solution were washed with PBS (thrice) and the cell count was adjusted to \( 5 \times 10^9 \) cells in 1.0 ml. Adult rats were injected IP with 0.2 ml of \( 5 \times 10^8 \) SRBC in PBS. Each received four such injections at weekly intervals. Ten days after the last injection the rats were bled and the serum was separated. The antiserum was inactivated at 57°C for 30 minutes and the titer of the antibody was detected by its ability to bind Fc receptor to macrophages as determined by the rosette assay.

Data for each rat, in the form of latex-positive macrophages or net rosettes, were assembled, group means and standard deviations calculated, and two-tailed t-tests (exposed vs. controls) performed. A level of significance of 0.05 or less was considered statistically significant.

VII. RESULTS

A. Epithelial Permeability

In a previous study, bronchoalveolar permeability to DTPA increased significantly over control levels in rats exposed for 4 hours on each day, up to 7 or 21 days, to the 7-component atmosphere at the EPRI (high) concentrations. In contrast, when the rats were exposed for 7 or 21 days to the atmosphere at the ARB (low) pollutant concentrations, the bronchoalveolar permeability was not significantly different from the controls (Table 2). Permeability in the exposed groups that were studied one day after the end of 7 day and 21 day exposures was similar to the controls, as was permeability in the exposed groups studied immediately at the end of the 7 day and 21 day exposure periods. In addition, this
### TABLE 2

**BRONCHO-ALVEOLAR MUCOSAL PERMEABILITY TO DTPA** at 1 and 24 hrs after a 7 day or 21 day 4 hr exposure of rats to 0.30 ppm O₃ + 1.2 ppm NO₂ + 2.5 ppm SO₂ + Fe₂O₃ + ammonium, ferric, and manganese sulfates.

<table>
<thead>
<tr>
<th>Labeled Molecule</th>
<th>Percent of Inoculum Transferred to Entire Blood Volume at Time Ti After Broncho-Alveolar Instillation of Labeled Molecules.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clean Air</td>
</tr>
<tr>
<td></td>
<td>1 HR</td>
</tr>
<tr>
<td>DTPA*</td>
<td></td>
</tr>
<tr>
<td>±0.246</td>
<td>±1.133</td>
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<tr>
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<td>n=5</td>
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<td>p=0.15</td>
<td>p=0.79</td>
</tr>
</tbody>
</table>

*DTPA: ⁹⁹ᵐTc-diethylenetriaminepentaacetate  
**Mean ± 1 standard deviation

### TABLE 3

**NASAL MUCOSAL PERMEABILITY TO DTPA** at 1 and 24 hrs after a 7 day or 21 day 4 hr exposure of rats to 0.30 ppm O₃ + 1.2 ppm NO₂ + 2.5 ppm SO₂ + Fe₂O₃ + ammonium, ferric, and manganese sulfates.

<table>
<thead>
<tr>
<th>Labeled Molecule</th>
<th>Percent of Inoculum Transferred to Entire Blood Volume at Time Ti After Broncho-Alveolar Instillation of Labeled Molecules.</th>
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<td>p=0.31</td>
</tr>
</tbody>
</table>

*DTPA: ¹¹¹In-diethylenetriaminepentaacetate  
**Mean ± 1 standard deviation
atmosphere did not effect the nasal mucosal permeability in rats exposed for 7 days or 21 days, and studied either immediately at the end of these exposures or a day later (Table 3).

B. Macrophage Studies

The effects of exposure to the 7-component atmosphere on Fc receptor activity are shown in Table 4. Seven days of exposure significantly reduced (p < 0.05) rosette formation immediately after the end of the exposure (day 0). Following the exposure this effect persisted until day 4; it was also observed that there was a significant increase in the percent of rosettes formed on day 4 as compared to the day 0 group. Continued exposure to the 7-component atmosphere for 21 days further reduced the rosettes formed up to day 4 (below the levels observed with the 7 day exposure group) with a significant increase in the day 4 group as opposed to the day 0 group. By day 7 the number of rosettes formed had returned to the control levels.

The effects of the 7-component atmosphere on the non-specific phagocytic activity of macrophages are shown in Table 5. It was observed that there was a significant (p < 0.05) depression of phagocytic activity immediately after 7 days of exposure and these effects persisted through day 4 without signs of recovery. There was a significant increase in phagocytic activity on days 2 and 4 as compared with the day 0 group. However, no changes were seen in the rats exposed to the atmosphere for 21 days.

VIII. DISCUSSION

A. Epithelial Permeability

We have previously reported (Bhall et al., 1986a) that the application of DTPA for detecting airway permeability provides a reliable means for studying epithelial injuries localized to various airway regions, i.e., nasal, tracheal and bronchoalveolar. A 2-hour exposure of resting rats to 0.8 ppm O₃ resulted in an approximately twofold increase in tracheal and bronchoalveolar permeability to DTPA. We have now extended these studies to evaluate permeability changes in the bronchoalveolar region of rats exposed under conditions that approach more realistic environmental exposures, i.e., exposure to atmospheres composed of multiple pollutants. Our previous results indicate an increase in permeability.
<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Effects of the 7-Component Atmosphere on the Percent of Rosettes* Formed by Pulmonary Macrophages</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purified Air Controls</th>
<th>Percent of rosettes formed on day D4 after pollutant exposure D0</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7-Component Atmosphere: 7 days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.6±2.3 n=9</td>
<td>10.3±2.7 n=6</td>
<td>12.9±4.3 n=6</td>
<td>---</td>
<td>18.0±1.8 n=6</td>
<td>---</td>
</tr>
<tr>
<td><strong>7-Component Atmosphere: 21 days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.4±1.0 n=13</td>
<td>3.0±0.8 n=6</td>
<td>4.1±1.2 n=5</td>
<td>---</td>
<td>15.1±1.3 n=6</td>
<td>19.2±1.7 n=6</td>
</tr>
</tbody>
</table>

* Values are the means ± SEM  
† P 0.05 vs control (two-tailed t-test)  
‡ P 0.05 vs D0 (two-tailed t-test)

<table>
<thead>
<tr>
<th>TABLE 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Effects of the 7-Component Atmosphere on the Phagocytic Activity of the Macrophages</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purified Air Controls</th>
<th>Percent* of macrophages with polystyrene latex particles on day D4 after pollutant exposure D0</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7-Component Atmosphere: 7 days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>78.9±1.8 n=9</td>
<td>57.5±2.8 n=6</td>
<td>68.6±5±2.4 n=6</td>
<td>---</td>
<td>73.4±2.8 n=6</td>
</tr>
<tr>
<td><strong>7-Component Atmosphere: 21 days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75.2±2.5 n=9</td>
<td>75.0±3.0 n=6</td>
<td>72.4±1.7 n=5</td>
<td>---</td>
<td>72.8±2.6 n=6</td>
</tr>
</tbody>
</table>

* Values are means ± SEM  
† p < 0.05 vs control (two tailed t-test)  
‡ p < 0.05 vs D0 (two tailed t-test)
immediately after a single exposure to O₃ (0.6 ppm), O₃ (0.6 ppm) + NO₂ (2.5 ppm) or the 7-component atmosphere at the EPRI concentrations. The magnitude of increase was approximately the same after exposure to O₃ alone or its combination with other pollutants. However, a significant increase in the persistence of enhanced permeability at 24 hrs after the exposure occurred after exposure to O₃ + NO₂ or to the EPRI 7-component atmosphere, but not after exposure to O₃ alone, suggesting extension of the duration of biologic effects by pollutant combinations. Increases in bronchoalveolar and nasal permeability were also observed upon repeated exposure of rats to the EPRI 7-component atmosphere for up to 20 days. However, permeability of nasal and bronchoalveolar mucosa was not affected in the present study when the rats were exposed to an atmosphere containing lower pollutant concentrations. Absence of effects may indicate that (a) the test atmosphere did not produce measurable permeability changes, (b) the animals may have adapted to this "less potent" atmosphere after repeated exposures, (c) normal repair processes were sufficient to protect against the low-level insult, or (d) the endpoint does not have the statistical power to allow the detection of very small permeability changes. In this endpoint the permeability must change by a factor of about 2 in order to produce a statistically significant result.

B. Macrophage Function

The rationale for these studies is based on the assumption that tests of immunological functions such as Fc receptor activity and phagocytic activity of pulmonary macrophages may serve as more sensitive methods to detect early damage to the bronchoalveolar region of the lung than has been the case for other endpoints.

Decreased phagocytic activity of the macrophages after exposure to the 7-component atmosphere for 7 days suggests a non-specific suppression of acid phosphatase, lysozymes and other oxidative enzymes. Reduction of acid phosphatase along with inhibition of macrophage Fc receptor binding induced by immune complexes has been reported by others (Mitchell et al., 1972). Suppression of phagocytic activity of alveolar macrophages was reported after exposure to diesel emissions (Castranova et al., 1985). Such reduction was also observed in other systems (Rao et al., 1983).

Pulmonary macrophages from rats exposed to the 7-component atmosphere for 7 days showed a reduction in both rosette formation and phagocytic activity. On the contrary, the macrophages from rats exposed to the same atmosphere for longer periods (21 days) did not show a decrease in phagocytic activity. This could be
due to recovery after initial damage caused by short-term (7 day) exposure. To understand the mechanism(s) involved in such changes associated with short and long term exposures, further studies at the sub-cellular level are necessary. (In contrast to the permeability endpoint, the macrophage endpoints were not utilized in the previous EPRI study.)

The recovery in the percent of macrophage rosettes with time after the end of exposure to pollutant atmospheres leads to a few speculations. One of them is that the Fc receptors destroyed or impaired by the pollutant atmosphere are reactivated or regenerated with time. Secondly, there may be an influx of a fresh population of pulmonary macrophages. It is unknown whether these newly recruited macrophages would have the same sensitivity as the initial lung macrophage population.

In conclusion, the pulmonary macrophage was a more sensitive target of the pollutant exposure than was the airway epithelium. A peer-reviewed paper that provides additional details regarding the macrophage response has been accepted by the Journal of Toxicology and Environmental Health. The paper is in press and the expected publication date is May 1988.

IX. REFERENCES


