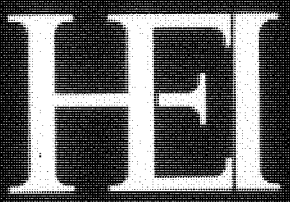

HEALTH EFFECTS INSTITUTE

Leukocyte-Mediated Epithelial Injury in Ozone-Exposed Rat Lung

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Includes the Commentary of the Institute's Health Review Committee

Research Report Number 44

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INVESTIGATORS' REPORT Kenneth Donaldson, Geraldine M. Brown, David M. Brown, Joan Slight, William M. Maclaren, and John M.G. Davis

HEALTH REVIEW COMMITTEE'S COMMENTARY Health Effects Institute

Leukocyte-Mediated Epithelial Injury in Ozone-Exposed Rat Lung

Kenneth Donaldson[1], Geraldine M. Brown, David M. Brown, Joan Slight, William M. Maclaren, and John M.G. Davis

ABSTRACT

Both epithelial injury and inflammation are characteristic findings in the centriacinar regions of the lungs of rats exposed to ozone. In humans such effects could lead to long-term lung damage and disease. In animals, neoplastic change in the lungs after exposure to ozone has been described previously. The possible relationships between inflammatory cell recruitment, epithelial injury, and hyperplasia, with special regard to the important role of repair processes in leading to increased incidence of tumors in some species, have been addressed in the present study. We have previously described that leukocytes from lungs inflamed by different agents can injure epithelial cells in vitro. We have suggested that this leukocyte-mediated epithelial injury could enhance epithelial turnover in ozone-exposed lungs and so enhance the likelihood of tumor development. We, therefore, set out to test the hypothesis that bronchoalveolar leukocytes from ozone-exposed lungs can injure epithelial cells in vitro. PVG rats were exposed to 0.2, 0.4, 0.6, and 0.8 parts per million[2] (ppm) ozone for seven hours per day for up to four days. On the morning following the last exposure, bronchoalveolar lavage was used to sample the bronchoalveolar leukocytes and the following parameters were assessed: total number, differential leukocyte count, production of oxidants, ability to degrade fibronectin, and ability to injure epithelial cells. In addition to these parameters, which were measured at all concentrations and time points in limited experiments, we also assessed macrophage size in short-term culture and inflammation in histological sections of lungs.

Total number of lavageable cells was not affected by ozone inhalation. However, the percentage of macrophages decreased with ozone treatment and the percentage of neutrophils increased on days 1 and 2 at 0.6 and 0.8 ppm ozone. There was no significant effect of ozone exposure on the ability of neutrophils to degrade fibronectin or injure epithelial cells. Production of superoxide anion in response to stimulation with phorbol myristate acetate was significantly decreased by exposure to 0.6 ppm ozone, as described in previous studies. Macrophages from the lungs of rats exposed to ozone were larger than control macrophages.

For histological examination, rats were exposed to ozone under conditions identical to those described above for the main study, and on the morning after exposure the lungs were fixed and processed in parafin wax. Inflammatory cells, in substantial numbers, could be seen at the one- and two-day time points with exposure to ozone at 0.8 ppm, decreasing as the exposure concentration became less. The accumulation of inflammatory cells was patchy and situated in the centriacinar region.

We conclude that inhalation of ozone causes low-level inflammation that is detectable in the bronchoalveolar lavage leukocyte population as an increase in neutrophils. In all of the assays, we found no stimulation of any measures of the ability of the leukocytes to cause injury to the epithelial cells or their associated extracellular matrix components. On the basis of the assays used here to determine the potential to cause epithelial injury, we conclude that epithelial injury by ozone, at the concentration used, is likely to be a direct effect of the ozone and not an indirect effect of inflammatory leukocytes. However, the use of different assays or time points may have yielded different results and should form the basis of studies in the future. The negative nature of the findings here should not detract from the obvious nature of the hazard associated with ozone exposure, acting via pathways other than those discussed here.

INTRODUCTION

Ozone is an atmospheric pollutant whose oxidizing activity makes it potentially injurious to the human lung, and epidemiological studies tend to confirm this potential (reviewed by Bresnitz and Rest 1988). In experimental studies ozone is, without doubt, injurious to the lungs of laboratory animals if the dose is sufficiently high.

In the short term, there is type I alveolar epithelial cell injury, with compensatory type II epithelial cell hyperplasia (Wright et al. 1988; Tepper et al. 1989) and substantial alterations to the epithelial cells of the terminal bronchioles, including hyperplasia, even at levels of ozone as low as 0.5 ppm (Tepper et al. 1989) or 0.25 ppm (Barry et al. 1988). With longer-term exposure there is an increase in interstitial mesenchymal cells and their products (Barry et al. 1985). Increased collagen synthesis in rat lungs exposed to ozone at 0.5 to 2.5 ppm has been described in one study (Last et al. 1983) but was not present in another study using

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[2] A list of abbreviations appears at the end of this report for your reference.

similar levels (Wright et al. 1988). Collagen that is structurally different from that found in normal lungs may also be laid down during ozone exposure (Reiser et al. 1987).

Recruitment of inflammatory leukocytes, macrophages, and neutrophils has been reported in several experimental studies, with severity of inflammation depending, in the main, on the exposure concentration used (Holtzman et al. 1983; Esterline et al. 1989; Hotchkiss et al. 1989b; Tepper et al. 1989). In recent experiments in humans, which tend to support the animal studies on the inflammatory effects of ozone, healthy volunteers inhaled 0.4 ppm ozone. They were lavaged before and after exposure and showed dramatic evidence of inflammation in the lavage profile after exposure (Koren et al. 1989). It has also been suggested that, in laboratory animals, inhalation of ozone causes an excess of lung tumors (Hassett et al. 1985; Mustafa et al. 1988). The present study is aimed at trying to determine whether the inflammatory leukocytes recruited to ozone-exposed lungs have the ability to cause epithelial injury. If such injury occurred, it could enhance the process of carcinogenesis because of the well-known relationship between repair processes and carcinogenesis. This effect would be in addition to the direct DNA-damaging effects of oxygen species such as ozone (Meneghini 1988). In addition, leukocytes recruited to ozone-exposed lungs may generate oxidants, which are likely to act as promoters, and could also generate lipid peroxidation products, which can themselves cause DNA damage (Copeland 1983; Vaca et al. 1988).

Previous work at the Institute of Occupational Medicine has been aimed at elucidating the relationship between inflammation, epithelial injury, and pathological change. Using pathogenic mineral dusts as model lung-injuring agents, we have demonstrated recruitment of inflammatory leukocytes (Donaldson et al. 1988a) that have the ability to injure epithelial cells in vitro via protease-mediated events (Donaldson et al. 1988c) and to attack components of the extracellular matrix (Brown and Donaldson 1988). As a working hypothesis, we have, therefore, suggested that epithelial injury leading to epithelial abnormalities occurs in dust-inflamed lungs and is mediated, to some extent, by the inflammatory leukocytes. The end results of these events are the epithelial abnormalities and type II cell hyperplasia that are characteristic of pneumoconiotic lungs (Gibbs et al. 1984) and lungs fibrosed by other etiologic agents (Kawanami et al. 1982). When the dust also has transforming ability, as in the case of asbestos, its ability also to cause hyperplasia could have a "promoting" effect.

Ozone injury has some similarities to that caused by certain industrial dusts that produce centriacinar fibrosis (Gibbs et al. 1984), epithelial injury (Schuyler et al. 1980), and neoplastic change (Becklake 1976). Although direct injury caused by ozone is very likely one important factor in ozone-mediated pathological change, it seems possible also that the inflammatory leukocytes recruited to ozone-exposed lungs could be involved in the type of epithelial injury and subsequent hyperplasia described above.

It is necessary to point out that the attraction of neutrophils into the lung in large numbers, as found with instillation of carbon, is not necessarily accompanied by autoradiographic evidence of marked epithelial injury (Bowden and Adamson 1984), although in this study there was an increase in mitogenesis in the interstitium. In our own studies using the inert dust titanium dioxide (TiO_2) we also see an influx of neutrophils, which is short-lived and causes no long-term pathological change (Brown et al. 1991). The mere presence of neutrophils is not, therefore, a guarantee of epithelial injury, but some other factor, such as neutrophil activation or persistence, is also likely to be required.

The ability to cause inflammation leading to hyperplasia is a property of many, but not all, tumor promoters (Miller and Miller 1981). Cell division is considered to be important for "fixing in" the transformed state, which, in turn, could be brought about by ozone because it is a known, albeit weak, mutagen and inducer of chromosome abnormalities (reviewed in Menzel 1984). The importance of increased cell turnover for expression of the transformed phenotype is highlighted by the well-known increased susceptibility of young animals, with high cell turnover rates, to chemical carcinogenesis (Kakunaga 1975). The hypothesis underlying this study is, therefore, that inhalation of ozone causes recruitment of inflammatory leukocytes, which cause epithelial injury and hence epithelial hyperplasia. This, in turn, may relate to the carcinogenic potential of ozone because hyperplasia increases the likelihood of expression of the transformed phenotype in the epithelium of ozone-exposed animals.

AIMS

The aims of the study were as follows:

1. To set up an ozone exposure facility.
2. To expose rats to 0.2, 0.4, 0.6, and 0.8 ppm of ozone for seven hours per day for up to four days.
3. To assess the following parameters in the bronchoalveolar lavage leukocytes from rats exposed as above: (a) total number of leukocytes, (b) differential cell count, (c) ability to produce oxidant, (d) ability to breakdown fibronectin, and (e) ability to cause injury to epithelial cells in vitro.
4. To measure the size of the macrophages exposed to 0.8 ppm ozone.
5. To assess the histopathological effects of ozone exposure for up to eight days.

6. To analyze the data to determine whether there were any statistically significant effects of ozone treatment on any of the above parameters.

The original project was envisaged as a three-year study, but because of the largely negative nature of the findings in the first two years, it was reduced to two years. In the modified two-year study, some of the original aims, mainly addressing the role of the extracellular matrix in epithelial detachment injury, were eliminated, and numbers 4 and 5 added after consultation with the Health Effects Institute.

MATERIALS AND METHODS

CHOICE AND VALIDITY OF ASSAYS

The assays were chosen to answer the essential research question contained in the study: Do the bronchoalveolar leukocytes from ozone-exposed lungs have the ability to injure epithelial cells? Bronchoalveolar lavage was used to obtain the leukocytes from the airspaces, and these were then used in the in vitro epithelial injury assay, because it detects the essential activity we were interested in. The oxidant and fibronectin proteolysis assays were chosen because oxidant and protease activities are considered to be the most likely processes through which the "innocent bystander" injury is caused by leukocytes. We have previously validated these assays and used them extensively to identify the potentially injurious activities of leukocytes within experimentally inflamed rat lungs. We have shown that bronchoalveolar lavage reliably detects and samples inflammatory leukocytes from lungs exposed to inhaled toxic substances (Donaldson et al. 1988a). The coculture of bronchoalveolar leukocytes with ^{51}Cr-labeled A549 epithelial cells demonstrates the potential of bronchoalveolar leukocytes from inflamed lungs to injure epithelial cells in vitro (Donaldson et al. 1988c). Previous studies by ourselves (unpublished) and others (Ayars et al. 1984) have revealed that, with regard to leukocyte-mediated injury, A549 epithelial cells show no essential differences from freshly derived rat type II epithelial cells. The fibronectin degradation assay reveals the ability of inflammatory leukocytes to cause proteolytic injury to components of the lung connective tissue (Brown and Donaldson 1988), and this type of injury correlates with the ability to cause epithelial injury (Donaldson et al. 1988c). The superoxide anion assay has been used extensively to measure the oxidative burst of bronchoalveolar leukocytes (Donaldson et al. 1988b).

OZONE EXPOSURE FACILITY

The early part of the study involved setting up an ozone generation and monitoring facility at the Institute of Occupational Medicine laboratories.

Ozone Generation

The ozone generator (laboratory ozonator type BA.023, Wallace Tiernan, Kent, England) was tested in the Institute's workshop and found to produce a stable ozone output. It was also ascertained that the output could be controlled by interfacing with a BBC microcomputer and an ozone monitor. The air supply to the ozone generator was drawn in, through a filter, from the roof of the building, which was in a countryside setting. The levels of ozone and oxides of nitrogen in the air supply were negligible. When a concentration of 0.8 ppm ozone was being generated, levels of oxides of nitrogen were also monitored in the exposure chamber and were found to be negligible.

Ozone Monitoring

The ozone monitor (427 photometric ozone analyzer, Analysis Automation, Oxford, England) was exhaustively tested and calibrated, both internally by the maker's own engineers upon commission, and externally with a similar analyzer in a nearby department (Institute of Terrestrial Ecology, Bush House Estate, Roslin, Midlothian). Results obtained with the two machines were very similar. The ozone analyzer was connected to a BBC microcomputer and the ozone levels analyzed were stored at regular intervals on floppy disk.

Ozone Exposure

After preliminary experiments revealed that the siting of the ozone sensor was critical and that ozone was reacting with plastic cages, the rats were finally exposed in stainless-steel cages with the ozone sensor centrally positioned just above rat head level in the exposure cages. In order to expose rats to ozone under safe conditions, both the ozone generator, within its own purpose-built chamber, and the rat exposure chamber were placed inside large secondary chambers at negative pressure to prevent access into the room of any leaked ozone. The ozonator was connected to the primary exposure chamber by a system of pipes of material known to resist attack by ozone. Ozone was kept at the required level by a three-term controller system. The stable supply of ozone from the ozonator was bled through a solenoid valve into the primary chamber air supply (15 L/min). The level of ozone in the primary exposure chamber was monitored by the ozone analyzer connected to the computer, and this was fed back to the solenoid valve, which corrected the percentage of the time that the valve was open. Air was withdrawn from the primary chamber and was scrubbed free of ozone before being vented to the out-

side of the building. In addition to the primary chamber, ozone was monitored in the secondary chamber, the room, and the scrubbed vented air. The computer was programmed to monitor the room regularly, and if the level exceeded 0.1 ppm for more than five minutes the ozone generator was switched off; in practice this never occurred.

CHOICE OF OZONE CONCENTRATION

Many previous studies have shown that, at high concentrations, ozone causes profound lung injury, as would be predicted on the basis of its powerful oxidizing activity. However, the objectives of these studies, as defined in Health Effects Institute RFA 86–1, were to seek to identify effects at environmentally plausible levels of ozone. In RFA 86–1, low-level exposure is defined as less than 1 ppm of ozone. Subsequently, we chose exposures of 0.2, 0.4, 0.6, and 0.8 ppm for these studies.

ANIMALS

Syngeneic, specific-pathogen-free rats of the PVG strain obtained from the Institute of Occupational Medicine's own breeding unit were used in these studies. This strain was chosen because of our previous extensive experience with them as models of lung injury caused by xenobiotics.

EXPOSURE OF RATS

Groups of three rats were placed in the primary ozone exposure chamber at 9 a.m. and were exposed to the required ozone concentration for seven hours; such an exposure is hereafter referred to as one day. The rats remained in the chamber overnight and were exposed for further days if required. On the morning after the end of the requisite number of days of exposure, rats were killed and subjected to bronchoalveolar lavage to obtain the bronchoalveolar leukocyte population. Control animals were not given a sham exposure but were kept in their usual accommodations and were killed and had their lungs lavaged at the same time as treated animals. We therefore treated the control groups as a single entity in statistical analyses, and this resulted in a partition of degrees of freedom (df) appropriate to 13 groups: 1 control group and 12 treatment groups (3 durations × 4 concentrations).

BRONCHOALVEOLAR LAVAGE OF RATS

Animals were killed by intraperitoneal overdose with phenobarbitone and the chest cavity was opened. The trachea was cannulated with a blunt 16G needle through a small incision. The lungs were dissected free of the chest cavity and lavaged with four 8-mL aliquots of sterile saline warmed to 36°C; lungs were massaged gently during each lavage to increase the cell yield. The pooled cells were placed immediately on ice before washing with phosphate-buffered saline (PBS) and dividing into aliquots that were delivered into different media, as described below, for the different assays.

BRONCHOALVEOLAR LAVAGE LEUKOCYTES

Cell Counting

Bronchoalveolar leukocytes were counted in a Neubauer chamber and then prepared for differential cell counting using a cytocentrifuge before staining with May Grunwald Giemsa stain.

Superoxide Anion Assay

As a measure of oxidant production, the release of superoxide anion in the presence or absence of the leukocyte trigger phorbol myristate acetate (PMA) (Sigma, Poole, England) was measured. Superoxide anion was measured according to the method of Johnston (1981). The reaction buffer PBS containing 1 mg/mL of cytochrome *C* and 2 mg/mL of dextrose was prepared and 1.5 mL was added to 30-mL petri dishes. Cells recovered through bronchoalveolar lavage were prepared at 5×10^6/mL in PBS and 50 µL of cells (0.25×10^6) was added to reaction buffer in dishes. Phorbol myristate acetate was freshly prepared and 10 µL was added to yield 1 µg/mL. After two hours of incubation, the supernatant was obtained, centrifuged to remove particulates, and then read at 550 nm in a scanning spectrophotometer (Pye-Unicam, Cambridge, England) with a reagent blank; this reading was converted to superoxide anion concentration (Johnston 1981). Superoxide dismutase (SOD) controls were always included, and superoxide anion was measured as the SOD-inhibitable cytochrome *C* reduction expressed as nanomoles of superoxide anion per 2.5×10^5 leukocytes in two hours.

Fibronectin Proteolysis Assay

To measure proteolytic activity, a functional assay of fibronectin degradation was used (Brown and Donaldson 1988). Fibronectin was iodinated by the method of McConahey and Dixon (1966), except that L-cysteine was substituted for sodium metabisulfite. Unbound iodine was removed by chromatography on a Sephadex G25 column (PD10; Pharmacia, Milton Keynes, England). The labeled protein showed activity of 5 to 25×10^6 counts per minute (cpm)/µg of protein. ^{125}I fibronectin was diluted in PBS and $4 \times 40{,}000$-cpm aliquots were dried onto Falcon Removawells (Becton Dickinson, Cowley, England). All assays of fibronectin proteoly-

sis were carried out in Newman and Tytell medium containing 2 percent bovine serum albumin (Gibco, Paisley, Scotland). To reduce background counts the Removawells, coated with ^{125}I fibronectin, were presoaked for two hours immediately before use with 200 μL of assay medium alone, and were then washed once with 300 μL of PBS. To assess fibronectin degradation, 1×10^5 cells were added to each ^{125}I fibronectin–coated Removawell in a final volume of 200 μL of medium, and matrix degradation was allowed to proceed for four hours at 37°C in 5 percent carbon dioxide (CO_2). To assay ^{125}I fibronectin degradation, the ^{125}I degradation products in 150 μL of supernatant medium were harvested from each well and counted by Rackgamma gamma counter (Pharmacia).

Epithelial Injury Assay

To assess alveolar epithelial cell detachment and lysis, cells of the A549 epithelial cell line (Lieber et al. 1976) were plated into Falcon microtiter plate wells (Becton Dickinson) as 5×10^4 cells per 100 μL of minimal essential medium (MEM) plus 10 percent heat-inactivated fetal calf serum and were incubated overnight in 74 kBq of ^{51}Cr per well. Monolayers were washed three times with PBS, and the bronchoalveolar lavage leukocytes from ozone-exposed or control lungs were added in 200 μL of Newman and Tytell medium to the required effector:target ratio of 10:1. Wells were incubated at 37°C in 5 percent CO_2 for four hours, at the end of which time soluble ^{51}Cr, as a measure of cell lysis, was obtained by aspirating 50 μL of supernatant and counting in a gamma counter. This figure was multiplied by 4 to give total free counts. Spontaneous release of ^{51}Cr was assessed by incubating labeled cells in medium alone and 100 percent lysis was obtained by lysing labeled cells with Triton X-100 (Sigma; 1 percent); spontaneous lysis at four hours was always 10 to 20 percent of the maximum value. Detachment of epithelial cells was assessed by removing the remaining 150 μL of supernatant, with accompanying detached cells, washing the wells with two 200-μL volumes of PBS, and pooling these for counting. The counts resulting from lysis in 150 μL of supernatant were subtracted from the pooled total to yield the counts of the detached cells alone. We have studied the ^{51}Cr labeling kinetics and effector:target ratio dependence of toxicity in this cell line using rat bronchoalveolar leukocytes (Donaldson et al. 1988a) and chose optimum protocols for this study consistent with these findings.

Assessment of Macrophage Size

Macrophage size was assessed by the method of Donaldson and associates (1984) modified to count the size of 200 cells on a cytocentrifuge preparation. Briefly, the maximum diameter of 200 May Grunwald Giemsa–stained macrophages was assessed using a microscope interfaced with a digitizing board and microcomputer; the counting rules exclude neutrophils, which can be identified morphologically. At an exposure regimen of 0.8 ppm ozone for one, two, and four days in each of two separate experiments, 200 cells were sized in the three exposed and three control rats.

HISTOLOGY

For histological examination of the effects of ozone on rat lungs, a separate series of short-term inhalation experiments was undertaken. This was because the main study involved pulmonary lavage, a procedure likely to mask or modify any minor pathological changes in the lung. Histological studies were undertaken after the main work on lavaged leukocytes was complete and it was known that maximum influx of inflammatory cells had appeared within the first 48 hours of exposure to ozone, with a consequent reduction over the remainder of the experimental period. The initial inhalation experiments for histology, therefore, concentrated on the highest dose examined, 0.8 ppm, and groups of three rats were exposed for seven hours per day for one, two, four, and eight days in two experimental series. On the morning after the last exposure, similar to the schedule used in the main part of the study, the ozone-exposed rats were killed along with three control rats and the lungs were taken for histological examination.

Lung tissue from animals treated with 0.8 ppm of ozone revealed pathological changes that reached maximum severity within 48 hours and were much less marked by eight days. In consequence, it was decided that the remaining histology would concentrate on determining whether recognizable pathology occurred in rat lungs at lower levels of exposure, and studies were undertaken at dose levels of 0.2, 0.4, and 0.6 ppm of ozone on two occasions with each dose; the groups of three rats were examined after 24 hours and 48 hours only. As before, three control animals were examined with the 24-hour exposure group on each occasion.

At the end of ozone exposure, animals were killed by an intraperitoneal injection of Nembutal and their lungs plus trachea were inflated with 4 percent formalin in saline. After fixation, tissue was embedded in paraffin wax and sections were stained with hematoxylin and eosin.

DESIGN OF OVERALL STUDY

The main study took the following design: (1) groups of three rats were exposed to ozone and groups of three rats were maintained in animal house air as controls; (2) animals were exposed to ozone for seven hours per day for one, two, or four days; (3) rats were killed and lavaged at 9 a.m. the

morning after the last day of exposure; (4) lavaged cells from each animal were kept separate for total cell count, percentage of leukocytes, fibronectin proteolysis and epithelial detachment and lysis assays; cells from all exposed or all control animals were pooled for assay of superoxide anion; (5) all experiments were repeated three times on separate occasions.

STATISTICAL ANALYSIS

The Main Study

A series of 32 experimental runs yielded information on eight response variables as shown in Table 1.

Table 1. Description of Response Variables

Response Variable Number	Description
1	Total count of leukocytes (10^6 cells per rat)
2	Percentage of macrophages (%)
3	Percentage of polymorphonuclear leukocytes (%)
4	Percentage of lymphocytes (%)
5	Fibronectin proteolysis (radioactivity in cpm)
6	Epithelial lytic injury (radioactivity in cpm)
7	Epithelial detachment injury (radioactivity in cpm)
8	Production of superoxide anion in the presence or absence of PMA (nmol/10^5 cells)

Table 2. Correspondence Between Specific Ozone Treatments and Experiment Numbers[a]

		Duration (days) and Concentration (ppm) of Ozone Exposure											
		1 Day				2 Days				4 Days			
Experiment Number	Control	0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8
182	x	x								x			
183	x	x								x			
187	x	x											
172	x		x										
174	x		x										
179	x		x								x		
163	x			x									
164	x			x									
165	x			x									
135	x				x								
141	x				x								
144	x				x								
185	x					x							
186	x					x							
188	x					x				x			
173	x						x						
175	x						x						
177	x						x						
166	x							x					
168	x							x					
170	x							x					
136	x								x				
143	x								x				
151	x								x				
180	x									x			
181	x									x			
167	x									x			
169	x											x	
171	x											x	
146	x												x
152	x												x
161	x												x

[a] See text for detailed explanation.

All eight responses were obtained under various conditions of ozone concentration (0.2, 0.4, 0.6, and 0.8 ppm) and duration of exposure (one, two, and four days). The eighth response was studied also in relation to another factor: the application (or not) of the "trigger" substance PMA. All but 4 of the 32 runs involved six rats: three control rats (not exposed to ozone) and three treated rats (exposed to ozone at a fixed concentration and for a fixed duration). The other four runs involved nine rats: three control rats as before, and six ozone-treated animals, all exposed to the same concentration of ozone, but for two durations (three rats on each duration).

The complete experimental design is shown in Table 2; each x in Table 2 represents three rats.

Data from triplets of rats assigned to particular treatment combinations were averaged prior to statistical analysis, so that each x in Table 2 also represents a single value of any of the response variables 1 through 7. In the superoxide assay, cells lavaged from both control and treated rats were further subjected to treatment by PMA (or not), so that in this case (response variable 8) each x in Table 2 represents two values of the response—one in which PMA has been applied and one in which it has not. Note, however, that experiment 181 provided no results for this response.

For responses 1 through 7, the means of the data were subjected to an analysis of variance. Classifying factors were experiment number, denoted briefly by EXPT in analysis of variance tables; TREAT, a two-level factor indicating whether or not rats had been exposed to ozone; CONC, a four-level factor indicating the concentration of ozone; and DAY, a three-level factor indicating the duration of exposure. In the analysis, the degrees of freedom were partitioned as shown in Table 3.

For response 8, there was an additional two-level factor: the application or not of the stimulating substance PMA to cells (denoted STIM in Table 4). Degrees of freedom were partitioned as shown in Table 4 (note that one of the experiments yielded no superoxide responses, which accounts for the reduction by 1 in the df column opposite EXPT).

Table 3. Partitioning of Degrees of Freedom for Analysis of Variance (Response Variables 1 Through 7)

Source of Variation	Degrees of Freedom
Experiments (EXPT)	31
Ozone treatment (TREAT)	1
Ozone concentration, within TREAT (TREAT.CONC)	3
Exposure duration, within TREAT (TREAT.DAY)	2
Interaction of CONC and DAY, within TREAT (TREAT.DAY.CONC)	6
Residual	24
Total	67

Table 4. Partitioning of Degrees of Freedom for Analysis of Variance (Response Variable 8)

Source of Variation	Degrees of Freedom
Experiments (EXPT)	30
PMA stimulation (STIM)	1
Ozone treatment (TREAT)	1
Ozone concentration, within TREAT (TREAT.CONC)	3
Interaction of TREAT and STIM (TREAT.STIM)	1
Exposure duration, within TREAT (TREAT.DAY)	2
Interaction of CONC and DAY, within TREAT (TREAT.DAY.CONC)	6
Interaction of DAY and STIM, within TREAT (TREAT.DAY.STIM)	2
Interaction of CONC and STIM, within TREAT (TREAT.CONC.STIM)	3
Three-way interaction of CONC, DAY, and STIM, within TREAT (TREAT.DAY.CONC.STIM)	6
Residual	76
Total	131

Thus, 13 treatment combinations (including control) were examined in relation to responses 1 to 7. For response 8, 26 such combinations were examined. The mean square for interaction between experiments and treatments was used as an estimate of error.

Because the experimental designs used were nearly balanced, the order in which terms such as TREAT.CONC or TREAT.DAY were added to statistical models had little effect on sums of squares in analysis of variance tables. The order chosen for each analysis of variance table was determined by considering the reductions in residual mean square, terms giving the greatest reduction being fitted first. Note that this did not apply to the term EXPT, which was always fitted before any treatment terms.

Examination of the distributions of untransformed data, and also examination of scatter diagrams of residuals against fitted values obtained from analyses of variance of both transformed and untransformed data, suggested that the transformations shown in Table 5 were appropriate.

Table 5. Transformations of Scale Used in Statistical Analysis of Eight Responses

Response Variable Number	Transformation
1	Logarithmic
2	Logistic
3	Logistic
4	Logistic
5	Logarithmic
6	Logarithmic
7	Untransformed
8	Logarithmic

Several zero values had been recorded for responses 3 and 4. The logistic transformation was modified slightly, by adding 0.1 percent to all data values before applying the function.

F tests derived from analysis of variance tables identified those factors that significantly affected the response variables ($p = 0.05$). In no case were any of the interaction terms statistically significant. Accordingly, the effects of treatments (ozone concentration, duration of ozone exposure, and, for response 8 only, PMA stimulation) were summarized in one-way tables of retransformed estimated treatment means, with 95 percent confidence intervals for the contrast between ozone concentration, or duration, and control. The interpretation of retransformed treatment differences depends on the transformation used: for the logarithmic, differences become ratios; for the logistic, antilogged differences are ratios, not of percentages, but of odds.

Although interactions between treatments were not statistically significant, estimated means were derived from models that included all interactions between treatment factors. These means were arbitrarily normalized to the level of experiment 135, the earliest of the series.

Analysis of Macrophage Size

The effect of ozone exposure on macrophage size was examined in a series of six experimental runs. The experimental design was similar to that used for the main study; Table 6 shows the layout in a format similar to that of Table 2. Note that a constant concentration of 0.8 ppm was used in these runs.

Statistical analysis was carried out along similar lines to the main study. Data were analyzed on the original scale of measurement, and treatment means were normalized to the level of experiment 160.

Table 6. Experimental Design for Examination of Effect of Ozone on Macrophage Size

Experiment Number	Days Exposed to Ozone			
	0 (Control)	1	2	4
160	x	x		
141	x	x		
151	x		x	
143	x		x	
152	x			x
146	x			x

RESULTS

OZONE EXPOSURE

Typical daily concentrations of ozone over 14 consecutive days of experimentation are shown in Figure 1 for the two extreme concentrations of ozone that were used (0.8 and 0.2 ppm). The data were obtained as means and SD of more than 2,500 measurements made at 10-second intervals throughout the seven hours of exposure. Other exposures were within the same range of variability. Typical quarter-hourly concentrations of ozone at 0.8 and 0.2 ppm over the seven-hour exposures are shown in Figure 2. Data are derived as the means and SD of more than 80 measurements taken at 10-second intervals. From these data it can be seen that the target levels of ozone were attained with precision.

LAYOUT OF RESULTS

In this section the results pertaining to each response are examined in turn. The raw data obtained from replicate experiments are first described informally, with reference to tables of means and SDs. This is followed by a discussion of the statistical significance of treatment effects, using analysis of variance. Finally, tables of estimated treatment means, and contrasts between treatments (with confidence intervals), are presented.

BRONCHOALVEOLAR LAVAGE LEUKOCYTES

Total Cell Counts

There was no obvious overall change in the total number of lavageable leukocytes with any of the treatments, the number remaining generally between 3 and 4 $\times$ 10^6 cells per rat throughout the study, as shown in Table 7.

Analysis of variance (Table 8) shows that the effect of

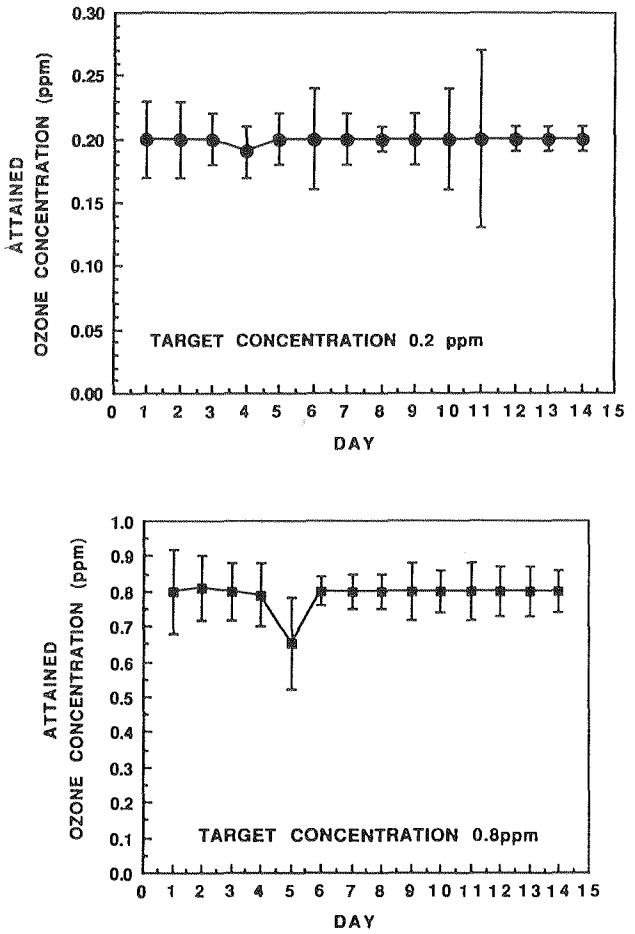

Figure 1. Attained ozone concentrations at the extremes of target concentrations sought in the study—0.2 and 0.8 ppm—in 14 consecutive experimental days chosen randomly from the entire sequence of experiments in the first six months. Each data point is derived as the mean ± SD of more than 2,500 measurements made at 10-second intervals throughout the seven hours of exposure on each day.

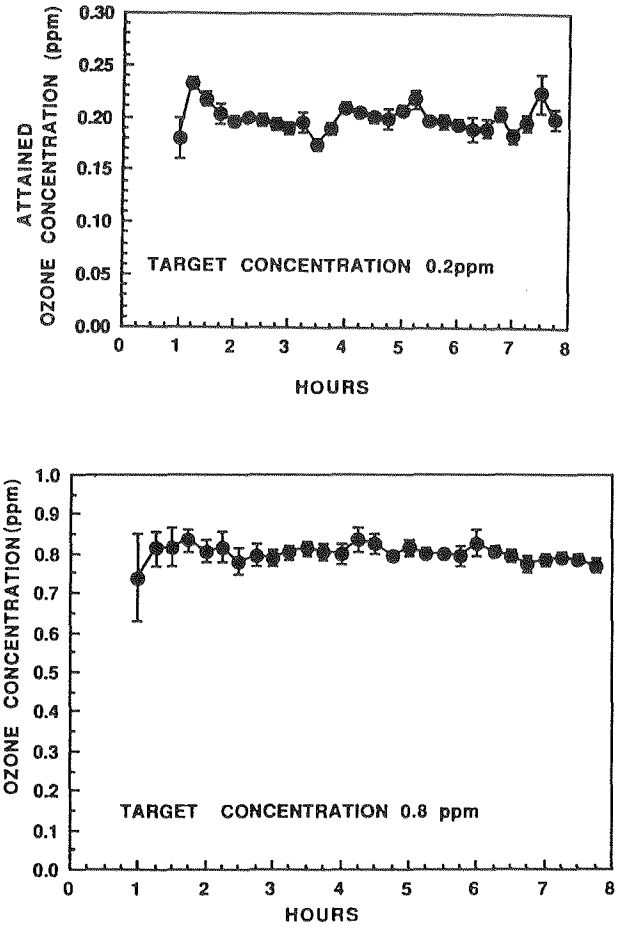

Figure 2. Typical quarter hourly ozone concentrations attained at the extremes of target concentrations used in the study—0.2 and 0.8 ppm—for randomly chosen single days. Each data point is derived as the mean ± SD of more than 80 measurements taken at 10-second intervals over each quarter-hour period.

ozone exposure just fails to reach statistical significance. Individual comparisons of treatments with control are given in Table 9. There is evidence of a raised total cell count after four days of exposure, but too much weight should not be attached to this in the light of the nonsignificant F test.

Percentages of Different Leukocyte Types

Macrophages. The proportion of macrophages in the bronchoalveolar lavage of control rats was 95 to 98 percent throughout the study. With the exception of the 0.8 ppm ozone exposure at one and two days, the same proportion was found in the ozone-exposed groups (Table 10). On these specified days, however, the proportion dropped to 89.9 and 93.6 percent, respectively.

Analysis of variance (Table 11) shows a statistically significant effect of ozone exposure, but no effects of concentration or duration. Individual comparisons between different concentrations and durations, and control, are presented in Table 12. For ozone-exposed rats, the estimated percentage of macrophages is 98.7 percent, averaged over concentrations and durations of exposure (not shown in Table 12). A t test, comparing this result to that of control animals (99.2 percent), is statistically significant at the 0.002 level but is unlikely to have biological significance except in so far as it relates to the proportions of neutrophils.

Neutrophils. Table 13 shows that in most of the ozone-exposed groups the proportion of neutrophils was raised relative to control, but this increase was substantial only at 0.8 ppm ozone for one and two days, when neutrophils reached a mean of 8.7 and 4 percent, respectively.

The presence of real effects of ozone exposure, depending on both concentration and duration, is shown by the analysis of variance in Table 14. There is, however, no evidence

Table 7. Total Leukocyte Cell Counts: Raw Data[a]

Ozone Concentration (ppm)	Days Exposed to Ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	3.6 (1.2)[b]	4.0 (1.4)	3.0 (0.6)	3.6 (0.5)	2.8 (0.6)[b]	3.3 (0.8)
0.4	3.2 (0.6)[c]	3.6 (0.6)	3.9 (0.7)	3.2 (0.6)	3.4 (0.6)[c]	3.7 (0.8)
0.6	2.8 (0.4)	2.6 (0.4)	3.4 (0.3)	3.6 (0.3)	2.5 (0.3)	3.3 (0.5)
0.8	3.7 (0.3)	3.2 (0.8)	4.0 (1.0)	4.6 (0.9)	2.4 (0.3)	3.7 (0.2)

[a] Total numbers of leukocytes that were obtained by bronchoalveolar lavage from the lungs of rats exposed to ozone for up to four days. Data are presented as means (SD) of millions of leukocytes per rat in three experiments, three rats per experiment.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see Table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data were obtained in experiments 172, 174, 179, 180, and 181, with experiment 179 contributing twice to the above information (see Table 2).

Table 8. Total Leukocyte Cell Counts: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	31	1.882	0.061	1.84	
+ TREAT	1	0.114	0.114	3.45	$0.05 < p < 0.1$
+ TREAT.DAY	2	0.084	0.042	1.28	$p > 0.1$
+ TREAT.CONC	3	0.087	0.029	0.89	$p > 0.1$
+ TREAT.DAY.CONC	6	0.370	0.062	1.88	$p > 0.1$
Residual	24	0.790	0.033		
Total	67	3.327	0.050		

[a] Data are presented as millions of leukocytes per rat, logarithmically transformed. Logarithms were calculated to the base $e = 2.718 \ldots$.

Table 9. Total Leukocyte Cell Counts: Estimated Treatment Means and Contrasts[a]

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Retransformed mean[b]	3.19	3.58	3.10	3.48	3.73
Ratio[c]	—	1.12	0.97	1.09	1.17
95% confidence interval[d]	—	(0.92,1.37)	(0.81,1.17)	(0.91,1.30)	(0.98,1.40)
	Duration of Exposure (days)				
	0 (Control)	1	2	4	
Retransformed mean	3.19	3.19	3.34	3.91	
Ratio	—	1.00	1.05	1.23[e]	
95% confidence interval	—	(0.86,1.16)	(0.90,1.22)	(1.06,1.42)	

[a] Values are expressed as millions of leukocytes per rat.

[b] The retransformed estimated mean is given by antilog (estimated treatment mean on log scale).

[c] Ratio of retransformed mean to control. Treatment differences on the log scale correspond to ratios of retransformed estimated treatment means.

[d] The 95% confidence intervals for ratios of retransformed treatment means are obtained by determining the antilog 95% confidence limits for differences between estimated means on the log scale.

[e] Significant differences ($p < 0.05$) from controls are indicated by 95% confidence intervals that do not bracket 1.

Table 10. Percentage of Macrophages in Lavaged Leukocytes: Raw Data[a]

Ozone Concentration (ppm)	Days Exposed to Ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	95.2 (4.2)[b]	96.4 (1.3)	98.8 (1.5)	98.2 (1.3)	95.8 (4.9)[b]	96.9 (2.2)
0.4	98.4 (0.8)[c]	97.6 (0.8)	97.9 (1.2)	96.7 (1.5)	98.0 (1.0)[c]	97.9 (0.8)
0.6	98.9 (0.4)	97.2 (2.1)	98.6 (0.2)	95.7 (2.3)	99.1 (0.2)	98.7 (0.7)
0.8	97.4 (1.6)	89.9 (7.2)	95.7 (2.3)	93.6 (2.3)	98.1 (0.4)	98.2 (0.8)

[a] Data are from rats exposed to ozone for up to four days, and are expressed as means (SD) of three experiments, three rats per experiment.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see Table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data were obtained in experiments 172, 174, 179, 180, and 181, with experiment 179 contributing twice to the above information (see Table 2).

Table 11. Percentage of Macrophages in Lavaged Leukocytes: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	31	33.08	1.07	3.62	
+ TREAT	1	4.17	4.17	14.12	$p < 0.001$
+ TREAT.DAY	2	0.73	0.36	1.24	$p > 0.1$
+ TREAT.CONC	3	0.31	0.10	0.35	$p > 0.1$
+ TREAT.DAY.CONC	6	1.99	0.33	1.12	$p > 0.1$
Residual	24	7.08	0.30		
Total	67	47.36	0.71		

[a] Logistic scale. The logistic transform of percentage macrophages (%M) is given by the formula: $L = \text{logit}\ (\%M) = \log_e [\%M/(100 - \%M)]$.

Table 12. Percentage of Macrophages in Lavaged Leukocytes: Estimated Treatment Means and Contrasts

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Retransformed mean[a]	99.2	98.9	98.9	98.6	98.5
Ratio (odds scale)[b]	—	0.71	0.70	0.54[d]	0.53[d]
95% Confidence interval[c]	—	(0.39,1.30)	(0.40,1.21)	(0.32,0.92)	(0.31,0.91)

	Duration of Exposure (days)			
	0 (Control)	1	2	4
Retransformed mean	99.2	98.5	98.5	99.1
Ratio (odds scale)	—	0.52[d]	0.53[d]	0.85
95% Confidence interval	—	(0.33,0.82)	(0.34,0.83)	(0.54,1.33)

[a] The retransformed mean for percentage of macrophages (%M) is given by the inverse of the logistic transformation (see footnote to Table 11) applied to the estimated treatment mean, L: $\%M = 100[\exp(L)/(1 + \exp(L))]$.

[b] If $\%M_o$ denotes the retransformed mean for control animals and $\%M_t$ that for treated animals, the ratio of the retransformed means, treated to control, on the odds scale is given by the formula: $[\%M_t/(100 - \%M_t)] / [\%M_o/(100 - \%M_o)]$. This quantity emerges as the antilog of a treatment difference on the logit scale. The quantities in square brackets are known as "odds."

[c] The 95% confidence intervals for ratios of retransformed means (those means being expressed as odds) are obtained by determining the antilog 95% confidence limits for differences between estimated means on the logistic scale.

[d] Significant differences ($p < 0.05$) from control are indicated by 95% confidence intervals that do not bracket 1.

Table 13. Percentage of Neutrophils in Lavaged Leukocytes: Raw Data[a]

Ozone Concentration (ppm)	Days Exposed to Ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	2.6 (4.1)[b]	2.0 (2.0)	0.1 (0.2)	0.4 (0.8)	2.6 (4.1)[b]	1.6 (2.4)
0.4	0.2 (0.4)[c]	0.9 (0.8)	0.4 (0.5)	1.3 (1.5)	0.7 (0.7)[c]	0.1 (0.2)
0.6	0.0 (0.0)	2.0 (1.5)	0.0 (0.0)	3.1 (2.2)	0.0 (0.0)	0.1 (0.2)
0.8	0.9 (0.8)	8.7 (7.0)	0.8 (0.5)	4.0 (1.8)	0.2 (0.4)	0.3 (0.0)

[a] Data are from rats exposed to ozone for up to four days, and are expressed as means (SD) of three experiments, three rats per experiment.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see Table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data were obtained in experiments 172, 174, 179, 180, and 181, with experiment 179 contributing twice to the above information (see Table 2).

Table 14. Percentage of Neutrophils in Lavaged Leukocytes: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	31	3.00	3.00	4.17	
+ TREAT	1	23.27	23.27	32.36	$p < 0.001$
+ TREAT.DAY	3	10.80	3.60	5.01	$0.005 < p < 0.01$
+ TREAT.CONC	2	10.02	5.01	6.97	$0.005 < p < 0.005$
+ TREAT.DAY.CONC	6	3.71	0.62	0.86	$p > 0.1$
Residual	24	17.26	0.72		
Total	67	158.06	2.36		

[a] Logistic scale. In this case, the logistic transformation was calculated from percentage of neutrophils + 0.1%, because values of 0.0% had been recorded as data.

Table 15. Percentage of Neutrophils in Lavaged Leukocytes: Estimated Treatment Means and Contrasts

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Retransformed mean[a]	0.05	0.14	0.08	1.18	0.67
Ratio (odds scale)[b]	—	1.58	1.23	8.63[c]	5.17[c]
95% Confidence interval[d]	—	(0.62,4.04)	(0.52,2.91)	(3.78,19.70)	(2.27,11.81)
	Duration of Exposure (days)				
	0 (Control)	1	2	4	
Retransformed mean	0.05	0.77	0.55	0.07	
Ratio (odds scale)	—	5.88[c]	4.38[c]	1.11	
95% Confidence interval	—	(2.91,11.88)	(2.16,8.90)	(0.55,2.23)	

[a] Retransformed estimated treatment means.

[b] Ratio of retransformed means to control (odds scale).

[c] Significant differences ($p < 0.05$) from controls are indicated by 95% confidence intervals that do not bracket 1.

[d] The 95% confidence intervals for ratios of retransformed means.

Table 16. Percentage of Lymphocytes in Lavaged Leukocytes: Raw Data[a]

Ozone Concentration (ppm)	Days Exposed to Ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	2.2(0.2)[b]	1.6 (0.8)	1.1 (1.6)	1.3 (0.6)	1.7 (1.2)[b]	1.6 (0.2)
0.4	1.3 (0.7)[c]	1.6 (0.8)	1.7 (0.9)	2.0 (0.6)	1.3 (0.3)[c]	2.0 (0.7)
0.6	1.1 (0.4)	0.8 (0.7)	1.4 (0.2)	1.2 (0.4)	0.9 (0.2)	1.2 (0.5)
0.8	1.7 (0.9)	1.4 (0.2)	3.6 (1.8)	2.4 (2.1)	1.7 (0.0)	1.4 (0.8)

[a] Data are from rats exposed to ozone for up to four days and are expressed as means (SD) of three experiments, three rats per experiment.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see Table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data were obtained in experiments 172, 174, 179, 180, and 181, with experiment 179 contributing twice to the above information (see Table 2).

Table 17. Percentage of Lymphocytes in Lavaged Leukocytes: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	31	22.395	0.722	1.70	
+ TREAT	1	0.062	0.062	0.15	$p > 0.1$
+ TREAT.CONC	3	1.688	0.563	1.32	$p > 0.1$
+ TREAT.DAY	2	0.614	0.307	0.72	$p > 0.1$
+ TREAT.DAY.CONC	6	2.082	0.347	0.82	$p > 0.1$
Residual	24	10.199	0.425		
Total	67	37.040	0.553		

[a] Logistic scale. In this case, the logistic transformation was calculated from percentage of lymphocytes + 0.1%, because values of 0.0% had been recorded as data.

of interaction between these factors. Table 15 shows that statistically significant increases ($p = 0.05$) occurred at concentrations of 0.6 and 0.8 ppm, and durations of one and two days.

Lymphocytes. The proportion of lymphocytes were 1 to 2 percent in the control rats (with the exception of a value of 3.6 percent in one group) and remained generally the same in the ozone-exposed group (see Table 16).

The impression of no effect of ozone exposure provided by the raw data is confirmed by the analysis of variance (Table 17), which shows no significant effects. Estimated contrasts between treatments and control are given in Table 18.

Production of Superoxide Anion

Inspection of experimental results (see Table 19) shows that PMA treatment caused a substantial stimulation of superoxide anion production in every case. There was, however, some variability in the production of superoxide anion, as we have found previously. There was no stimulation of oxidant production with any ozone treatment, but there was evidence of a reduction in superoxide anion production in bronchoalveolar leukocytes from rats exposed to ozone at 0.6 ppm.

Table 20, the analysis of variance, shows significant effects of PMA stimulation and ozone treatment. The effect of ozone depended on concentration, but not duration, and there was no evidence of interaction between these aspects of ozone exposure, or between ozone exposure and PMA stimulation. The estimated effects of treatments are shown in Table 21, where it can be seen that a statistically significant lowering of superoxide production occurred at an ozone concentration of 0.6 ppm.

Fibronectin Proteolysis

There was some variability in the ability of bronchoalveolar leukocytes to break down fibronectin, mostly due to half-life effects in the ^{125}I fibronectin matrix. Tabulation of experimental results shows no evidence of any systematic effects of treatment in comparisons made with the controls on each day (Table 22).

Table 18. Percentage of Lymphocytes in Lavaged Leukocytes: Estimated Treatment Means and Contrasts

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Retransformed mean[a]	0.97	1.32	1.25	0.75	0.58
Ratio (odds scale)[b]	–	1.34	1.27	0.79	0.63
95% Confidence interval[c]	–	(0.65,2.75)	(0.66,2.45)	(0.42,1.49)	(0.34,1.20)
	Duration of Exposure (days)				
	0 (Control)	1	2	4	
Retransformed mean	0.97	0.74	0.90	1.17	
Ratio (odds scale)	–	0.79	0.94	1.19	
95% Confidence interval	–	(0.46,1.35)	(0.55,1.62)	(0.70,2.04)	

[a] Retransformed estimated treatment means.

[b] Ratio of retransformed means to control values.

[c] The 95% confidence intervals for ratios of retransformed means.

Table 19. Production of Superoxide Anion: Raw Data[a]

Ozone Concentration (ppm)	PMA Stimulation	Days Exposed to Ozone					
		1		2		4	
		Control	Ozone	Control	Ozone	Control	Ozone
0.2	No	5.8 (2.7)[b]	6.4 (2.9)	8.9 (1.3)	8.3 (1.4)	6.1 (3.0)[b]	6.0 (1.7)
	Yes	21.4 (6.7)[b]	25.5 (11.1)	28.4 (3.5)	27.3 (1.2)	23.9 (8.2)[b]	24.3 (6.4)
0.4	No	6.0 (0.6)[c]	5.3 (1.7)	7.8 (0.5)	7.1 (1.5)	9.7 (6.0)[c]	9.8 (4.0)
	Yes	23.0 (2.7)[c]	16.3 (2.2)	35.6 (4.8)	30.5 (2.6)	21.9 (4.0)[c]	26.2 (9.8)
0.6	No	5.2 (0.5)	4.8 (0.5)	5.8 (0.2)	3.9 (1.0)	7.7 (1.1)	6.6 (0.4)
	Yes	26.2 (1.5)	15.4 (2.3)	28.6 (3.7)	16.0 (3.7)	33.3 (9.1)	31.4 (9.9)
0.8	No	4.4 (6.4)	3.5 (4.1)	2.7 (3.2)	2.3 (1.9)	6.6 (4.8)	5.1 (3.9)
	Yes	15.4 (19.0)	13.2 (15.4)	10.5 (9.1)	9.4 (6.7)	14.5 (7.8)	14.8 (9.4)

[a] Data for production of superoxide anion by bronchoalveolar leukocytes are from control rats and rats exposed to ozone. Data are expressed as nanomoles of superoxide anion per 10^5 cells per two hours.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data for the 0.4-ppm, 1-day treatment group were obtained from experiments 172, 174, and 179. Control data for the 0.4-ppm, 4-day treatment group were obtained from experiments 179 and 180 only, with experiment 181 contributing no data on production of superoxide anion. Thus, data from control animals in experiment 179 contributed twice to above information. As already noted in the footnotes to Table 7, this duplication of data is used only in descriptive tables.

Analysis of variance, in Table 23, shows no statistically significant effects. Estimated contrasts between treatments and control are shown in Table 24.

Epithelial Lytic Injury

Experimental results are shown in Table 25. They suggest that bronchoalveolar leukocytes from rats inhaling ozone did not have altered ability to cause lytic injury to the target epithelial cells compared to control rats. An effect of ozone exposure, dependent on duration but not on concentration, was suggested by the analysis of variance (Table 26), although it just failed to reach statistical significance. Table 27 shows that the suggested effect was due to the contrast between one and two days of exposure (estimated ratio, 1 day:2 day, is 1.09; 95 percent confidence interval, 1.01,1.18).

Epithelial Detachment Injury

There was some variability in the ability of leukocytes to cause detachment injury between experiments due to the differences in labeling efficiency of the target cells. The

Table 20. Production of Superoxide Anion: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	30	63.373	2.112	33.43	
+ STIM	1	61.225	61.225	968.80	$p < 0.001$
+ TREAT	1	0.392	0.392	6.20	$0.01 < p < 0.025$
+ TREAT.CONC	3	0.554	0.185	2.92	$0.025 < p < 0.05$
+ TREAT.STIM	1	0.048	0.048	0.76	$p > 0.1$
+ TREAT.DAY	2	0.078	0.039	0.61	$p > 0.1$
+ TREAT.DAY.CONC	6	0.716	0.119	1.89	$0.05 < p < 0.1$
+ TREAT.STIM.DAY	2	0.051	0.025	0.40	$p > 0.1$
+ TREAT.STIM.CONC	3	0.048	0.016	0.25	$p > 0.1$
+ TREAT.STIM.DAY.CONC.	6	0.283	0.047	0.75	$p > 0.1$
Residual	76	4.803	0.063		
Total	131	131.570	1.004		

[a] Data are expressed as nanomoles of superoxide anion per 10^5 cells per two hours, logarithmically transformed.

Table 21. Production of Superoxide Anion: Estimated Treatment Means and Contrasts[a]

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Retransformed mean[b]	17.4	18.2	16.1	12.9	17.0
Ratio[c]	—	1.04	0.92	0.74[d]	0.97
95% Confidence interval[e]	—	(0.86,1.26)	(0.76,1.12)	(0.63,0.87)	(0.82,1.15)
	Duration of Exposure (days)				
	0 (Control)	1	2	4	
Retransformed mean	17.4	15.6	15.1	17.0	
Ratio	—	0.90	0.87	0.98	
95% Confidence interval	—	(0.78,1.03)	(0.75,1.00)	(0.84,1.13)	
	PMA Stimulation				
	Absent	Present			
Retransformed mean	8.3	31.0			
Ratio	—	3.8[d]			
95% Confidence interval	—	(3.4,4.2)			

[a] Data are expressed as nanomoles of superoxide anion per 10^5 cells per two hours.

[b] Retransformed estimated treatment means.

[c] Ratios of retransformed means (concentrations and durations) to control, and of PMA present to PMA absent.

[d] Significant differences ($p < 0.05$) from controls are indicated by 95% confidence intervals that do not bracket 1.

[e] The 95% confidence intervals for ratios of retransformed means (concentrations and durations) to control, and of PMA present to PMA absent.

results (shown in Table 28) were suggestive of increased detachment injury on days 1 and 4 at 0.8 ppm ozone and of decreased detachment injury at 0.4 ppm ozone on all days.

The analysis of variance showed an effect of ozone, which was concentration dependent (Table 29). Table 30, which displays estimated treatment effects, shows that detachment injury was decreased at 0.4 ppm and that the suggestion of increased injury at 0.8 ppm was not statistically significant.

Macrophage Size

Macrophages obtained from the lungs of rats inhaling ozone at 0.8 ppm were large and vacuolated compared to control macrophages. When sizes were estimated there was a clear increase in size of the ozone-exposed cells compared to the control cells (Table 31). This difference in size appeared to be time-related, reducing from 3.4 μm on average on day 1 to 0.9 μm by day 4.

Table 22. Fibronectin Proteolysis: Raw Data[a]

Ozone Concentration (ppm)	Days Exposed to Ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	4,247 (3,168)[b]	3,872 (2,559)	2,210 (810)	2,305 (498)	4,133 (2,427)[b]	3,741 (2,427)
0.4	3,833 (1,123)[c]	4,034 (877)	4,322 (689)	4,422 (1,195)	5,668 (1,605)[c]	5,691 (857)
0.6	3,880 (121)	4,094 (369)	4,173 (609)	4,296 (739)	3,628 (177)	3,390 (284)
0.8	6,418 (2,849)	6,830 (3,258)	6,165 (2,783)	6,042 (2,998)	4,059 (2,491)	4,119 (2,689)

[a] Proteolysis of ^{125}I fibronectin matrix by cells from control and ozone-exposed lung. Results are expressed as mean (SD) counts per minute of ^{125}I triplicate wells in three separate experiments.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see Table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data were obtained in experiments 172, 174, 179, 180, and 181, with experiment 179 contributing twice to the above information (see Table 2).

Table 23. Fibronectin Proteolysis: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	31	12.5936	0.4062	49.46	
+ TREAT	1	0.0028	0.0028	0.34	$p > 0.1$
+ TREAT.DAY	2	0.0041	0.0021	0.25	$p > 0.1$
+ TREAT.CONC	3	0.0027	0.0009	0.11	$p > 0.1$
+ TREAT.DAY.CONC	6	0.0193	0.0032	0.39	$p > 0.1$
Residual	24	0.1971	0.0082		
Total	67	12.8197	0.1913		

[a] Data are expressed as counts per minute of degraded ^{125}I fibronectin, logarithmically transformed.

Table 24. Fibronectin Proteolysis: Estimated Treatment Means and Contrasts[a]

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Retransformed mean[b]	3,156	3,196	3,268	3,165	3,166
Ratio[c]	—	1.01	1.04	1.00	1.00
95% Confidence interval[d]	—	(0.92,1.12)	(0.94,1.13)	(0.92,1.10)	(0.92,1.10)
	Duration of Exposure (days)				
	0 (Control)	1	2	4	
Retransformed mean	3,156	3,274	3,181	3,141	
Ratio	—	1.04	1.01	1.00	
95% Confidence interval	—	(0.96,1.12)	(0.93,1.09)	(0.92,1.07)	

[a] Data are expressed as counts per minute of degraded fibronectin.

[b] Retransformed estimated treatment means.

[c] Ratio of retransformed means to control values.

[d] The 95% confidence intervals for ratios of retransformed means to control values.

Table 25. Epithelial Lytic Injury: Raw Data[a]

Ozone Concentration (ppm)	Days Exposed to Ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	3,765 (508)[b]	3,937 (615)	8,115 (1,261)	7,754 (1,384)	5,839 (3,152)[b]	5,787 (3,142)
0.4	4,469 (2,359)[c]	4,565 (3,073)	8,500 (4,808)	6,996 (3,013)	6,764 (1,149)[c]	6,826 (1,189)
0.6	4,032 (381)	4,419 (835)	2,987 (413)	3,152 (473)	3,309 (430)	3,277 (419)
0.8	6,438 (3,055)	7,190 (3,493)	5,493 (3,200)	5,445 (3,102)	6,172 (1,120)	6,035 (894)

[a] Lytic injury caused to epithelial cells by coculture with bronchoalveolar leukocytes from control and ozone-exposed rats. Data are expressed as mean (SD) cpm of released ^{51}Cr.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see Table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data were obtained in experiments 172, 174, 179, 180, and 181, with experiment 179 contributing twice to the above information (see Table 2).

Table 26. Epithelial Lytic Injury: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	31	12.2297	0.3945	85.05	
+ TREAT	1	0.0000	0.0000	0.00	$p > 0.1$
+ TREAT.DAY	2	0.0250	0.0125	2.70	$0.05 < p < 0.1$
+ TREAT.CONC	3	0.0264	0.0088	1.90	$p > 0.1$
+ TREAT.DAY.CONC	6	0.0182	0.0030	0.65	$p > 0.1$
Residual	24	0.1113	0.0046		
Total	67	12.4107	0.1852		

[a] Data are expressed as counts per minute of released ^{51}Cr, logarithmically transformed.

Table 27. Epithelial Lytic Injury: Estimated Treatment Means and Contrasts[a]

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Retransformed mean[b]	9,955	9,903	9,380	10,377	10,216
Ratio[c]	—	0.99	0.94	1.04	1.03
95% Confidence interval[d]	—	(0.92,1.07)	(0.88,1.01)	(0.98,1.11)	(0.96,1.10)
	Duration of Exposure (days)				
	0 (Control)	1	2	4	
Retransformed mean	9,955	10,478	9,601	9,825	
Ratio	—	1.05	0.96	0.99	
95% Confidence interval	—	(0.99,1.11)	(0.91,1.02)	(0.93,1.04)	

[a] Lytic injury caused to epithelial cells by bronchoalveolar leukocytes. Data are expressed as counts per minute of released ^{51}Cr.

[b] Retransformed estimated treatment means.

[c] Ratio of retransformed means to control values.

[d] The 95% confidence intervals for ratios of retransformed means to control values.

Table 28. Epithelial Detachment Injury: Raw Data[a]

Ozone Concentration (ppm)	Days Exposed to Ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	756 (1,051)[b]	927 (1,166)	4,860 (4,319)	4,667 (3,201)	4,002 (5,138)[b]	4,273 (5,391)
0.4	1,168 (2,023)[c]	978 (1,463)	4,243 (2,929)	3,723 (2,624)	3,085 (2,193)[c]	2,217 (1,547)
0.6	1,271 (1,623)	1,272 (1,517)	125 (69)	92 (90)	153 (205)	160 (229)
0.8	1,705 (1,114)	2,148 (960)	2,065 (2,170)	1,842 (1,780)	1,613 (1,311)	2,357 (1,293)

[a] Detachment injury caused to epithelial cells by coculture with bronchoalveolar leukocytes from control rats and rats exposed to ozone. Data are expressed as mean (SD) counts per minute of ^{51}Cr in detached cells in triplicate wells in three experiments.

[b] Control data for the 0.2-ppm, 1-day treatment group were obtained in experiments 182, 183, and 187 (see Table 2). Control data for the 0.2-ppm, 4-day treatment group were obtained in experiments 182, 183, and 188. Thus, data from control animals used in experiments 182 and 183 contributed twice to the above information. This duplication of control data is used only in descriptive tables; analyses of variance are in accordance with the slightly unbalanced plan shown in Table 2.

[c] Control data were obtained in experiments 172, 174, 179, 180, and 181, with experiment 179 contributing twice to the above information (see Table 2).

Table 29. Epithelial Detachment Injury: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Significance
EXPT	31	354,820,672	11,445,828	71.61	
+ TREAT	1	6,637	6,637	0.04	$p > 0.1$
+ TREAT.CONC	3	1,600,002	533,334	3.34	$0.025 < p < 0.05$
+ TREAT.DAY	2	751,994	375,997	2.35	$p > 0.1$
+ TREAT.DAY.CONC	6	894,322	149,054	0.93	$p > 0.1$
Residual	24	3,836,314	159,846		
Total	67	361,909,952	5,401,641		

[a] Data are expressed as counts per minute of ^{51}Cr in detached cells.

Table 30. Epithelial Detachment Injury: Estimated Treatment Means and Contrasts[a]

	Ozone Concentration (ppm)				
	0.0 (Control)	0.2	0.4	0.6	0.8
Mean[b]	2,822	2,974	2,309	2,813	3,143
Difference[c]	—	153	−513[d]	−8	321
95% Confidence interval[e]	—	(−290,595)	(−918, −108)	(−397,381)	(−67,710)
	Duration of Exposure (days)				
	0 (Control)	1	2	4	
Mean	2,822	2,943	2,540	2,948	
Difference	—	121	−282	126	
95% Confidence interval	—	(−211,452)	(−616,52)	(−204,455)	

[a] Detachment injury caused to epithelial cells by bronchoalveolar leukocytes. Data are expressed as counts per minute of ^{51}Cr in detached cells.

[b] Estimated treatment means.

[c] Estimated difference between treatment means and control.

[d] Significant differences ($p < 0.05$) are indicated by 95% confidence intervals that do not bracket 0.

[e] The 95% confidence intervals for the differences between treatment means and control.

Table 31. Macrophage Size: Raw Data[a]

	Days Exposed to Ozone		
	1	2	4
Control	16.25 (2.61)	19.31 (1.35)	18.04 (2.55)
Ozone	19.61 (2.55)	21.96 (3.77)	18.96 (1.93)

[a] Macrophage size in bronchoalveolar lavage samples from rats exposed to 0.8 ppm ozone for up to four days. Data are expressed as means (SD) of major diameters in micrometers over three experiments.

Analysis of variance showed a statistically significant effect of ozone, which did not vary with duration of exposure (Table 32). The size of effect is shown in Table 33. For ozone-exposed rats, the estimated mean macrophage size is 20.4 μm. A t test comparing this result to the control value of 18.1 μm is significant at $p = 0.05$.

HISTOLOGY

The major pathological changes present in the lungs of rats treated with ozone at a concentration of 0.8 ppm were observed in the trachea, bronchial tubes, and particularly in the area of the terminal and respiratory bronchioles, including the alveolar ducts. No changes were visible at any time in alveoli situated well away from the respiratory bronchioles. In this main area of pulmonary parenchyma the alveolar walls appeared normal at all time points with no discernible rounding of type I epithelial cells, and the number of pulmonary macrophages did not appear increased although some of those present showed an increased cytoplasmic area with a foamy appearance. Close to some terminal and respiratory bronchioles, however, there was marked accumulation of inflammatory cells after 24 hours of exposure (Figures 3 and 4). Most of these cells were pulmonary macrophages, often with a foamy cytoplasm, but smaller cells of monocytic type were also present as well as some neutrophil leukocytes. In most cases clusters of inflammatory cells were present within alveolar spaces, either leading off from alveolar ducts or in the close vicinity of these, with little evidence of interstitial accumulation of cells in the alveolar walls. The surfaces of the alveolar ducts themselves, however, did show a thickening in many cases that was due partly to a rounding of epithelial cells but also to the interstitial accumulation of small mononuclear cells, which were probably young macrophages, monocytes, or young fibroblasts (Figures 3 and 4). In the heaviest exposures, occasional neutrophils were present in the interstitial space. These changes were dose-related, being much more marked in animals treated with 0.8 ppm of ozone, although thickening of the walls of alveolar ducts could still be detected with animals treated with 0.2 ppm.

Table 32. Macrophage Size: Analysis of Variance[a]

Source of Variation	Degrees of Freedom	Mean Square	Variance Ratio	Significance
Experiments	5	10.546		
+ Ozone	1	16.013	15.42	$0.025 < p < 0.05$
+ Duration of exposure	2	1.569	1.51	$p > 0.1$
Residual	3	1.038		
Total	11	6.818		

[a] Macrophage size is expressed in micrometers.

Table 33. Macrophage Size: Estimated Treatment Means and Contrasts[a]

	Duration of Exposure (days)			
	0 (Control)	1	2	4
Mean[b]	18.08	21.43	20.73	19.00
Difference[c]	—	3.35	2.66	0.92
95% Confidence interval[d]	—	(0.10,6.60)[e]	(−0.59,5.91)	(−2.33,4.17)

[a] Data are expressed as lengths of the major diameters in micrometers.

[b] Estimated treatment means.

[c] Estimated differences between treatment means and control values.

[d] The 95% confidence intervals for the differences between treatment means and control values.

[e] Significant differences ($p < 0.05$) are indicated by 95% confidence intervals that do not bracket 0.

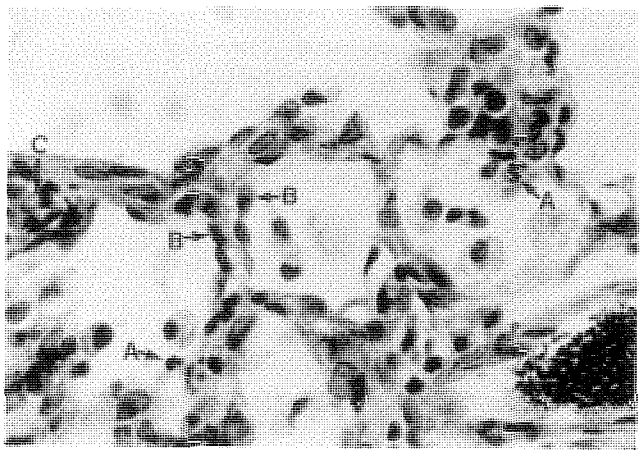

Figure 3. Alveolar duct wall and adjacent alveoli from the lung of a rat treated with ozone at 0.8 ppm for 24 hours. The alveoli contain enlarged pulmonary macrophages with foamy cytoplasm, and some neutrophil leukocytes are present (arrow A). Alveolar walls are covered by rounded epithelial cells (arrow B). The surface of the alveolar duct itself has rounded epithelial cells and there is evidence of interstitial accumulation of inflammatory cells (arrow C). Magnification is × 550.

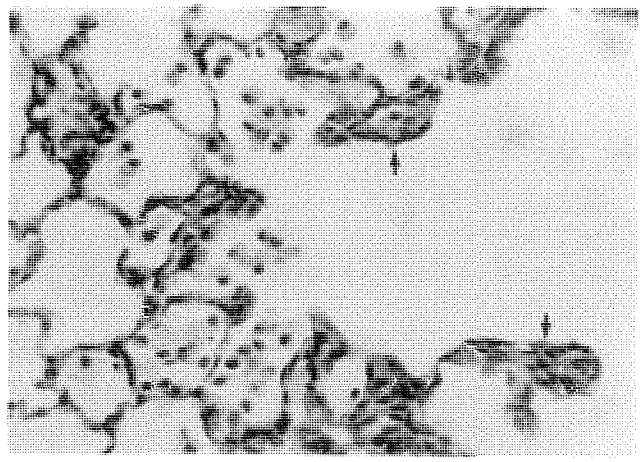

Figure 4. Alveolar duct region of a rat lung treated with ozone at 0.8 ppm for 24 hours. The proximal alveoli contain accumulations of inflammatory cells that are mainly enlarged pulmonary macrophages with a few neutrophils. The surfaces of the alveolar duct are lined with rounded epithelial cells (arrows). Magnification is × 300.

Although the epithelium of most alveoli remained normal in animals treated with ozone at 0.8 ppm, the epithelial cells of those alveoli closest to the respiratory bronchioles were frequently rounded, and occasional alveoli appeared to be lined completely, with the thickened epithelium of almost cuboidal type, similar to type II pneumocytes (Figure 3).

Ciliated cells in the terminal bronchioles appeared very sensitive to high levels of ozone, and in many areas of rat lungs treated with ozone at 0.8 ppm cilia appeared either absent or vestigial by 48 hours (Figure 5). Some Clara cells still showed cytoplasmic projections on the luminal surface, but many of these showed signs of rupture or disintegration. Once again, these changes were dose-related, and most respiratory bronchioles in animals treated with 0.2 ppm of ozone were relatively normal after 48 hours, although some apparent damage to cilia could still be found (Figure 6).

In the larger bronchial tubes and trachea, epithelial damage could be noted but was extremely patchy, with many

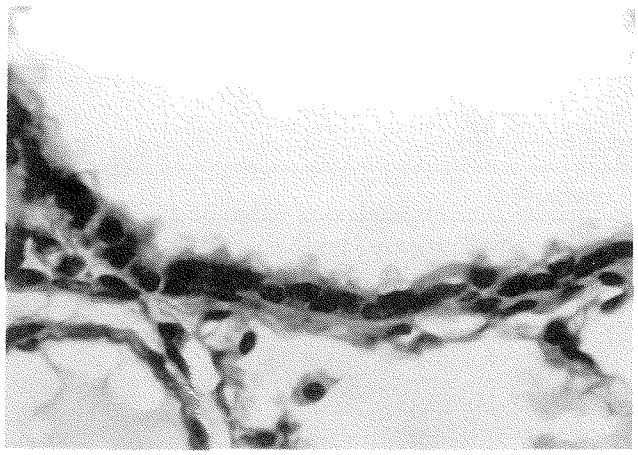

Figure 5. Terminal bronchiole wall of a rat lung treated with ozone at 0.8 ppm for 48 hours. Some luminal projections of Clara cells are still present, although these seem less pronounced than in a normal lung. Cilia are not visible in an area where they would normally occur. Magnification is × 550.

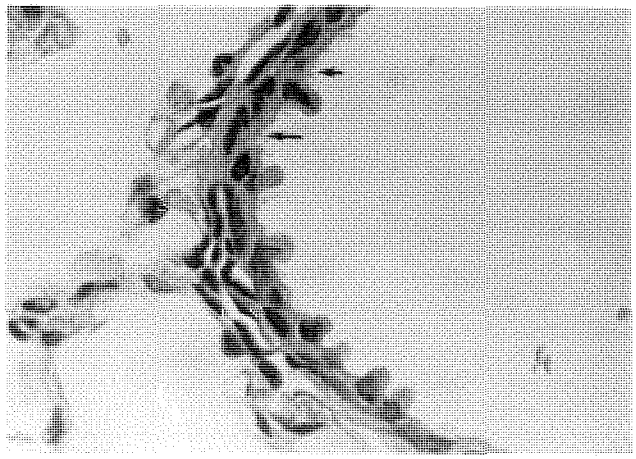

Figure 6. Terminal bronchiole wall of a rat lung treated with ozone at 0.2 ppm for 48 hours. Clara cell luminal projections are marked. Some ciliary structures are present, although with the light microscope they appear shorter and less regular than in normal lungs (arrows). Magnification is × 550.

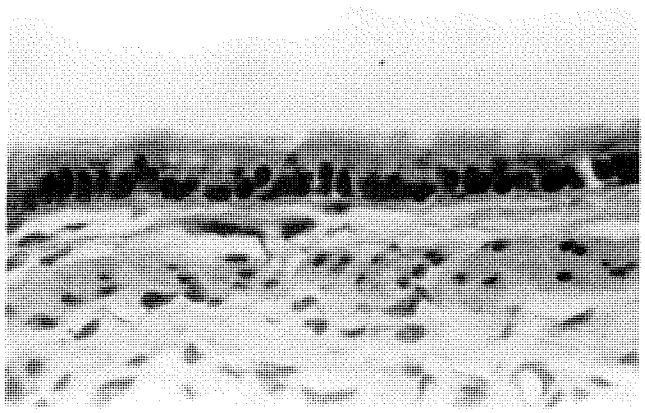

Figure 7. Ciliated epithelium of the wall of a large bronchial tube from the lung of a control rat. Magnification is × 400.

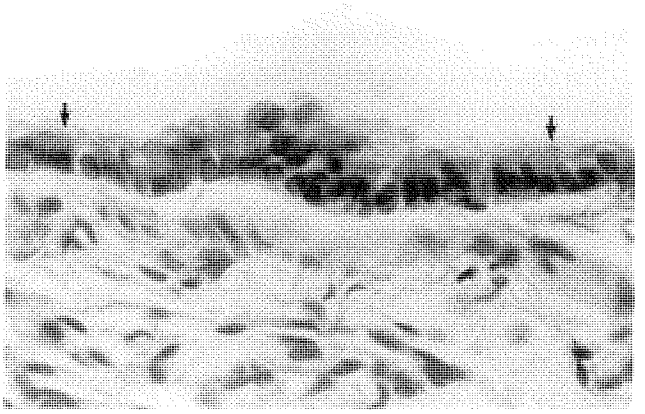

Figure 8. Epithelial surface of the wall of a large bronchial tube from a rat lung treated with ozone at 0.8 ppm for 48 hours. Cilia, where present, are irregular and form a less complete carpet than normal (arrows). In the center of the photograph, multiple layers of cells are present. Some may be adherent pulmonary macrophages, but there is also evidence of some desquamation of epithelial cells. Magnification is × 400.

areas of ciliated epithelium appearing normal at all doses (Figure 7). In other areas, however, some cells appeared to have lost their cilia or only a few shortened cilia were visible by 48 hours. At the same time, the neat columnar appearance of the epithelial cells was disturbed by a pseudostratified pattern present in places, with evidence of some cell desquamation (Figure 8). Damage to ciliated epithelium in the larger airways was most marked after treatment at 0.8 ppm.

After treatment with ozone at 0.8 ppm, the accumulation of inflammatory cells, including neutrophil leukocytes, was most marked after 24 hours of exposure. Fewer cells appeared by 48 hours, and thereafter there was a marked reduction, until by eight days, neutrophils were absent and the number of pulmonary macrophages appeared scarcely raised above normal, although some of those present still had an enlarged foamy cytoplasm. Epithelial changes in the alveolar duct region were also reduced with time after 48 hours, although some ducts and alveolar spaces lined by rounded cells were still present at the end of the exposure period. Similarly, areas of damage to the epithelial linings of the larger bronchial tubes and trachea were still found in animals treated with ozone for eight days.

Treatment of rats with 0.6, 0.4, and 0.2 ppm of ozone produced progressively less pathological change in rat lung tissue over 24 or 48 hours. With 0.6 ppm, some accumulation of inflammatory cells occurred in alveoli close to the respiratory bronchioles, but this was less marked than that following 0.8 ppm treatment and there was also less identifiable interstitial accumulation of these cells. At 0.4 ppm, the inflammatory cell accumulation was very slight, and at 0.2 ppm, cell numbers appeared normal, with only pulmonary macrophages present. Epithelial changes were also reduced with decreasing doses of ozone, but even at 0.2 ppm some alveolar ducts could be found where the epithelial lining cells appeared more rounded than usual, with some evidence of interstitial thickening of the duct walls. Areas of damage to the ciliated epithelium of the bronchioles, larger bronchial tubes, and trachea were visible in animals treated at 0.6 ppm, 0.4 ppm, and 0.2 ppm of ozone for 48 hours but were progressively reduced with dose, and relatively few areas of ciliary damage were found following treatment at 0.2 ppm.

Throughout this study, at all dose levels and treatment times, it was found that rat lung tissue had been damaged in a most irregular way by the inhalation of ozone. Even following treatment at 0.8 ppm, the terminal and respiratory bronchiole regions of many acini appeared normal, although within a few millimeters, others showed marked cellular accumulation and damage. The same pattern was found in the linings of the larger bronchial tubes and trachea, with many areas of normal ciliated epithelium always being present.

DISCUSSION

The main aim of this study was to assess whether the bronchoalveolar leukocytes from the lungs of rats inhaling ozone at plausible environmental levels were able to injure epithelial cells in vitro. Bronchoalveolar leukocytes lavaged from rats the day after exposure to 0.2, 0.4, 0.6, and 0.8 ppm of ozone did not produce elevated levels of leukocyte prod-

ucts known to mediate injury (oxidants or fibronectin-degrading activity), nor did they injure epithelial cells when incubated with them at an effector:target ratio of 5:1.

The studies did reveal that ozone had the ability to cause inflammation in the lung, detectable by bronchoalveolar lavage, as shown by increased proportions of neutrophils in the lungs at the highest concentrations and earliest time points. These effects were, however, small, and there was no overall increase in the total number of lavageable leukocytes in ozone-exposed lungs compared to control lungs. It remains possible that neutrophils may have been present in vivo, and may have been in contact with epithelial cells at higher neutrophil:epithelial cell ratios than those produced in vitro, and so epithelial injury in vivo remains a possibility.

Many studies (see Introduction section) have described low-level inflammation with macrophage and neutrophil accumulation following ozone exposure; epithelial injury in the centriacinar region, increases in interstitial mass, and concomitant septal thickening of the proximal alveoli are also described. Our results are, therefore, in agreement with previous studies finding a low level of inflammation with ozone exposure.

Our results suggest that the epithelial injury that is characteristic of ozone exposure (Wright et al. 1987; Tepper et al. 1989) is unlikely to be caused by the leukocytes themselves, but most likely is a direct toxic effect of ozone. At the low levels of inflammation that were detected in ozone-exposed lungs, no substantial epithelial injury would be anticipated, because our own previous studies have indicated that epithelial injury occurs only when there is a substantial neutrophil recruitment to the rat lung (Donaldson et al. 1988c) and an overall expanded leukocyte population. Neutrophil-enriched inflammatory leukocyte populations have the ability to cause detachment injury when the proportion is greater than approximately 5 percent (unpublished observation), although the injury is substantial only at higher proportions of neutrophils. The relatively low level of neutrophil recruitment seen with ozone exposure in the present study is, therefore, unlikely to lead to epithelial injury. It is possible that neutrophil-mediated injury to epithelial cells, or production of oxidant and protease fibronectin–degrading activity, may have been masked by the high relative proportions of macrophages. However, the proportions of neutrophils and the low total cell numbers available precluded separation of neutrophils into an enriched population and the testing of their activity. It is also possible that using different time points may have yielded different activational states of leukocytes, which could have produced different results. It was not possible within the scope of the study to do further work, however.

The statistically significant decrease in epithelial detachment produced with leukocytes from rats exposed to 0.4 ppm ozone may be due to the release of growth factors by the leukocytes, although this was not present at other concentrations and was not further investigated.

Alveolar macrophages, as well as neutrophils, have the ability to produce increased amounts of fibronectin-degrading activity when suitably stimulated in inflammatory lungs (Brown et al. 1989) and so macrophages might have been expected to be a source of epithelium-injuring activity. However, because such macrophages are derived from inflamed lungs, with neutrophils present, they may have taken up neutrophil elastase (Campbell et al. 1979) that they then release to cause the injury. Because there was no substantial increase in neutrophils in the ozone-exposed lung, this would not be a mechanism in any injury expressed by alveolar macrophages from ozone-exposed lungs.

The modest neutrophil recruitment that was present in the ozone-exposed lungs was probably related, in part at least, to the neutrophil chemotaxin released by alveolar macrophages treated with ozone in vitro, which has been described by Driscoll and Schlesinger (1988). However, the large amount of chemotaxin produced by alveolar macrophages exposed to 0.8 ppm ozone in vitro in that study could be related to the relatively high dose experienced by macrophages because of the in vitro exposure technique.

Exposure in vitro does not take into account the fact that ozone at 0.8 ppm in the atmosphere is likely to be attenuated substantially by solution as it passes down into the deep lung. The accumulation of protein in the alveolar space, described by Hu and colleagues (1982) in rats exposed to ozone, may also contribute to the chemotactic attraction of the neutrophils found in our study, although we did not measure protein accumulation in the alveolar space.

We could not find functional evidence of leukocyte activation in ozone-exposed bronchoalveolar leukocytes, but the macrophages did appear to be activated, so we measured their size, which we have found previously to be increased with activation (Donaldson et al. 1984). This confirmed that with the highest concentrations of ozone at short time points, there were increases in macrophage size; the macrophages were also markedly vacuolated. It is likely that these cells have altered activities based on this altered morphology, which is suggestive of macrophage activation. A similar increase in size and vacuolation has been described by other workers in alveolar macrophages from ozone-exposed rats (Hotchkiss et al. 1989a).

It is important to note that the present study utilized leukocytes obtained by bronchoalveolar lavage, a process that does not obtain all leukocytes but samples those that are accessible. The failure to detect increased numbers may mean

that leukocytes had accumulated in inaccessible regions. The interstitial accumulation of small mononuclear cells seen in histological sections could have represented a population of potentially lytic monocytic cells. These could be sampled only by techniques of lung disruption and should be the subject of future studies. The authors also point out that the overall negative findings in the present study, with regard to the injurious effects of ozone-elicited epithelial cells, should not detract from the well-known hazard associated with ozone exposure caused by other well-described pathways.

There was clear evidence of adaptation to the harmful effects of ozone, the inflammation being present at the early time points with a return to normal bronchoalveolar lavage profile by day 4. This is in agreement with previous reports on adaptive responses to ozone in terms of protein accumulation (Hu et al. 1982) and other indices of lung injury (Boorman et al. 1980; Wright et al. 1987). The basis of this adaptive response may be induction of antioxidant defenses (Menzel 1984; Wright et al. 1987) but this was not investigated in the present study.

Histological examination of lung tissue from rats treated with ozone in the present study has, in general, confirmed previously published reports. Pathological changes can be detected in rat lung tissue following treatment with a dose as low as 0.2 ppm for as little as two days but are very obvious following treatment at 0.8 ppm. Ozone damage is most obvious in the center of the acinus. The respiratory bronchioles and alveolar ducts are particularly affected, but the epithelial lining of larger airways may also be affected. Whereas proximal alveoli showed marked changes, the more distal alveoli appeared normal in all our experiments. Other studies have used transmission electron microscopy and scanning electron microscopy to examine ozone-treated lung tissue, and this has permitted a more precise description of morphological changes. However, most of what has been reported is visible by light microscopy even if the exact structural patterns of cells cannot be determined. The study using the protocol closest to our own was reported by Schwartz and colleagues (1976), who treated rats for seven days with ozone at doses between 0.2 and 0.8 ppm. Changes seen were dose-related and damage to ciliated cells was reported, particularly in the terminal bronchiolar region, with a reduced height or even elimination of Clara cell luminal projections. Accumulations of macrophages with some neutrophils were reported in the alveolar duct region, and the thickened epithelial lining of proximal alveoli was shown to consist of cells with characteristics between type I and type II pneumocytes. Some infiltration of uncharacterized mononuclear cells was found in the interstitium of proximal alveolar walls. In a separate study (Boorman et al. 1980) it was shown that by 20 days of exposure to 0.8 ppm of ozone, pathological changes were less than at 7 days, but the numbers of inflammatory cells in the centriacinar regions still showed a fivefold increase compared to controls. Similar changes to the airways and centriacinar region of the lungs were found in monkeys exposed to 0.2 and 0.35 ppm of ozone for seven days (Castleman et al. 1980).

Adaptive changes, reducing the harmful effects of ozone, have been reported by other workers and have already been described in this report. Where the present study appears at variance with these findings is that in our experiments the maximum inflammatory response developed very quickly (one to two days) and the number of inflammatory cells present had already fallen by four days. By eight days, even at 0.8 ppm, the number of inflammatory cells seen by light microscopy appeared little above control levels. Changes to the epithelium of proximal alveoli were also most obvious after only two days and were less marked by eight days, although damage to ciliated cells showed less obvious reduction by this time point.

One factor raised by the present study has been the apparent inability of pulmonary lavage techniques to obtain populations of inflammatory cells, at least in some instances. Certainly at the higher doses tested, accumulations of inflammatory cells were present around many of the respiratory bronchiolar and alveolar duct regions of treated rats by 24 hours. In spite of this, the number of lavageable cells was not significantly raised, although a transient increase in neutrophils was present. It may be that because only small areas of the pulmonary parenchyma showed an accumulation of inflammatory cells, overall numbers within the lungs were not significantly raised. It is also possible that, in areas of inflammation, particularly where epithelial damage is present, cells like pulmonary macrophages may become more adhesive than usual and less available to lavage techniques. In animals treated by inhalation with toxic dusts such as quartz, pulmonary accumulation of macrophages and neutrophils is more uniformly spread throughout the lungs with all alveoli involved, and this may explain why cell numbers obtained by pulmonary lavage are very greatly increased above control levels.

The finding of a decreased ability of bronchoalveolar lavage leukocytes from ozone-exposed lungs to produce superoxide anion is a phenomenon that has been described previously. Amoruso and colleagues (1981), Ryer-Powder and colleagues (1986), and Esterline and colleagues (1989) described the reduction in the oxidative potential of alveolar macrophages exposed to ozone. This effect appears to be related to the loss of activity of a cytochrome b_{558} caused by the direct effect of ozone demonstrable in vitro (Ryer-

Powder et al. 1986). Both Amoruso and Ryer-Powder described ozone dose-dependent inhibition of oxidant production in their studies. We, however, found no significant inhibition at 0.8 ppm, and significant inhibition at 0.6 ppm. We can identify no reason for the lack of a dose-response correlation, although altogether the control and experimental production of superoxide anion was less at 0.8 ppm ozone than at any of the other doses. This variability is inexplicable in retrospect, but we have encountered this type of variability before; we believe that it is unlikely to mask any effect of ozone on the cells. It is interesting to note that in the study of Ryer-Powder and colleagues (1986), 0.8 ppm also failed to cause inhibition, but the ozone concentration was then increased and revealed inhibition at higher doses and not lower doses. It is possible that 0.8 ppm in our study caused protein leakage into the alveolar space (Hu et al. 1982) sufficient to protect the leukocytes or induce antioxidant defenses. It was not, however, possible retrospectively to examine further this somewhat anomalous finding. The diminished oxidative potential could be related to the increased infection found in ozone-exposed animals (Aranyi et al. 1983), although oxidant damage to the epithelial cells of the mucociliary escalator could interfere with clearance of bacteria and bring about any observed increase in infection.

CONCLUSION

The study has fulfilled its aims and objectives, which were (1) to develop an ozone exposure system; (2) to expose rats to defined low levels of ozone; (3) to assess inflammatory responses in the bronchoalveolar region; (4) to measure the production of harmful substances by the bronchoalveolar leukocytes; and (5) to assess the short-term injurious activities of unfractionated bronchoalveolar leukocytes against epithelial cells in vitro at an effector:target ratio of 5:1. That the rat system was a relevant one and responded similarly to previous studies was indicated by the fact that there was inflammation, adaptation of the inflammatory response, increased size and appearance of alveolar macrophages, and decreased oxidative activity of the ozone-exposed bronchoalveolar leukocytes; all of these have been reported in previous studies with ozone. The hypothesis addressed in this study was that the bronchoalveolar leukocytes from ozone-exposed rats had the ability to injure epithelial cells. There was no evidence that whole, unfractionated bronchoalveolar leukocytes collected on the morning following the cessation of exposure had increased production of superoxide anion or fibronectin-degrading activity, which might be capable of mediating injury to epithelial cells. Nor was there evidence that these leukocytes could injure the epithelial cells when incubated with them. Leukocytes collected at different time points, earlier or later, or at different ratios of cells separated into pure macrophage or neutrophil populations, might have shown different activities, but the opportunity to extend the study to include these different aspects was not available.

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ABOUT THE AUTHORS

Kenneth Donaldson was educated at the University of Stirling and graduated with a B.Sc. in Biology in 1978. He then undertook a Ph.D. degree in the Department of Surgery in the University of Edinburgh on the subject of the immunological and inflammatory effects of asbestos, and graduated in 1982. He subsequently became involved in the experimental pathology program in the Institute of Occupational Medicine, gradually increasing his interest in the cell biology of the dust-related lung diseases. In 1984 he took over as head of the Unit which is now the Toxicology and Immunopathology Unit of the Institute of Occupational Medicine. Since then, the interests of the Toxicology and Immunopathology Unit have expanded to embrace research into the cellular basis of lung disease caused by a range of occupational and environmental agents. Kenneth Donaldson is an Honorary Fellow in the Department of Medicine of the University of Edinburgh, a part-time lecturer and examiner in toxicology and immunology in Napier Polytechnic of Edinburgh, and an Assistant Editor of the Annals of Occupational Hygiene.

Geraldine Brown began her scientific career in the Biochemistry Department of the Veterinary College of the University of Edinburgh. She moved to the Institute of Occupational Medicine in 1975, became involved increasingly in cell biology studies, and developed new assay systems for detecting subtoxic effects of mineral dusts. She was awarded a Ph.D. from the University of Edinburgh in 1990 for research into the role of connective tissue protease in dust-related lung injury, and is currently involved in several projects relating to lung inflammation.

David Brown joined the Institute of Occupational Medicine in 1974 and trained in histopathological techniques, gaining a Higher National Certificate in Medical Laboratory Technology in 1978. He moved to the Toxicology and Immunopathology Unit in 1987, and learned diverse techniques in cell biology and immunology. He is completing a B.A. with the Open University and is currently studying toward a Ph.D. on the subject of pulmonary response to organic dust.

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William M. Maclaren graduated from Glasgow University in 1971 with a B.Sc. in Mathematics. There followed a year's postgraduate work in the Department of Medical Cardiology at Glasgow Royal Infirmary, where he studied applications of computers to the analysis of pressure waveforms obtained at cardiac catheterization. After teacher training at Jordanhill College of Education, Glasgow, he spent the next three years at Paisley College of Technology lecturing in mathematics and statistics to engineering and social science students. His interest in medical applications led to a one-year contract at University Hospital, Edmonton, Alberta, where he adapted Glasgow Royal Infirmary's electrocardiograph interpretation program for routine use on the hospital's own computer. He gained an M.Sc. in Medical Statistics from the London School of Hygiene and Tropical Medicine in 1978 and, following a short spell at the Medical Research Council's Cytogenetics Unit, also in Edinburgh, he joined the Institute of Occupational Medicine in 1980. Since then he has engaged in a wide variety of statistical work, in both epidemiological and experimental fields. His main interest currently is in survival analysis.

John M.G. Davis graduated in Pathology from the University of Cambridge in 1957, and subsequently undertook research into the cellular effects of radiation at the Cambridge University Department of Radiotherapy. In 1961 his research interest changed to the biological effects of asbestos, on which he worked at the Cambridge University Department of Pathology until 1971, where he became Assistant Director of Research. In 1971, he moved to Edinburgh to set up an experimental pathology unit at the Institute of Occupational Medicine. Work on asbestos and other mineral fibers continued at Edinburgh, but research interests were expanded to include the harmful effects of other dusts, particularly coal and quartz, and some environmental factors. Dr. Davis left the staff of the Institute in 1990 and became a private consultant in occupational pathology. In this capacity he is still involved with the Institute's program of research into the harmful effects of mineral fibers.

ABBREVIATIONS

ANOVA	analysis of variance
CO_2	carbon dioxide
cpm	counts per minute
^{51}Cr	chromium-51
df	degrees of freedom
^{125}I	iodine-125
PBS	phosphate-buffered saline
PMA	phorbol myristate acetate
SD	standard deviation
SOD	superoxide dismutase

INTRODUCTION

In the summer of 1986, the Health Effects Institute (HEI) issued a Request for Applications (RFA 86-1) soliciting proposals for "Genotoxic, Carcinogenic and Cocarcinogenic Effects of Ozone." In response to this RFA, Drs. John M.G. Davis and Kenneth Donaldson from the Institute of Occupational Medicine, Edinburgh, Scotland submitted a proposal entitled, "Leukocyte-Mediated Epithelial Injury in Ozone-Exposed Lung." The HEI approved the 29-month project, which began in August 1987. Total expenditures were $204,644. The Investigators' Report was received at the HEI in February 1990. A revised report was received in September 1990 and was accepted by the Health Review Committee in October 1990. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of the HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h), and Sections 207(c)(4), (5), and (6) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants), and, in some cases, provide the EPA with limited discretion to modify those requirements.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. The current primary and secondary NAAQS for ozone is 0.12 parts per million (ppm)[1], which means that the maximum hourly average concentrations of ozone should not exceed 0.12 ppm on more than one day per year. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

Appropriate standards for emissions of oxidants and their chemical precursors are determined, in part, according to the health risks that they present. Thus, research into the health effects of ozone in studies such as this one is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

Ozone is a highly reactive gas and a major constituent of photochemical smog. Although not directly emitted by motor vehicles, ground-level or tropospheric ozone is formed in the atmosphere by a complex series of photocatalyzed reactions among oxides of nitrogen and volatile organic compounds that are released directly by mobile and stationary combustion sources. Ozone formation requires the presence of both the appropriate pollutant precursors and sunlight, a combination that occurs frequently during the summer months in southern California, in metropolitan centers across the United States, and along the Northeast corridor. Ozone pollution is not confined to urban areas or industrialized countries; recent satellite measurements indicate that large quantities of tropospheric ozone are present over southern Africa and the adjacent eastern tropical South Atlantic Ocean, reportedly because of widespread biomass burning in Africa (Fishman et al. 1991).

The current NAAQS promulgated by the EPA for ozone is 0.12 ppm for one hour, not to be exceeded more than once per year. Many areas of the United States routinely fail to meet this standard (U.S. Environmental Protection Agency 1991). Summertime peak hourly ambient levels of ozone range from 0.05 ppm in rural sections of the country to as high as 0.33 ppm in some urban areas. The EPA estimated that in 1989, 66.7 million people lived in counties in which the levels of ozone in the air violated the NAAQS (U.S. Environmental Protection Agency 1991). In the Los Angeles basin, the ozone standard is exceeded for several hours per day approximately 150 times per year. On smoggy days, high ozone levels tend to persist for eight or more hours in many areas of the country (Lioy and Dyba 1989).

Because of ozone's high chemical reactivity, it has a great potential for causing adverse health effects. Data from animal, human, and epidemiological studies indicate that, even at concentrations near 0.12 ppm, exposure to ozone ex-

[1] A list of abbreviations appears at the end of the Investigators' Report for your reference.

erts numerous effects on the respiratory system (U.S. Environmental Protection Agency 1986, 1988a,b; Lippmann 1989). Ozone is a respiratory irritant to humans, and acute exposures can produce cough and shortness of breath, as well as transitory changes in lung function. Animal studies that use higher ozone concentrations than those used in human investigations indicate that exposure to ozone produces inflammation and cell damage in different regions of the respiratory tract. These changes are of concern, not only because they may affect pulmonary function, but also because they may be related to carcinogenesis. Drs. Davis and Donaldson hypothesized that ozone exposure induces an inflammatory response that may affect bronchoalveolar leukocytes (white cells) in such a way that they damage the lung epithelial tissues, possibly leading to neoplasia. The following discussion will focus on the effects of ozone on inflammatory processes in the lung and how ozone-induced inflammation might be related to carcinogenesis.

PULMONARY INFLAMMATORY RESPONSES

Pulmonary immune responses depend on a complex, highly regulated network of cells, cell products, and tissue to defend the lung against inhaled foreign material. Several types of white cells, or leukocytes, are responsible for recognizing and reacting to substances that the immune system recognizes as foreign. In the lungs, phagocytic cells such as macrophages and neutrophils ingest and destroy microorganisms. Although phagocytes play a central defensive role in clearing inhaled particulate material from the lung, they can also inflict injury on lung tissue when regulatory mechanisms go awry (Sibille and Reynolds 1990). The alveolar macrophages, which are located at the airway-tissue interface, form the first line of cellular defense against inhaled particles and microorganisms. A second type of phagocytic cell, the polymorphonuclear neutrophil, normally resides in the lung vasculature. When foreign organisms or material are inhaled, alveolar macrophages are stimulated not only to ingest and destroy the substance, but also to release chemotactic factors that initiate the inflammatory response and subsequently attract neutrophils.

In order to carry out their microbicidal function, activated phagocytic cells release numerous secretory products, such as free radicals, enzymes (lysozyme, elastase, and collagenase), enzyme inhibitors, cytokines, and prostaglandins (Fantone and Ward 1982; Klebanoff 1988; Sibille and Reynolds 1990). Although these substances assist the macrophages in their defense role, their release also has pathophysiologic implications. For example, an imbalance between proteolytic enzymes and their inhibitors leads to damage to connective tissue components and may contribute to tissue destruction. The respiratory burst, which is required for optimum microbicidal activity, results in the production of highly reactive oxygen species, such as the superoxide anion, hydrogen peroxide, and the hydroxyl radical. (These free radical species also are produced by ozone when it reacts with cellular targets.) Although these free radical species and their secondary reaction products protect the lung by killing bacteria, they also damage epithelial cells and the cell matrix components, thus contributing to cell death and the subsequent regenerative response.

Much of what is known about the pathogenic consequences of lung inflammation comes from studies of mineral dusts, such as silica and asbestos. Researchers have developed a paradigm for pulmonary inflammatory responses from studies of dust-related disease (Doelman et al. 1990; Rom et al. 1991). In many respects, this paradigm is also applicable to ozone-mediated injury. Inhaled mineral dusts give rise to an acute inflammatory response, followed by injury to the surrounding epithelium and subsequent cell proliferation. Alveolar macrophages play a central role in dust-induced diseases, not only as the initial responding cells, but also by modulating the response of incoming neutrophils and lymphocytes (Driscoll et al. 1990). Inhaling dusts alters the cellular and biochemical profile of the bronchoalveolar lavage fluid (Henderson et al. 1985) and stimulates the phagocytic cells to release increased amounts of free radicals (Cantin et al. 1988; Kehrer et al. 1988; Doelman et al. 1990), proteolytic enzymes (Donaldson et al. 1988c; Brown and Donaldson 1989), cytokines (Driscoll et al. 1990), and chemotactic factors (Brown et al. 1991).

Studies of the pathogenesis of the pulmonary immune response have focused on its role in lung diseases such as fibrosis or emphysema. However, overstimulated inflammatory and immune responses may also play a role in carcinogenesis (Weitzman and Gordon 1990). Activated phagocytic cells produce strand breaks, mutations, and chromosomal alterations (Weitzman et al. 1985). Phagocytes also can activate exogenous carcinogens into mutagenic derivatives. Esterline and coworkers (1989) reported activation of a metabolite of benzo[*a*]pyrene to its genotoxic derivative by neutrophils isolated from rats exposed to 2 ppm ozone for four hours. This suggests that ozone exposure may increase carcinogenic risk by altering the metabolism of inhaled xenobiotics. Thus, phagocytes may play a role in carcinogenesis, not only by increasing the free radical burden of the lung, but also by introducing a potential xenobiotic-activating capability (Harris et al. 1978; Trush et al. 1985; O'Brien 1988; Weitzman and Gordon 1990).

OZONE AS AN INFLAMMATORY AGENT

Inflammation is one of the characteristics of oxidant injury to the respiratory tract (U.S. Environmental Protection Agency 1986, 1988a,b; Lippmann 1989; Schlesinger 1989). Seltzer and coworkers (1986) demonstrated that leukocytes move into the alveolar space of human subjects after an acute exposure to ozone. They reported that a two-hour exposure of healthy human subjects to ozone (0.4 or 0.6 ppm) produced a ninefold increase in the percentage of neutrophils in samples of bronchoalveolar lavage fluid recovered from the subjects three hours after the end of exposure. Increased levels of neutrophils and other biomarkers of inflammation were found in bronchoalveolar lavage samples of exercising human subjects 18 hours after they were exposed either for two hours to 0.4 ppm ozone (Koren et al. 1989) or for 6.6 hours to 0.10 or 0.08 ppm ozone (Devlin et al. 1991). Ozone exposure induced small changes in the phagocytic capacity of the alveolar macrophages in the lavage fluid from human subjects exposed to ozone, but had no influence on superoxide production by either stimulated or unstimulated macrophages (Devlin et al. 1991).

Exposure of animals to ozone leads to an accumulation of macrophages and neutrophils in the pulmonary airspaces and interstitial tissue (Zitnik et al. 1978; Boorman et al. 1980; Eustis et al. 1981; Barry et al. 1985; Gross and White 1987). During the first few days of ozone exposure, the functional state and distribution of lung leukocytes is in a constant state of flux. The relative contribution of different cell types to the measured response depends on a number of factors: the species and strain of the laboratory animal; the concentration and duration of the ozone exposure; and the time points for lavage sampling and response measurement after exposure.

The interval between the end of an ozone exposure and the isolation of the bronchoalveolar lavage fluid is a critical factor in determining the profile of leukocytes in the lavage fluid. If lavage samples are obtained immediately after an acute exposure, ozone will have little effect on the volume of lavage fluid recovered, the total number of cells obtained, or the types of cells present (Veninga and Evelyn 1986; Hotchkiss et al. 1989). However, morphological and functional evaluations of cells in the lavage fluid, such as the macrophages, show marked differences in properties such as structure (Dormans et al. 1990), adherence (Veninga and Evelyn 1986), free radical production (Amoruso et al. 1981; Ryer-Powder et al. 1988; Esterline et al. 1989), phagocytosis (Van Loveren et al. 1988), and metabolism (Mochitate and Miura 1989) between cells isolated from ozone-exposed animals and those exposed to air.

At later time points after an acute exposure to ozone, or immediately after a multiday exposure, there is a marked accumulation of neutrophils in the bronchoalveolar lavage fluid of rats (Bassett et al. 1988; Esterline et al. 1989; Hotchkiss et al. 1989; Mochitate and Miura 1989), mice (Kleeberger et al. 1990), and rabbits (Driscoll et al. 1987). The exact point at which the neutrophils infiltrate the lower airways has not been established, but it appears to depend on the exposure protocol and the animal species. For example, Hotchkiss and coworkers (1989) reported no neutrophils in the lavage fluid of Fischer-344 rats exposed to 0.12 ppm ozone for six hours when lavage samples were taken at intervals from 0 to 66 hours after exposure. Neutrophils infiltrated rapidly, however, after exposure to higher doses of ozone; they were found 42 hours after exposure to 0.8 ppm ozone and three hours after exposure to 1.5 ppm ozone. Thus, the bronchoalveolar lavage samples obtained immediately after an acute exposure to low or moderate levels of ozone (less than 1 ppm) appear to consist predominantly of resident alveolar macrophages. Infiltrating neutrophils populate samples taken at later time points or within a few hours after exposure to moderate to high levels of ozone (greater than 1 ppm).

Just as the profile of inflammatory cells in the alveolar spaces after acute ozone exposure is affected by dose and time, the spectrum of oxygen metabolites released by the infiltrating inflammatory cells also changes over time. Two hours after exposure of Sprague-Dawley rats to high levels of ozone (2 ppm), the production of superoxide anion by bronchoalveolar cells stimulated by 12-*O*-tetradecanoylphorbol-13-acetate was inhibited and remained inhibited for three days (Esterline et al. 1989). One day after exposure, when the neutrophil influx was at its peak, peroxidase-mediated reactions were at their highest levels.

Because of the well-documented link between oxidant exposure and reduced resistance to microbial infections (Pennington 1988), and because the pulmonary alveolar macrophage is the primary mediator of lung bactericidal activity, the effect of ozone on this cell population has been examined extensively. In most studies, investigators have found that in vivo exposure to moderate or low concentrations of ozone (less than 1 ppm) reduces the phagocytic activity and killing function of cells in the lavage fluid samples of rabbits (Coffin et al. 1968; Driscoll et al. 1987) and rats (Van Loveren et al. 1988). However, one study reported that ozone exposure did not affect the phagocytic activity of mouse alveolar macrophages (Ryer-Powder et al. 1988), and another study even reported enhanced phagocytosis in rat pulmonary macrophages (Christman and Schwartz 1982). The conflicting results may be due to differences in exposure conditions or differences in the methodology used to isolate the inflammatory cells and evaluate phagocytosis.

Examination of the ability of phagocytic cells in the lavage fluid to produce reactive oxygen species showed that brief exposures (three to four hours) to relatively high concentrations of ozone (1 to 3 ppm) inhibited superoxide anion production in bronchoalveolar lavage cells isolated from rats (Amoruso et al. 1981; Esterline et al. 1989). There appear to be marked species differences in the sensitivity of rodent macrophages to ozone. Although exposure to ozone caused a dose-dependent decrease in superoxide production by alveolar macrophages isolated from both Swiss Webster mice and Sprague-Dawley rats, this effect occurred in mice at a much lower level of ozone than in rats (Ryer-Powder et al. 1988).

Recently, investigators have come to appreciate the marked difference in the ozone-induced inflammatory response among different animal species and even among different strains from the same species. Slade and coworkers (1991) reported that the ozone-induced neutrophil influx was greater in the bronchoalveolar lavage fluid of Sprague-Dawley rats than in the Fischer-344 rat strain. These rat strain differences in the ozone-induced inflammatory response are consistent with the results of a screen of nine inbred strains of mice, showing interstrain differences in the airway inflammation that developed six hours after a brief exposure to 2 ppm ozone (Kleeberger et al. 1990).

OZONE AS A CARCINOGEN OR COCARCINOGEN

Whether or not there is a relationship between exposure to air pollutants, such as ozone, and lung cancer is an important question, and one for which there is, as yet, no definitive answer (Witschi 1988, 1991). Ozone is a highly reactive molecule and theoretically could affect the development of lung tumors by either genotoxic or nongenotoxic mechanisms.

Genotoxicity and Carcinogenicity

Studies of the effects of ozone exposure on isolated cells and tissues provide limited evidence that cellular genetic material may be at risk of attack by oxidizing air pollutants (Zelac et al. 1971a,b; Merz et al. 1975; Tice et al. 1978; Guerrero et al. 1979; Rasmussen 1986). Recently, Rithidech and coworkers (1990) observed chromatid gaps and chromatid deletions in pulmonary alveolar macrophages isolated from ozone-exposed rats. Brief in vitro exposure of mammalian cells to relatively high concentrations of ozone resulted in the transformation of primary hamster embryo cells and mouse fibroblasts to a neoplastic phenotype (Borek et al. 1986), but cultured rat tracheal epithelial cells were not affected by single 40-minute exposures to either 1 or 10 ppm ozone (Thomassen et al. 1991). However, multiple exposures to 0.7 ppm ozone (over a period of five weeks) increased the frequency of transformation of the tracheal epithelial cells approximately twofold. When tracheal epithelial cells isolated from rats exposed in vivo to ozone (0.14 to 1.2 ppm, six hours/day, five days/week for one, two, or four weeks) were placed in tissue culture, they showed no increase in the frequency of preneoplastic transformation when compared with cells isolated from unexposed rats. The latter experiment illustrates a basic paradox in studies of ozone carcinogenicity. Although the findings of in vitro experiments are consistent with the hypothesis that ozone is genotoxic, the results of in vivo studies, especially those conducted at environmentally relevant concentrations of ozone, are largely negative.

Animal Carcinogenesis

There are early reports of neoplasia and inflammation in laboratory animals exposed to pollutant mixtures containing relatively high concentrations of ozone (estimated to be 1 to 4 ppm) generated from artificial smog (Kotin and Falk 1956; Kotin et al. 1958; Nettesheim et al. 1975) or air (Werthamer et al. 1970). Two recent studies in laboratory animals have more direct relevance to the issue of ozone carcinogenicity. In two independent studies, A/J mice, a strain with a high spontaneous incidence of pulmonary adenomas, were exposed to ozone (0.4 to 0.8 ppm) for four to six months; this exposure resulted in a statistically significant increase in tumor incidence and tumor frequency (Hassett et al. 1985; Last et al. 1987). However, ozone had no effect on lung tumor development in Swiss-Webster mice, a strain that does not display a high incidence of spontaneous lung tumors (Last et al. 1987). Interpreting the A/J strain results is difficult, not only because the positive effect was observed in a strain of mice known to be highly susceptible to the development of lung tumors but also because in the two experiments in which a positive effect of ozone was reported the abnormally low baseline values for tumors in the control animals may have contributed to the positive finding.

Multistage Carcinogenesis

During the past decades, a number of scientific findings have led to the concept that carcinogenesis is a multistage process involving at least three different stages: initiation, promotion, and progression (Weinstein 1988; Weinberg 1989). Genotoxic events include critical mutations and alterations in regulatory genes, such as the activation of protooncogenes or the inactivation of tumor suppressor genes. There are, however, nongenotoxic carcinogens that do not react with DNA, but can act as tumor promoters or result in the production of tumors when administered at high

doses (Butterworth 1990). Although some chemicals can be classified easily as either genotoxic or nongenotoxic, the distinction is less clear for other chemicals. For example, some agents induce mutations at both low and high doses, but also enhance cell proliferation at high doses. Ozone is a highly reactive molecule with the potential for both genotoxic and nongenotoxic activity, especially in the lungs, which are the primary target of injury (Kehrer et al. 1988; Weitzman and Gordon 1990; Berger 1991).

When ozone reacts with tissue, it can initiate a cascade of free radicals (molecular species that contain one or more unpaired electrons) that can damage DNA in a number of ways: by reacting with unsaturated hydrocarbons in cell membranes to form reactive intermediates that decompose to yield free radicals, by initiating reactions in aqueous environments to produce the extremely reactive hydroxyl radical, or by causing cellular injury and subsequent recruitment of phagocytic cells that produce free radicals when activated (Van der Zee et al. 1987; Pryor 1991). There are a number of mechanisms by which free radicals can alter genes, including the production of mutations, DNA strand breaks, chromosomal alterations, and the induction of transformation in cell lines. Thus, the intracellular or extracellular production of these reactive oxygen species may be important in producing some types of cancers (Marnett et al. 1987; Weitzman and Gordon 1990). There is also strong evidence from investigations in the mouse skin cocarcinogenesis model (Slaga et al. 1981) and studies of neoplastic transformation in cell lines (Nakamura et al. 1985) that oxygen free radicals act as tumor promoters (Goldstein et al. 1983; Floyd 1990).

Recently, there has been renewed interest in how nongenotoxic mechanisms, such as cell proliferation, contribute to the promotion and progression stages of carcinogenesis. Cell replication or mitogenesis is necessary for the fixation of mutagenic events. Dividing cells then may be at greater risk of mutation than quiescent cells. Therefore, stimulation of cell division may increase the probability of genetic damage and the conversion of a cell to a neoplastic phenotype (Ames and Gold 1990a,b; Cohen and Ellwein 1990; Preston-Martin et al. 1990). Ozone has biological properties similar to a number of chemical and physical agents that enhance cell proliferation and are risk factors for carcinogenesis (Ames and Gold 1990a; Butterworth 1990; Preston-Martin 1990).

As the preceding discussion indicates, ozone has the potential to contribute to carcinogenesis by more than one route. It might inflict direct damage on the respiratory epithelial cells by means of ozone-mediated free-radical reactions; it also might initiate compensatory cell proliferation, thereby increasing the rate of epithelial cell replication. In addition, ozone might initiate the same genotoxic or nongenotoxic events indirectly by recruiting inflammatory cells that release free radicals and other mediators into the airways. The latter mechanism is the one under investigation in the current study.

JUSTIFICATION FOR THE STUDY

The HEI solicited proposals under RFA 86-1 for studies that would provide information on the genotoxic, carcinogenic, or cocarcinogenic potential of ozone. The HEI was particularly interested in animal bioassays and studies related to mechanisms of carcinogenesis. In the latter area, the HEI Health Research Committee identified free radicals, inflammation, genotoxicity, injury repair, and immunosuppression as possible candidates for research.

Drs. Davis and Donaldson proposed to assess the ability of leukocytes derived from bronchoalveolar lavage fluid of ozone-exposed rats to injure pulmonary epithelial cells in vitro, and to examine the mechanism of this response. The investigators hypothesized that the pulmonary inflammatory response induced by exposure to ozone might play a role in carcinogenesis, either by mediating cytotoxic and subsequent hyperplastic events, or by increasing the permeability of epithelial cells to carcinogens.

In support of their application, the investigators cited their experience in previous studies of the role of leukocytes in lung diseases. They proposed using an experimental approach to study the effect of ozone on lung inflammation and epithelial cell injury similar to one they had previously used to evaluate the pathogenesis of mineral dust exposure in laboratory animals. Exposing rats to mineral dusts elicits an intense pulmonary inflammatory response that is characterized by an increase in neutrophils in the bronchoalveolar lavage fluid (Donaldson et al. 1988a). When the investigators added bronchoalveolar lavage samples from dust-exposed animals to an alveolar epithelial cell line in vitro, the epithelial cells detached from their support (Donaldson et al. 1988c) and degraded a radiolabeled fibronectin matrix (Brown and Donaldson 1988, 1989). The investigators speculated that proteases and superoxide anion may have played a role in mediating the epithelial detachment injury (Donaldson et al. 1988b,c). As a basis for their proposal to HEI, they speculated further that the pulmonary leukocytes elicited by exposure to ozone could release mediators that would be capable of damaging epithelial cells and thus could contribute, either directly or indirectly, to lung tumorigenesis.

Given what was known about the ability of ozone to cause inflammation and the capacity of phagocytic cells to re-

lease free radicals and other cytotoxic factors, the proposed approach was a reasonable one. Two other researchers funded by the HEI under RFA 86-1 (Dr. Carmia Borek and Dr. David Thomassen) focused on the direct genotoxic potential of ozone by examining the ability of ozone to induce malignant transformation of target cells. The three studies, taken together, were expected to provide information about the carcinogenic or cocarcinogenic potential of ozone. Also, it was assumed that any positive findings would provide information about whether ozone exerts its effect directly on the epithelial cells of the respiratory tract or indirectly through leukocyte-mediated events.

OBJECTIVES

The investigators' overall objective was to determine whether leukocytes recruited into ozone-exposed lungs can cause epithelial injury that could contribute to tissue damage and perhaps to carcinogenesis. Their immediate goal was to examine the effects of exposure to ozone on the morphological and functional properties of rat bronchoalveolar leukocytes. (Although the term leukocytes encompasses lymphocytes, neutrophils, and macrophages, only the latter two types of white cells comprised the population of white cells under investigation.) This study did not directly examine the carcinogenic or cocarcinogenic activity of ozone.

SPECIFIC AIMS AND STUDY DESIGN

The specific aim of this study was to assess the ability of the bronchoalveolar leukocytes from PVG rats exposed to either air or ozone to elicit epithelial injury by using a series of in vitro measurements on cells lavaged (washed out) from the animals' lungs. In vitro assays included an epithelial detachment assay that measured the ability of the isolated leukocytes to detach alveolar epithelial cells from a solid support, and selected assays of leukocyte secretory and effector functions.

The study design was as follows: (1) set up an in vivo ozone exposure and monitoring system; (2) expose PVG rats to 0, 0.2, 0.4, 0.6, and 0.8 ppm ozone for seven hours/day, for one, two, or four days; (3) perform bronchoalveolar lavage the morning after the last exposure to assess total number of leukocytes; differential counts (composition of the cells in the lavage fluid); ability of the isolated leukocytes to cause alveolar epithelial cell lysis and detachment (in vitro injury assays); production of superoxide anion and hydrogen peroxide; and ability to break down fibronectin, a component of the extracellular matrix, in vitro (fibronectin proteolysis assay); (4) measure the size of the macrophages (0.8 ppm ozone only); and (5) examine the histopathological effects of exposure to 0.8 ppm ozone (seven hours/day) for one, two, four, or eight days, or to 0.2 or 0.6 ppm ozone (seven hours/day) for one or two days. (This last procedure was added primarily to provide in vivo confirmation of ozone-induced inflammation.)

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The objectives of this study generally were met. The investigators were successful in developing a facility in which they exposed PVG strain rats to specified ozone levels and durations and measured the parameters described above. The results of the study clearly are limited to the ozone dose schedule, the animal strain, the specific time period after ozone exposure chosen for examination of the various endpoints, and the conditions selected for the in vitro assays.

ASSESSMENT OF METHODS AND STUDY DESIGN

The investigators did an excellent job of developing a reliable ozone exposure facility specifically for this project. They overcame difficulties regarding appropriate placement of the ozone sensor and problems with the plastic caging. Proper attention was directed toward the inevitable problems associated with monitoring ozone, including the requirements for careful testing and calibration of both the ozone generator and analyzer, as well as continuous monitoring of ozone concentrations in the exposure chamber.

The study design is reasonably straightforward, although more attention to the timing of the bronchoalveolar lavage would have been valuable. The investigators chose 15 ozone exposure conditions (0, 0.2, 0.4, 0.6, or 0.8 ppm ozone, seven hours/day, for one, two, or four days), but only one time point (approximately 16 hours) after the cessation of the last exposure for performing the bronchoalveolar lavage. The evolution of lung pathology follows a specific time course after ozone exposure; it is possible that direct ozone toxicity to epithelial cells or extracellular matrix may occur at a different time period than toxicity caused by inflammatory leukocytes. Therefore, a study design with fewer alternative exposures but additional sampling times for bronchoalveolar lavage might have allowed more confidence in the findings. For example, in keeping with the investigators' hypothesis, it is possible that the inflammatory leukocytes recruited into the airways during seven hours of exposure, as well as those activated by the inhaled ozone, do release substances that damage lung epithelial cells. However, dur-

ing the approximately 16 hours between cessation of the ozone exposure and performance of the bronchoalveolar lavage, such activated leukocytes might die or be significantly diluted by a wave of leukocytes recruited after cessation of the exposure. Furthermore, leukocytes entering the airways and alveolar spaces after cessation of ozone exposure might have characteristics different from those subjected to ozone exposure. The choice of only one time point after ozone exposure for obtaining samples of bronchoalveolar lavage leukocytes is a limitation of this experimental design.

The sensitivity of the in vitro assays to detect cytotoxicity and mediator release also may limit the interpretation of the findings. These assays were conducted using mixed populations of cells, predominantly macrophages, from the bronchoalveolar lavage fluid. The investigators took advantage of assays that they had used previously to monitor neutrophil-mediated epithelial injury following exposure to mineral dusts. If neutrophils are mediating injury in the ozone model, they may not be present in sufficiently high numbers to produce detectable levels of mediators or to effect epithelial cell injury. Alternatively, if ozone-induced injury to respiratory epithelial cells is mediated by alveolar or interstitial macrophages, different assay conditions, longer incubation times, or higher effector:target ratios may be required to produce observable cytotoxic effects.

The investigators used the epithelial detachment assay and the cell lysis assay to examine the isolated leukocytes' ability to damage epithelial cells. In both assays the A549 tumor cell line was used as a surrogate for alveolar epithelial cells. The relevance of the A549 cell line to alveolar cells is questionable because this cell line does not express many of the known specific functions of type II cells, such as phospholipid composition and ultrastructure (Mason and Williams 1980). Preparations of type II cells are readily grown in culture and would have been preferable for the cytotoxicity assays.

The experimental design is also limited because the investigators did not examine the direct effect of ozone on alveolar epithelial cells or the combined effect of ozone-induced macrophage activation and direct ozone damage. Exposing the test cells and extracellular matrix material to ozone would have been desirable. However, this was beyond the scope of the project because such studies would have required the design and construction of a specialized in vitro exposure apparatus.

STATISTICAL METHODS

The statistical design, presented clearly in Table 2 of the Investigators' Report, is a strength of this study. Exposures were conducted in a systematic, controlled scheme that varied ozone concentration and duration. A single pool of control animals was drawn upon for comparison with each exposed group. A small number of well-defined endpoints were measured and analyzed, one by one, for effects of ozone.

A thorough, consistent procedure for data analysis was applied within the context of a statistical model that correctly describes the experiment as conducted. For each endpoint, a coordinated set of analyses was displayed in a uniform, tripartite format. This integrated, efficient presentation allows the reader to examine whichever rendering of the data is most easily comprehensible and to reconcile the data with the authors' accompanying interpretation. The results for each endpoint are presented in a set of three tables that include (1) descriptive statistics, which permit the reader to examine real data; (2) analysis of variance (ANOVA) tables, which succinctly and definitively address the major hypotheses; and (3) a set of quantitative contrasts between control values and various levels of exposure, which are marked where statistically significant.

The ANOVA is somewhat unconventional for the analysis of these data because the control animals were not given a sham exposure. The data analysis reflects the fact that the control animals remained in their original housing and shared only a common date of lavage and assay with their experimental counterparts. The authors partitioned the degrees of freedom (df) in a manner appropriate for a one-factor ANOVA with 13 experimental groups: one group of control animals and 12 groups exposed to various levels of ozone for various durations. In this design, all control animals were treated as equivalent; distinctions regarding exposure dose and duration apply only to the treated animals.

Some animals were used as controls for two different exposure groups. The appropriate use of control values, given this design, is somewhat tricky. The footnotes to Table 7 of the Investigators' Report give the full details of how the double-duty controls were treated, emphasizing that the sample size was 68 (not 72) in all analyses. Also, although the control values in question are displayed twice in the raw data tables, they were used only once in the ANOVA and construction of contrasts, as is appropriate.

The investigators used logistic transformation, which gives the data better statistical properties but may be relatively unfamiliar to many scientists. The logistic formula and the concept of odds are presented lucidly in the footnotes to Table 12. The mechanics of re-transforming the estimates and confidence intervals, following statistical analysis of the logistic values, are also well explained.

The authors' coordinated data tables, ANOVA tables, and contrast tables generally give the same results. For example,

Table 20 shows a statistically significant effect of ozone concentration on the production of superoxide anion ($F = 2.92$, $p < 0.05$), and Table 21 shows 0.6 ppm ozone acting differently from 0.2, 0.4, and 0.8 ppm. In some cases, however, the reader may come away confused by apparent inconsistencies. The following discussion of how these tables were constructed may help readers who are not familiar with the statistical procedures use the data effectively. Tables 11 and 12 are examples. Table 11 shows no statistically significant effect of ozone concentration on the percentage of macrophages in bronchoalveolar lavage leukocyte population ($F = 0.35$, not significant). Yet Table 12, with confidence intervals derived mathematically from the same ANOVA, shows an apparent dose response: footnote symbols (d) indicating statistical significance are attached to higher-dose ozone (0.6 to 0.8 ppm) but not to lower-dose ozone (0.2 to 0.4 ppm). Which table is telling the truth?

The reason for these discrepancies is benign, and relates to the particular way the authors formulated their ANOVA and constructed their contrasts, rather than any flaw or true inconsistency in their analysis. The remainder of this section will be devoted to explaining this issue, so that the reader will be better able to read Tables 7 through 33 accurately.

A conventional ANOVA would divide the major sources of variation as shown in Table 1 of this Commentary. This would be the natural way to express the design if the control animals were given a sham exposure (for example, four days in an exposure chamber with no ozone). The conventional ANOVA would test the following two main-effects hypotheses independently:

Does y(0 ppm) = y(0.2 ppm) = y(0.4 ppm) = y(0.6 ppm) = y(0.8 ppm)?

Does y(1 day) = y(2 days) = y(4 days)?

where y is a generic endpoint.

Instead of the conventional ANOVA, the authors split off a single indicator variable representing the presence or absence of ozone (treatment), irrespective of dose or duration.

Table 1. Conventional Two-Factor Analysis of Variance

Source of Variation	Levels	Degrees of Freedom
Ozone concentration	0 ppm, 0.2 ppm, 0.4 ppm, 0.6 ppm, 0.8 ppm	4
Duration of exposure	1 day, 2 days, 4 days	2
Interaction		8

As indicated above, this formulation reflects the absence of any sham exposure. In this case, the controls form a homogeneous group, distinguished by their pairing with certain laboratory animals but not by any common period of residence in an exposure chamber. The treatment variable (1 df) is supplemented with two quasi-main effects, concentration and duration, which vary nominally within treatment, but in fact varied only within the exposed group (Table 2 of the Commentary). The three hypothesis tests that result from this partitioning of df are:

Does y(ozone) = y(control)?

Does y(0.2 ppm) = y(0.4 ppm) = y(0.6 ppm) = y(0.8 ppm)?

Does y(1 day) = y(2 days) = y(4 days)?

This analysis begins with a crude but highly pertinent question: Did ozone have any effect at all? The finer question of dose response is reserved for the latter two tests, which ask whether various nonzero dose levels differ from one another and not whether they differ from control values.

The table of contrasts, which follows each ANOVA table, addresses a different set of hypotheses, even though it is mathematically part of the same statistical analysis that produced the ANOVA. The authors have tabulated the set of quantities shown in Table 3 of the Commentary. Each contrast is individually noted if it is significantly different from zero. The tests reported in this table ask whether various nonzero dose levels differ from control values, as opposed to differing from each other, as tested in the ANOVA.

The apparent discrepancy between Table 11 and Table 12 of the Investigators' Report can be explained on this basis. Table 11 shows a significant overall difference between control and ozone exposures, but no difference among concentrations. Table 12 suggests that the overall difference is attributable to the 0.6 ppm and 0.8 ppm responses. The insignificant ANOVA test for difference among concentrations might be interpreted as showing that, should the random variation happen to go the other way, the 0.2 and 0.4 ppm exposures could be responsible for the overall ozone effect. Biologically, however, the higher concentrations are more likely to produce an effect, and the attribution of cause to 0.6 and 0.8 ppm is plausible.

RESULTS AND INTERPRETATION

Ozone exposure, especially at the two higher concentrations (0.6 to 0.8 ppm), induced an inflammatory response in PVG rats, as evidenced by the histological lesions and the increased number of neutrophils in the bronchoalveolar lavage fluid. However, the investigators found no evidence

Table 2. Analysis of Variance as Constructed by the Investigators

Source of Variation	Symbol	Levels	Degrees of Freedom
Presence of ozone	TREAT	Yes/no	1
Ozone concentration	TREAT.CONC	0.2 ppm, 0.4 ppm, 0.6 ppm, 0.8 ppm	3
Duration of exposure	TREAT.DAY	1 day, 2 days, 4 days	2
Interaction	TREAT.DAY.CONC		6

Table 3. Contrasts Reported by the Investigators

y(control)	y(0.2 ppm) – y(control)	y(0.4 ppm) – y(control)	y(0.6 ppm) – y(control)	y(0.8 ppm) – y(control)
y(control)	y(1 day) – y(control)	y(2 days) – y(control)	y(4 days) – y(control)	

that the leukocytes obtained from PVG rats exposed to ozone were able to injure lung epithelial cells or extracellular matrix components in vitro.

The histopathological studies support other published reports of ozone-induced inflammation, including the focus of ozone toxicity in the terminal and respiratory bronchioles and damage to the Clara cells lining the small airways. There was a patchy distribution of lesions in the lungs of rats exposed to ozone at all dose levels. The authors point out the apparent discrepancy between the histological evidence of increased lung inflammation and the lack of increase in the total number of inflammatory cells in the lung lavage fluid. The investigators suggest that the patchy nature of lung lesions, with many parts of the lung appearing normal, might lead to no detectable increase in lavage leukocyte number. They also suggest that the leukocytes elicited by ozone exposure may be particularly adherent and thus difficult to lavage. Other researchers, using different rat strains, have observed increases in the number of inflammatory cells in the bronchoalveolar lavage fluid from rats exposed to the same range of ozone concentrations (Bassett et al. 1988; Esterline et al. 1989; Hotchkiss et al. 1989; Mochitate and Miura 1989).

In the present study, Drs. Donaldson, Davis, and coworkers found that ozone exposure did not affect the ability of the lavaged leukocytes to degrade fibronectin or injure epithelial cells. Ozone exposure also had no effect on the nonstimulated production of superoxide anion by the lavaged leukocytes; production of superoxide anion by cells stimulated with phorbolmyristate acetate (PMA) was decreased in leukocytes collected from rats exposed to 0.6 ppm ozone. A number of factors may have contributed to the essentially negative functional findings:

- The assays were carried out on crude mixtures of cells that contained only a small fraction of neutrophils. Although unlikely, it is possible that the selected assays may not have been sufficiently sensitive to detect neutrophil activity, or the activity may have been inhibited by mediators produced by the macrophages.
- In vitro assay variables, such as choice of target cells, labeling procedures, effector:target ratios, and kinetics were not examined in a fully systematic manner. Different results might have been obtained if different concentrations of bronchoalveolar lavage cells, different incubation times, or different assays had been selected.
- Mediators produced in vivo may have been diluted by the lavage procedures.
- The failure to observe a fully dose-responsive effect on superoxide anion production may reflect some inherent variability in this assay, particularly because the production of superoxide anion by resting cells and by PMA-stimulated cells from animals chosen as controls for the 0.8 ppm ozone study was aberrantly low compared with other control animals.
- The PVG rat may not be highly sensitive to ozone toxicity. Little is known about the response of this strain to ozone; most investigators have used the Sprague-Dawley, Fischer-344, or Wistar rat strains for ozone toxicity studies.
- As discussed earlier, bronchoalveolar leukocytes obtained at different intervals after the cessation of ozone exposure might have shown different results.

The histopathology findings suggesting that exposure to 0.8 ppm ozone leads to activation of lung macrophages are

of interest. There was a statistically significant increase in leukocyte size, and many macrophages showed microscopic changes consistent with activation.

As pointed out by the investigators, and as with any study in which the findings are negative, the results are directly pertinent only to the assays and time points studied. The findings and interpretations might have differed if other time points for lavage had been chosen, or if different methods had been selected to evaluate the effector status of bronchoalveolar lavage leukocytes. However, the results of this study are consistent with the hypothesis that ozone, or an ozone-derived reactive species, injures pulmonary epithelial cells directly, rather than indirectly through leukocyte-mediated events.

IMPLICATIONS FOR FUTURE RESEARCH

The role of leukocytes in ozone-induced effects on lung epithelial cells or extracellular matrix remains uncertain. The results of this study suggest that it is more likely that there is a direct toxic effect of ozone or ozone-derived oxidizing species on lung epithelial cells or extracellular matrix rather than macrophages or neutrophils contributing to ozone toxicity. However, additional research is needed before leukocyte-mediated effects can be ruled out. The following variables should be considered in designing future studies: sensitivity of the animal model to ozone toxicity, timing of the bronchoalveolar lavage, appropriate assays of leukocyte-mediated toxicity, separation of macrophages from neutrophils, and quantitative testing of both cell types.

An additional factor that needs to be considered is the possibility that a direct effect of ozone on lung epithelial cells or extracellular matrix could alter their susceptibility to cytotoxic factors released by macrophages. In order to test this hypothesis, cells or substrates that have been previously exposed to ozone would be required. Improved approaches for performing in vitro ozone exposures that more closely mimic in vivo inhalation would be of value in further studies of the effect of ozone on normal epithelial cells or studies of the ozone-induced potentiation of macrophage effects.

CONCLUSIONS

This study addressed an important issue in ozone toxicology: do the leukocytes that collect in the airways after exposure to ozone cause injury to the epithelial cells and structural material of the lungs and thereby contribute to lung disease? The investigators studied leukocytes obtained by bronchoalveolar lavage after exposure of PVG rats to either air or ozone. The ozone exposures were conducted under rigorously controlled conditions according to a protocol that varied ozone concentration (0.2 to 0.8 ppm) and duration (seven hours per day for one to four days). The investigators measured a number of well-defined end points and their careful and consistent procedures for data analysis are a strength of the study. The major limitation is that the effects were measured on a mixed population of leukocytes collected at only one time point (approximately 16 hours) after exposure. Different results might have been obtained if the leukocytes had been purified or collected at different time points.

As in other studies using different animal species and different rat strains, the investigators found that exposure of PVG rats to ozone produced a patchy inflammatory response in the centriacinar region of the lungs. The percentage of neutrophils in the bronchoalveolar lavage fluid increased in ozone-exposed animals, an effect that was most pronounced one and two days after exposure to 0.8 ppm ozone. There was, however, no evidence that the leukocytes in bronchoalveolar lavage fluid of ozone-exposed rats differed from leukocytes of animals exposed to air in their ability to damage A549 tumor cells (which served as a surrogate for lung epithelial cells), to degrade fibronectin, or to produce free radicals. These findings are directly pertinent only to the particular experimental protocol under investigation. Different assays or time points, as well as different animal species or exposure regimens, conceivably could produce different results. Such studies would provide better information about the mechanism by which ozone injures the lungs and the significance of inflammation and epithelial cell injury in the development of lung diseases such as emphysema, fibrosis, and cancer.

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Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research (Supplement)	January 1988

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