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EFFECT OF OZONE ON MEAN LINEAR INTERCEPT IN THE LUNG OF YOUNG BEAGLES

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Although photochemical air pollutants are believed to be associated with respiratory illness, there is also a need to consider their possible effects on postnatal lung maturation. The purpose of this study was to determine whether the maturation of lungs of young beagle dogs might be altered by an inhalation exposure to ozone that represents a severe 5-d episode of photochemical oxidant air pollution. Exposures were at 6 wk of age to purified air, 1 or 2 ppm ozone for 4 h/d on 5 consecutive days. After holding for 6 wk in clean air, lungs were removed and weighed, and the left lung was fixed both by inflation at 30 cm pressure and immersion using buffered formalin. Histologic sections were used for morphometric measurements. Statistical analysis showed that the mean linear intercept (inversely related to lung surface area) was greater than controls (up to about 5%) in the 1 ppm ozone-exposed group. This effect was not seen at 2 ppm ozone, apparently due to large variations in mean linear intercept. No significant differences were seen in body weight, chest girth, lung weight, or volumes of the fixed, inflated lungs. It is concluded that if anatomic maturation of the lung was retarded by this brief regimen of ozone exposure, the effect was small and not likely to have major health consequences.

INTRODUCTION

Although birth and the transition to air breathing are associated with major physiologic and anatomic changes in the mammalian cardiopulmonary system, subsequent events in the differentiation and de-

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velopment of the lung may be equally significant to normal lung function in adult life. The pattern of postnatal lung development is similar in humans, dogs, rats, mice, cats, and rabbits (Thurlbeck, 1975; Boyden and Tompsett, 1965; Boyden, 1977; Hislop and Reid, 1974; Dunnill, 1962; Emery, 1969; Burri et al., 1974; Crocker et al., 1970). Although most investigators conclude that the full number of tubular airways are present at birth, there is a significant increase in the number of alveoli throughout the period of early maturation after birth (Kerr et al., 1975; Thurlbeck, 1977; Reid, 1977; Jeffery and Reid, 1977; Burri and Weibel, 1977; Brody and Vaccaro, 1979).

In humans the number of alveoli present at birth, estimated at about 20–70 million, increases over about the first 5–10 years of life to an adult complement of about 200–500 million (Thurlbeck, 1975; Reid, 1977); the rate of increase in number steadily decreases with age after birth. Great variability in the number of alveoli is seen at all ages, presumably due to genetic and environmental factors superimposed on differences in counting techniques as well as the difficulty in identifying immature forms of alveoli. The sites of alveolar development, as elucidated in human and laboratory animal studies, include the distal bronchioles (Thurlbeck, 1975, 1977), where saccular protrusions in the bronchiolar wall, which is initially covered by cuboidal epithelial lining cells, are eventually replaced by thin alveolar epithelium (Boyden and Tompsett, 1961). The process of formation of alveoli appears to proceed along these airways in a direction from the distal airways toward more proximal ones.

The question of possible interference with this sequence of alveolar development by environmental, disease, or toxic factors has been addressed (Thurlbeck, 1975, 1977; Reid, 1977; Burri and Weibel, 1977; Taeusch and Avery, 1977; Emery, 1970). In summary, development of a full complement of alveoli may be disturbed by early infection, congenital diaphragmatic hernia, childhood onset kyphoscoliosis (spinal curvature), hyperoxia, and possibly hypophysectomy. Few studies have been conducted on the potential effects of common airborne pollutants on postnatal lung development. Bartlett et al. (1974) exposed initially 3- to 4-wk-old rats to about 0.2 ppm ozone for 30 d. Because the rat lung may be largely past the period of formation of new alveoli at age 3–4 w, the study did not clearly address the effect of ozone on lung parenchymal development. In fact, after the exposure no differences between exposed and control rats were found with respect to body weight, wet or dry lung weights, and alveolar number. The ozone-exposed group did have greater average lung volumes as measured in pressure–volume inflation and deflation experiments using both air and saline. Accordingly, the decrease in lung elasticity was associated with greater mean linear intercept values for the ozone-exposed group. The authors point out that other exposure schedules and other

levels of ozone have been seen by others to increase rather than decrease lung elasticity, indicating that in any experiment in which mean linear intercept is an endpoint, lung volumes should be carefully documented.

Freeman et al. (1972) reported that lifetime continuous 10–20 ppm NO_2 exposure of rats initially 1 mo old led to loss of alveoli and alveolar surface. Greater volumes of fixative were required to fill the NO_2-exposed lungs to 25 cm pressure. However, the effects of direct destruction of already formed alveolar tissue by this gas obscured any potential interference with the sequence of alveolar development. In a subsequent study, Freeman et al. (1974) looked at the effects of continuous exposure to 10 and 15 ppm NO_2. The animals were born in the exposure atmosphere. After 62 d exposure to 10 ppm NO_2, body weight and length were diminished (poor nutrition and maternal care may have influenced this result) but no significant difference in "alveolation" was found. Exposure to 15 ppm NO_2 produced a significant "temporary lag, or delay in pulmonary maturation" between exposure d 10 and 45; by d 75 no significant difference was found. Although the separate 10-ppm and 15-ppm experiments differed in several ways (numbers of rats, killing times, and quantitative assessment methods), it appeared that 15 ppm NO_2 produced a delay in lung maturation while 10 ppm did not.

Airborne oxidants such as ozone and nitrogen dioxide have marked effects on the transitional zone between terminal bronchioles and alveolar ducts as well as on alveolar septa, and thus can potentially interfere with postnatal lung development. Documentation of the effects of ozone on this distal airway region is abundant (Committee on Medical and Biologic Effects of Environmental Pollutants, National Research Council, 1977; Stern, 1977; Coffin and Stokinger, 1977). A study by Zitnik et al. (1978) illustrates the effects of relatively low levels of this gas. Mice were exposed to 0.5 ppm ozone continuously for up to 35 d. In a 7-d exposure group, minimal changes were seen in bronchiolar epithelium, but the "proximal alveoli of alveolar ducts contained accumulations of alveolar macrophages, and interalveolar septae were thickened by accumulations of mononuclear cells." It is clear that ozone exposure may damage the same anatomical region of the lung in which new alveoli are developing in the young mammal.

METHODS

Animals

Male and female purebred beagles were whelped and continuously housed in an indoor kennel supplied with purified (ozone-free) air. Beagle dogs were selected because alveolar development occurs over a

convenient period of a few months (Boyden and Tompsett, 1961; Anderson, 1970) and because we have experience in breeding, handling, and exposing this breed by inhalation. Each pup of 10 litters was randomly assigned to a sham-exposure or an ozone-exposure group. Exposures were conducted when the pups were weaned at 6 wk of age. Two dogs, 1 exposed to clean air and 1 to 1.0 ppm ozone, were sacrificed immediately after the 5-d exposure in order to examine the degree of lung injury at this concentration.

Experimental Procedure and Tissue Processing

Animals reared in purified ozone-free air until 6 w of age were randomly placed in chambers for exposure to either clean air (sham) or ozone (1 or 2 ppm) for 4 h. The exposure was repeated daily for 5 consecutive days. Before and between exposures and after the final one, animals were housed in their indoor kennel. At age 12 wk, animals were deeply anesthetized with sodium pentobarbital, subjected to a variety of body-size measurements, and killed by exsanguination. The lungs were removed and trimmed free of the heart, esophagus, major vessels, and lymphatic tissue and weighed. The left lung was ligated and separated distal to the tracheal bifurcation, weighed, cannulated with polyethylene tubing, and fixed by inflation of the airways with 10% neutral buffered formalin at a pressure of 30 cm of a column of fixative solution (Dungworth et al., 1976). In view of the critical importance of accurately knowing the lung volume at a known inflation pressure, a custom-design fixation system with precise pressure control was used (McClure et al., 1982). The inflated lung was also immersed in fixative during 72 h of fixation, after which time the cannula was clamped and the inflated lung remained immersed in the fixative for an additional 48 h. Following 5 d of fixation, the left-lung volumes were measured by the method of Scherle (1970), which involves weighing a beaker of water with and without a submerged supported lung and which was found to be more precise than simple liquid displacement methods. The left cardiac and diaphragmatic lobes were removed and transected along the lobar bronchus. The two halves were cut into eight pieces at equally spaced intervals. These tissues were processed for paraffin embedding and sectioned at 6 μm. Representative sections from each tissue were stained with hematoxylin and eosin.

Morphometry

The method of Weibel (1963b) for morphometric determination of the mean chord length in lung slices was used. In this method lines of known length L are superimposed on microscope fields of sections of the lung. The number of times (M) that alveolar septa cross these lines is recorded. The procedure is repeated N times (200 per dog) and the

mean chord length, or mean linear intercept, is calculated by the relationship

$$L_m = \frac{NL}{\Sigma M_i} \qquad i = 1\text{–}N$$

In order to eliminate possible bias due to compression during sectioning, two perpendicular lines (1 mm each in length) were used for counting septal crossings. If the lung had been randomly sampled and the volume of the total respiratory portion of the lung, V_L, known, the overall surface of the alveolar membrane would be given (approximately) by

$$S = \frac{4V_L}{L_m}$$

In our analysis the sections were taken from two lobes only and were selected from the same sites for each dog within those lobes. While random sampling from the entire lung was not done, with the result than an estimate of the total alveolar surface area of the lung could not be made, the careful selection of sections from similar anatomical sites in each lung maximized comparability between animals and decreased the statistical variance in the measured chord lengths. Additionally, complete random sampling of the entire lung would have greatly increased the morphometric effort.

Exposure Methods

Exposure of groups of dogs to clean air and to ozone were simultaneous, two adjacent chambers being used. Exposures were in stainless-steel exposure chambers of 1 m^3 volume. Exposure to 1 ppm ozone and matched shams were completed before the 2-ppm study was begun. Purified air of controlled humidity (41 ± 5% SD) and temperature (24 ± 0.7°C) was supplied at a rate of 0.3 or 0.6 m^3/min depending on the ozone concentration desired. Each dog was placed in an open-mesh wire stainless-steel cage for the 4-h exposure. Neither food nor water was provided during exposures. Animals were always exposed on a single tier of the chamber; a maximum of four animals was exposed at a given time. The activity level of each animal was recorded every 10 min using a scoring system (apparent sleep = 0, awake but lying down = 1, standing still = 2, and moving about = 3). Mean group activity scores were calculated from the observational data.

Atmosphere Generation and Characterization

Ozone was generated by metered passage of medical grade oxygen through an electrical ozone generator (Sander, Osterberg, West Germany). The concentration was measured continuously by a Dasibi (Dasibi Environmental Corp., Glendale, Calif.) ozone monitor by way of an

inert sampling line (Teflon, Dupont) at the center of the animal breathing zone. The monitor was calibrated using a factory calibrator both before and after each exposure. The calibration procedure was checked both at the Dasibi factory and at the University of California Air Monitoring Station (Department of Engineering) and was found to be in agreement with methods at both locations.

Data Analysis

Data for paired animals were statistically analyzed using the two-tailed *t*-test. Animals were paired for statistical analysis in order to control for sex, body size, and fixation batch. Each pair comprised littermates of the same sex, matched with respect to body weight as measured at sacrifice. The parameters measured were body weight at time of exposure, body weight at sacrifice, body length at sacrifice, chest diameter at sacrifice, weight of lungs with trachea at sacrifice, left lung volume after fixation, and mean linear intercept of lung parenchyma. In each case the value for the ozone-exposed dog was subtracted from that for the sham-exposed animal. The null hypothesis tested was that the mean difference between exposed and sham animals was not significantly different from zero, at a significance level of 0.05 or less. Rejection of the null hypothesis then implies a 95% probability that the pairs differed, with only a 5% chance that the null hypothesis was really true. Mean linear intercepts were also examined using a repeated measures analysis of variance. Because one sham-exposed outlier pair (number 12) had an unusually large difference in mean linear intercept (more than 3 SD from mean), the analysis of variance was repeated omitting its pair of values.

RESULTS

Exposure Levels

The mean exposure level for sham exposure was less than 0.01 ppm ozone, the detection limit for that gas. The corresponding mean values and standard deviations for ozone exposures were 1.0 ± 0.05 and 2.0 ± 0.04 ppm ozone.

Body and Gross Lung Characteristics

Table 1 shows the group means and standard deviations for sham- and ozone-exposed animals. The overall groups are not significantly different in any of the measured gross characteristics. The more sophisticated statistical analysis using paired same-sex littermates indicates that one parameter, body length, may be different, the 1 ppm ozone-exposed group being shorter by about 1.4 cm and the 2 ppm ozone-exposed group being longer by about 1.4 cm. This result is

TABLE 1. *Body and Gross Lung Characteristics for Dogs Exposed to Ozone or Clean Air (±SD)*[a]

Exposure	Body weight at exposure (kg)	Body weight at sacrifice (kg)	Body length at sacrifice (cm)	Chest diameter at sacrifice (cm)	Lung weight (g)	Left lung volume (cm^3)
1 ppm Ozone						
Shams	1.59 ± 0.53	4.69 ± 1.14	52.1 ± 2.8	35.8 ± 4.4	49.3 ± 10.9	105.6 ± 31.1
Exposed	1.53 ± 0.41	4.53 ± 0.98	50.8 ± 3.6	35.9 ± 2.8	45.6 ± 8.0	105.0 ± 17.8
Difference	0.06 ± 0.27	0.16 ± 0.81	1.40 ± 2.3	−0.1 ± 2.7	3.7 ± 7.5	0.6 ± 25.1
Significance (*p*)	NS	NS	0.1	NS	NS	NS
2 ppm Ozone						
Shams	1.82 ± 0.37	5.07 ± 0.83	51.9 ± 2.4	38.9 ± 2.8	55.0 ± 7.9	122.4 ± 27.5
Exposed	1.82 ± 0.29	5.35 ± 0.44	53.2 ± 1.5	39.4 ± 1.6	55.7 ± 3.3	117.7 ± 15.7
Difference	−0.01 ± 0.10	−0.28 ± 0.54	−1.4 ± 1.9	−0.6 ± 2.4	−0.6 ± 6.0	4.6 ± 14.6
Significance (*p*)	NS	NS	0.1	NS	NS	NS[b]

[a] The *p* values are for a two-tailed *t*-test using 0.1 as the significance level; $N = 7$ except as noted.
[b] $N = 4$, as 3 pairs of lungs were inadvertently sectioned for making slides prior to the planned volume measurements.

probably spurious, and in any case the effect is small. It is important to note that the lung weight and volume at fixation (30 cm fixative pressure head) are not significantly different in sham- and ozone-exposed groups using either group or paired analyses.

Gross and Microscopic Examination of Lungs

All examinations were performed blind in order to prevent bias. Lungs of the animals at sacrifice were normal upon gross examination, except for up to about a dozen 1-mm-diameter flat, erythematous spots per lobe on the pleural surfaces of several lungs. Seven sham-exposed and eight ozone-exposed lungs had these spots, which were apparently unrelated to ozone exposure. Histologically, these were areas of lymphocyte accumulations within thin-walled lymphatic vessels with no significant lymphocytic infiltration into adjacent respiratory tissues. In dogs sacrificed immediately after 5 d of exposure to 1 ppm ozone, histologic examination showed only thickening and heavier generalized staining of alveolar septal walls with increased cell numbers especially at septal junctions. Such thickening was not seen in animals sacrificed at age 12 wk, 6 wk post ozone exposure.

Mean Linear Intercept Measurements

Quantitative measurements on the lungs of sham-exposed and ozone-exposed groups (Table 2) identified a mean difference in the mean linear intercepts between paired littermates which was statistically significantly different from zero at the 95% confidence level using a two-tailed t-test for the 1 ppm ozone-exposed group ($p = < 0.02$) but not the 2 ppm ozone-exposed group.

Comparison of mean linear intercepts between paired sham- and ozone-exposed animals was made by pooling data from both levels of ozone exposure by a repeated measures analysis of variance. The paired difference between all sham-exposed and all ozone-exposed dogs was not significantly different from zero ($p = 0.25$). Moreover, the actual difference in mean linear intercept observed in the 1 ppm ozone experiment was larger than that in the 2 ppm ozone experiment. However, this analysis was strongly influenced by the presence of the outlier pair, data corresponding to pair number 12 (Table 2). Reanalysis with this matched pair being omitted indicated a difference between the sham and pooled ozone exposure for which $p = < 0.02$. In this reanalysis, similar paired differences were seen at the two concentrations of ozone exposure, but the effect at 2 ppm still lacked statistical significance ($p = 0.17$).

TABLE 2. Mean Linear Intercepts (L_m) of Clean Air-Exposed (Sham) and Ozone-Exposed Matched (Sex and Litter) Pairs of Dogs[a]

Pair number	Sex of pair	L_m (μm) (sham−ozone)	Difference in L_m (μm)
1 ppm Ozone			
1	M	63.22−68.61	−4.94
2	M	66.99−70.33	−3.34
3	M	63.14−70.43	−7.29
4	F	74.29−75.24	−0.95
5	F	63.59−68.18	−4.59
6	M	69.08−67.77	+1.31
7	M	67.84−72.07	−4.23
Mean difference			−3.43
SD			2.82
SE			1.07
t			3.21
p			0.02
2 ppm Ozone			
8	M	63.60−74.21	−10.61
9	M	63.04−65.89	−2.85
10	M	61.50−59.49	+2.01
11	M	61.85−59.91	+1.94
12	F	87.72−71.58	+16.14
13	M	75.56−86.51	−10.95
14	M	82.74−85.41	−2.40
Mean difference			−0.96
SD			9.20
SE			3.48
t			0.28
p			NS

[a] Cardiac and diaphragmatic lobes were measured in each dog and the data combined. The *p* given is for a two-tailed *t*-test.

DISCUSSION

The change in mean linear intercept seen in 1 ppm ozone-exposed animals was small but statistically significant. Although no attempt was made to randomly sample the entire lung, this difference implies a diminished lung surface area in the ozone-exposed group of about 4% when compared to their sham-exposed matched littermates. It is not likely that such a decrement would have been measurable functionally (as by CO diffusion). Neither is it likely that such a biological change would impair physiologic performance.

The absence of a significant effect of 2 ppm ozone exposure on

mean linear intercept is puzzling, especially in view of the high level of significance seen for the effect after exposure to 1 ppm. Among the possible explanations, two that can be addressed using our data are (1) that 2 ppm ozone produced responses such as decreased physical activity during exposure that led to protection of the lung tissue and (2) that changes in lung stiffness induced by the 2-ppm exposure led to underinflation of the lungs during fixation. As mentioned previously, behavior was recorded periodically for all dogs during each exposure. Although the group behavioral score for the sham-exposed group was highest (1.49 ± 0.58, SE), followed by the 1 ppm-exposed group (1.41 ± 0.60), followed by the 2 ppm-exposed group (1.38 ± 0.43), these differences were small and statistically insignificant. Furthermore, the ozone exposure did not lead to excess burying of noses in the animal's own fur.

In order to quantitatively estimate the influence of a change in fixed lung volume on mean linear intercept, Eq. (3.94) from Chapter III of Weibel's *Morphometry of the Human Lung* (1963a) was used. An assumption is similarity between alveolar shape in the human and dog lung. The constants in Weibel's equation were evaluated by substituting in our average experimental values for mean linear intercept and for lung volume for the shams for the 2-ppm ozone experiment. The resulting relationship between mean linear intercept and lung volume is

$$L_m \quad \text{(micrometers)} = 14.3(V_L)^{1/3} \quad (\text{cm}^3)$$

Using this equation, and the observed (not statistically significant) difference in lung volume between the 2 ppm ozone-exposed group and their shams, one calculates that the mean linear intercept of the exposed group could be increased by about 0.9 μm to correct for volume. If one takes the uncertainty of the lung volumes into account, one sees that a difference in lung volume probably did not obscure a significant effect of 2 ppm ozone on the measured mean linear intercept. (Even if 2 SE is added to the ozone exposed lung volumes, the effect on mean linear intercept, although in the direction for a significant effect, does not pass a *t*-test.) Thus, differences in inflation volume do not explain the lack of significant effect at 2 ppm. The finding remains unexplained.

The probable anatomical retardation in formation of new alveoli reported here was not accompanied by grossly or histologically observable defects in lung morphology at time of sacrifice (at 12 wk of age). This would tend to diminish the potential significance of the observed changes as evidence of a toxic hazard from exposure of young individuals to ozone on the schedule used in this study. On the other hand, a morphologic change in a developing structure cannot be ignored, and further study of this phenomenon is warranted. Further, as

the space to house dogs indoors was severely limited, sufficient numbers were not available for serial sacrifice after exposure. Therefore, the question of whether the effect of 1 ppm ozone was permanent or reversible with age could not be addressed.

The simulation of ambient exposure to photochemical oxidant pollution used here was based on the model of peak oxidant pollution occurring over 4-h periods in the afternoons during 5-d episodes but did not include the lower base level of oxidant that prevails during the remaining part of each day in such episodes. The episode simulation involved in this study precluded long-term chronic exposure that might have produced a larger effect.

This study suggests additional laboratory animal work leading to better understanding of the possible risks faced by growing humans. Such studies could include (1) use of lower levels of ozone, (2) use of younger animals, (3) serial assessments to determine whether or not recovery occurs, and (4) longer-term exposures.

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