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CHRONIC EXPOSURE TO OZONE CAUSES RESTRICTIVE LUNG DISEASE

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INTRODUCTION

Due to the adverse health effects of ozone (O_3) EPA has set a National Ambient Air Quality Standard (NAAQS) for this pollutant (0.12 ppm O_3, hourly average). During re-evaluation of the criteria for the O_3 NAAQS, it became clear that there were several major uncertainties in the health data base. Most important of these was the potential for O_3 to produce chronic health effects. Examination of the chronic effects of O_3 in the rat is the subject of this paper.

General conclusions drawn from the entire data base of published studies were that O_3 causes chronic lung disease and other effects on lung morphology, biochemistry, and host defense systems after long-term exposures. Quantitative interpretation of these few chronic studies for EPA regulatory purposes was inhibited severely because most of the studies were conducted several years ago using methods less sensitive than are presently available or because they were designed to address issues not directly related to EPA needs. Therefore, O_3 chronic studies relevant to regulatory issues were necessary.

Data from the National Aerometric Data Bank (NADB) of the Office of Air Quality Planning and Standards (OAQPS), EPA, were used to assess diurnal patterns for O_3. The intent of these analyses was to focus on days when pollution levels were elevated and discern the nature of the diurnal pattern. For the chronic toxicological studies, the typical pattern was mimicked, but the absolute concentrations of the O_3 in such episodes was not necessarily maintained. The exposure pattern chosen represented frequently occurring worst cases for urban summer environments.

Although a multitude of chronic studies were possible, this study was designed to address several specific objectives to determine the effects of chronic exposure to O_3 in animals that could be quantitatively extrapolated to man. These objectives were as follows: (1) to determine the progression of chronic lung disease in animals exposed to O_3 as measured physiologically, morphometrically, and biochemically; (2) to determine the correlations between these 3 experimental disciplines; and (3) to determine the reversibility or progression of lung disease during exposure and clean air post-exposure periods.

EXPERIMENTAL METHODS

Exposure Facility

The exposure chambers used were of two types: (a) identical, walk-in environmental rooms (Forma Scientific, Inc.) are described in detail by Davies et al.[1] and (b) Hazelton 2000 exposure chambers ($2m^3$), two for each study group (O_3, and control). All chamber environments were monitored and controlled by mechanical systems providing temperatures of 74 ± 3°C and relative humidities of 60±10%, while preventing possible system failures that would affect study data. The chambers were operated at a ventilation rate of 9-10 air changes per hour. The delivery of O_3 to the chambers was regulated by mass flow controllers, which could be controlled either automatically or manually. Ozone was generated by passing cylinder-supplied oxygen through a

silent arc O_3 generator (Ozone Research and Equipment Corporation Mode 03V-0) Redundant components were incorporated to back-up all critical chamber systems.

Computer control of the exposures was incorporated into the system in order to achieve: 1) continuous and unassisted control of the exposure levels, 2) time sequencing of the pollutant concentrations to simulate environmental patterns, 3) repeatable exposure over the entire study period, 4) detection of abnormal events, and 5) automatic storage of data.

Ozone in the chamber was monitored using continuous chemiluminescent analyzers (Bendix 8101C for NO_x and Bendix Model 8002 for O_3) according to EPA reference methods (RFOA-0176-007 for O_3). The analyzers were calibrated biweekly to a UV-based standard following EPA procedures (EPA, 975, 1979).

A wet/dry bulb hygrometer provided continuous monitoring of the chamber temperature and relative humidity. Chart recorders were used to continuously record the temperature and humidity values.

Prior to the study, the spatial distribution of the pollutant in the exposure chamber (with and without animals) was tested to determine gas homogeneity. These test data were used to select the location of the analyzer probes to obtain a representative sample indicative of the chamber concentration. The analyzer probe was placed in the cage to sample O_3 from the breathing zone of the rats. Spatial testing was performed to cover the range of gas concentrations to be used during the study. The requirements were that the range of concentrations within a chamber shall not exceed ± 8% of the target concentration.

Exposure Regimen

The O_3 exposure consisted of a background level of 0.06 ppm for a period of 13 hours, an exposure spike from 0.06 ppm to 0.25 ppm and back to 0.06 ppm over 9 hours, and a downtime of 2 hours for servicing the facility (Figure 1). Integration of the spike portion of the curve shows that the exposure was equivalent to a square wave average of 0.19 ppm. The background exposure level was maintained on weekends. These concentrations, as well as the pattern of exposure, do occur in the ambient air. In order to determine the progression and/or reversibility of lung disease, various exposure lengths and recovery periods were chosen (Table 1).

Table 1 Exposure regimen for O_3 Chronic Study

EXPOSURE PERIOD	RECOVERY PERIOD	EXAMINATION TIMES
1 wk (5 days)	none	immediate
3 wk	none	immediate
3 mo	1.5 mo	immediate and 1.5 mo
12 mo	6.0 mo	immediate and 6 mo
18 mo	4.0 mo	immediate and 4 mo

RESULTS

Structural Studies

A wide range of acute responses occurred in the proximal alveolar region (PAR) of rat lungs. After 1 wk of exposure the volume of alveolar tissue was increased 32%. This increase consisted of a 16% increase in Type I epithelium, 60% increase in Type II epithelium, 52% increase in interstitial cell volume and 33% increase in interstitial matrix. The volume of alveolar macrophages was nearly three fold that of control animals. Qualitative evaluation of the alveolar septum indicated that the increase in interstitial matrix resulted mainly from edema. The number, size, and surface area of the alveolar cells were not significantly changed except for a 32% increase of the number of Type I epithelial cells in the O_3-exposed rats.

After 3 wk of exposure the morphometric parameters from PARs were not significantly different from controls. Tissue volume was no longer increased, however, the number of Type I cells was still elevated (31%).

Epithelial and interstitial injuries were again evident after three months of exposure to O_3. The volume of the proximal alveolar tissue increased 17%. These volume changes included a 34% increase in Type I cell epithelium, a 44% increase in Type II cell epithelium, a 14% increase in interstitial cells and an 18% increase of interstitial cell matrix. The numbers of Type I and Type II epithelial cells were elevated 40% and 18%, respectively.

Persistence of epithelial injury and progression of interstitial fibrosis were observed in the PARs of the O_3-exposed rats at 18 mo. A 23% increase in alveolar tissue volume was measured that consisted of elevated volumes of Type I epithelium (17%), Type II epithelium (90%), cellular interstitium (20%), and interstitial matrix (35%). Large bundles of collagen were evident in the interstitium. The number of Type I epithelial cells was 47% higher in exposed rats. These cells covered an alveolar surface that was 38% smaller than in control animals. The number of Type II epithelial cells increased 65%. The characteristics of these Type II cells remained unchanged.

Lung reactions to O_3 in the proximal alveolar region occurred in a biphasic manner. An acute response involving epithelial hyperplasia and hypertrophy, interstitial edema and alveolar macrophage influx was observed 1 wk after O_3 exposure. These reactions largely resolved after 3 wk of exposure. Epithelial and interstitial reactions were observed again after 3 mo of exposure and these changes either persisted or intensified during the 18 mo exposure period. The increase in interstitial matrix and the 30% increase of Type I epithelial cells seen at 3 mo exposure was not fully resolved after 1.5 mo in clean air. However, the majority of epithelial and interstitial injuries observed after 18 mo exposure to O_3, disappeared, if not totally resolved, after 4 mo recovery in clean air.

Labeling indices were also measured from PARs of control and exposed rats. Alveolar cell labeling was increased approximately 2 fold in exposed rats as compared to controls. Prolonged exposure (3 and 18 mo) did not appear to increase labeling density. The rate of Type II cell differentiation appeared

to have increased after O_3 exposure, however, no change in the rate of Type II cell proliferation was observed. These results correspond to findings in the morphometric studies. In addition, rates of fibroblast turn over appear to have increased in the exposed group without a net increase in the size of the cell population. These results suggest that interstitial cell turn over may occur earlier than was previously suspected.

The morphometric and cytodynamic studies of rat lungs exposed chronically to O_3 indicated that the alveolar tissue responded to the initial insult of O_3 with inflammation and edema. This acute reaction subsided and was not detectable after 3 wk of O_3 exposure. Progressive tissue changes involving both epithelial and interstitial elements developed on prolonged exposure and persisted in the presence of O_3. Deposition of interstitial matrix was observed after 3 mo of exposure and intensified slightly as the exposure continued to 18 mo. There was an elevated cell turnover in the PAR throughout the chronic exposure. However, when rats were placed in clean air, the majority of both epithelial and interstitial changes were reversed. The extent of recovery may be dependent on the length of recovery time in clean air.

Pulmonary Functional Studies

Pulmonary function tests were designed to elucidate functional changes indicative of structural abnormalities. Quiet, resting tidal breathing was analyzed, and included a measure of tidal volume, frequency, airway flow and breath timing parameters. No changes were observed in any of these parameters throughout the study. Static lung volumes, such as vital capacity, residual volume, total lung capacity and functional residual capacity, which historically are used as indicators of lung damage in the clinic were also measured. Statistically significant reductions in total lung capacity and residual volume were detected in the O_3-exposed rats after 3 months of exposure. These depressions in lung volume persisted thereafter through the 18-month examination point. These changes suggest the presence of a restrictive structural lesion, such as would be expected in a 'stiff' lung. However, when the animals were removed from ozone and placed in clean air for 1.5 mo, the animals recovered and the lung volumes returned to normal.

The distribution of air within the ventilated lung was assessed using the nitrogen washout test. At 3 and 12 months, this test exhibited ventilatory evidence of a stiffened lung. However, this measure returned to control values after 18 months probably as the result of compensatory shifts in regional ventilation.

The distensibility, or compliance of the lung was evaluated using the slow-inflation pressure-volume curve. Alveolar diffusing capacity, an index of gas exchange was evaluated by measuring the uptake of carbon monoxide. Small airway integrity was evaluated by analysis of the maximal expiratory flow-volume (MEFV) curve. Wet and dry lung weights were recorded to detect changes in tissue growth or edema. None of these parameters were different from control at any of the examination times.

We speculate that the structural alterations indicated by this set of unctional data lie as restrictions in the extremes of lung volume. This would

explain why no changes were observed in the lung compliance measure, as this is determined at intermediate lung volumes, where elastic properties theoretically dominate. Furthermore, it appears from these data that some degree of functional degradation persists as long as the animal is continually being exposed to ozone. This functional lesion is reversible, however, as affected pulmonary function returns to normal upon removal of the animal from O_3.

Lung Biochemistry Studies

The general concept of the toxic effects of ozone is that it exerts its effect by oxidation of unsaturated fatty acids in membrane phospholipids and/or oxidation of the thiol groups and sensitive amino acid residues of proteins. In designing a study which would be able to detect biochemical changes in the respiratory tract resulting from exposure to low concentrations of ozone, we attempted to sample those cells and fluids which would be in closest contact with the oxidant challenge. These include: alveolar macrophages, which make up the largest portion of free lavagable cells in the lung; lung surfactant, which is present in the gas exchange regions; and pulmonary epithelial cells, present in the whole lung homogenate. This study sought to find evidences that the cells or fluids in the lung are oxidized or otherwise altered by the chronic ozone exposure. This was done by analyzing antioxidant compounds and enzymes that have been shown to be sensitive indicators of pulmonary damage.

Following the time course of the study from 1 wk thru 18 mo of exposure the most noticeable change observed was in the aging of both air and ozone exposed rats. Age alone caused significant treatment-related increases or decreases in many of the endpoints studied. Following 1 wk, 3 wk, and 3 mo of ozone exposure no significant changes were observed as compared to controls. Since no changes were observed, the protective mechanisms of the lung (antioxidants, antioxidant enzymes, glutathione, vitamin C and vitamin E) are assumed to have been able to more than compensate for the oxidative stress placed on these animals. However, after 12 months of ozone exposure selenium dependent GSH-peroxidase, GSH-reductase, and NADPH cytochrome C reductase showed significant increases in activity. In addition, GSH-transferase, GSH-peroxidase (non-selenium), superoxide dismutase and glucose-6-phosphate dehydrogenase also showed increases in activity although not statistically significant. After 18 mo of exposure all of these parameters were back to control levels except for superoxide dismutase which was still slightly elevated. All of these changes seem to indicate a transient increase in the susceptibility of the pulmonary cells to the oxidative stress of ozone between 3 mo of exposure and 12 mo. These changes may be attributable to the aging of the animal and a lessening of it's ability to produce and/or replace the necessary amounts of antioxidants in the lung tissues to protect against ozone attack. The reason for the apparent return to normal levels of these enzymes at 18 mo is not fully understood.

The permeability of the gas exchange region was assessed by measurement of protein in the lung lavage fluid. There was a general decrease in protein concentration in lung lavage fluid with increasing age of the rats. Rats older than 12 mo had half the protein concentrations of 3 mo old rats. The O_3

exposure increased the protein concentration in the lung lavage fluid by 40% initially in 1 and 3 wk exposed rats and by 20% after 3, 12, and 18 mo of exposure. This increase in lavagable protein dissipated when the animals were allowed to reside in clean air. Under conditions of acute O_3 exposure, an increase in lavagable protein often is thought to indicate the leakage of plasma from the blood into the air spaces, due to damage to the blood-air barrier of the deep lung.

Phospholipids, which form a surfactant lining of the gas exchange region of the lung, fluctuated widely in concentration with the age of the rats, reaching an apparent peak at 3 mo. Ozone exposure caused deviations from this fluctuation which represented at most about a 15% change from control. O_3 exposed animals had higher phospholipid levels at 1 wk, lower levels at 3 wk and 3 mo, and higher levels at 12 mo. At 18 mo, phospholipid concentration is were equal to controls.

Phospholipids in the surfactant of the lung contain carbon-carbon double bonds which are highly reactive with O_3. We examined the content of the major saturated as well as unsaturated fatty acids in the lung lavage fluid in order to determine whether we could detect oxidation caused by O_3 exposure. It was evident from the data that after 3 mo of exposure, O_3 lowered the content of all of the fatty acids measured to between 60 and 80% of control. This change was not observed at 12 and 18 mo.

Antioxidants, ascorbic acid (vitamin C), uric acid and tocopherol (vitamin E), form a lining in the respiratory tract through which oxidants must pass before they can reach the tissues. Tocopherol normally protects carbon-carbon double bonds in the lung lipids from oxidation. We measured the tocopherol in a manner which detected only the non-oxidized vitamin. Total lavagable tocopherol in the cell fraction of lung lavage fluid was increased by 34%. The ratio of tocopherol to phospholipid in the whole lung homogenate was increased by 55% after 18 mo of exposure (no similar measurement was made at 12 mo). The fact that tocopherol did not decrease in the lavaged cells and whole lung suggests that the mechanisms for regeneration of this vitamin in the tissues are sufficient to cope with the oxidant challenge. The results also indicate that the lung may even over-compensate by increasing the tocopherol content above the normal level.

Chronic ozone exposure caused a lowering of lung surfactant tocopherol to 40% of control at 12 mo ($p = 0.01$) and to 69% of control ($p = 0.15$) at 18 mo. A decrease in tocopherol in the lining layer of the lung would indicate an impaired defense against further oxidant challenge. The fact that the extracellular tocopherol was decreased suggests that mechanisms for regeneration extracellularly may be weaker than those in cells, and thus not able to compensate for the oxidant exposure.

After 18 mo of O_3 exposure, ascorbic acid in the extracellular fluids lining the inner lung was increased to 233% of control ($p = 0.0001$). There was no change in ascorbic acid in the whole lung homogenate at 12 mo (no lung measurement was made at other times). The ascorbic acid/protein ratio in the cells lavaged from the lung was increased to 185% of control ($P = 0.001$) at 12 mo and to 251% of control ($p = .02$) at 18 mo. Glutathione and uric acid were

measured in the lung lavage fluid supernatant and cells at 18 mo, but no changes were observed in these antioxidants due to O_3 exposure.

Although the present study indicated that most of the evidence of O_3-induced lung alteration did not persist during a post exposure period in clean air, the true reversal of the structurally remodeled connective tissues is not clear. Since the thickened interstitium of the alveolae did not completely regress to control levels during this period, the question has been raised whether collagen or elastin fibers/matrix may show irreversible disorganization or hypertrophy if examined specifically. Hence, with the tissue sections previously studied still intact, these connective tissues will be quantitated morphometrically at the deposition target of O_3, the bronchoalveolar junction, in an effort to address this question.

CONCLUSIONS

Through this multifaceted study we have been able to show structurally and functionally that chronic exposure to O_3 causes restrictive lung disease that progresses with continued exposure. The initiation and development of a focal interstitial fibrosis and a persistence of alveolar epithelial injury was observed over the 18 mo exposure period. The progression of lung injury occurred in a biphasic manner. Initially an acute inflammatory response was observed after 1 wk of exposure, which subsided and was undetectable after 3 wk of exposure. However, epithelial and interstitial reactions were again observed after 3 mo of exposure to O_3 and the lesions persisted or increased in intensity up to 18 mo of exposure. Functional studies showed persistence of decreased total lung capacity and residual volumes at 3, 12 and 18 mo, suggestive of the presence of a restrictive structural lesion as would be expected with a 'stiff' lung. These functional results correlated well with the structural findings of interstitial fibrosis, a restrictive lung disease. Collagen biochemistry studies were not conclusive at any time point thus could not be correlated to the structural and functional results.

The overall consensus of observations was both a persistence and a progression of injury with continuous O_3 exposure. However, when the animals were allowed to reside in clean air post exposure, all major lesions were resolved. This was supported by O_3 challenge experiments which showed that chronic exposure to O_3 altered the responsiveness of the airways to O_3. This alteration was observed as an attenuation of the normal response to O_3 challenge. As with other parameters studied, the tolerance phenonema was not observed after a recovery period in clean air.

Several significant changes were observed in the biochemical defense system of the lung. There appeared to be a persistence of increased epithelial permeability up to 18 mo of exposure which, like the structural and functional changes, resolved after a clean air recovery period. Another significant finding was the lack of persistence or progression of the antioxidant biochemical lesions. Changes that were observable at 3 or 12 mo of exposure were not observed after 18 mo of exposure, apparently resolving or compensating between 12 and 18 mo.

The primary conclusion of this study is that continuous low level exposure to O_3 causes restrictive lung disease as characterized by development of a focal interstitial fibrosis. Removal of the animals from the O_3 environment to an environment of clean, filtered air appeared to reverse the disease state to one of normal structural and functional being. However, one must remember that people do not breath clean, filtered air, thus O_3 exceedance conditions that exist in numerous cities in the U.S. would appear to promote a causal relationship for the potential development of pulmonary interstitial fibrosis.

REFERENCES

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