FINAL REPORT

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CORRELATIVE AND SENSITIVE DISCRIMINANTS FOR AIR QUALITY CONTROL

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Principal Investigator: Russell P. Sherwin, M.D.
Hastings Professor of Pathology
Department of Pathology
USC School of Medicine
Los Angeles, California 90033

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1.ABSTRACT

The investigation tested the effects of a variable daily dosage of 0.25ppm nitrogen dioxide (NO_2) on the lungs of weanling mice. Image analysis was used to quantitate Type 2 Cells, elastic tissue, and walls of alveoli. Effects of NO_2 exposure were increases in Type 2 Cell **number**, Type 2 Cell **field** and **mean areas, alveolar alveolar wall area** with and without the exclusion of Type 2 Cell area, and relationships (ratios) of Type 2 Cells to alveolar wall area, including **numbers** per wall area and **area** per wall area (with or without Type 2 Cell area excluded). There were also increases in elastic tissue fiber numbers, mean fiber area, and numbers of elastic fibers to alveolar wall area (minus elastic tissue area). A number of the statistically significant increases had not reversed by the final test period.

The effects that have been produced are well recognized as early lesions and common denominators for diverse kinds of human lung disease. Type 2 Cell increases in number (hyperplasia) and size (hypertrophy) are generally responses to Type 1 Cell damage and loss. The Type 1 Cell is primarily responsible for gas exchange in the lung, and is also a major factor in the prevention of air space replacement by watery fluids (edema). Hypertrophy and hyperplasia of Type 2 Cells not only implies damage to the Type 1 Cell but in addition may reflect damage to the Type 2 Cell itself. There may also be a need for increased Type 2 Cell work, in particular the secretion of detergent like substances (surfactants) that prevent the air spaces from collapsing (atelectasis and condensation fibrosis). Alterations of the alveolar wall impact on vital aspects of gas exchange. Integrity of the connective tissue of the wall is critical to appropriate expansion and recoil of the lung (compliance), to proper blood vessel perfusion and fluid drainage, and to the maintenance of a scaffolding that permits appropriate regeneration of alveolar lining cells when they are damaged and lost. The results of the elastic tissue and alveolar wall measurements imply suboptimal repair processes subsequent to NO_2 exposure and thus a depletion of respiratory reserves. The persistence of the cell and wall alterations 32 weeks after NO_2 exposure was stopped further implies some degree of irreversible depletion of structural and/or functional lung reserves.

2. ACKNOWLEDGMENTS

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3. DISCLAIMER

"The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products."

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6. SUMMARY-CONCLUSIONS

The experiment of the contractual period was designed to test the effects of intermittent 0.25 ppm nitrogen dioxide (NO_2) exposure on the lungs of weanling male mice. The specific aim was to search for NO_2 induced alterations of two major components of the respiratory units of the lung, the air space linings (alveolar epithelial cells) and the elastic tissue of the air space walls (alveoli, alveolar ducts, respiratory bronchioles).

Of 392 mice, 196 were exposed to 0.25 ppm NO_2 for a total of 210 hrs over a six week period, and the remaining 196 served as controls. Identical environmental chambers, supplied with filtered air, housed the animals. There were two postexposure test periods of 10 and 32 weeks. Image analysis quantitation of frozen sections of lung was used to measure numbers and area of alveolar Type 2 Cells, alveolar wall area, alveolar wall linear intercepts (reflecting "pieces" and surface area), and alveolar wall perimeters. Image analysis quantitation of formalin fixed and paraffin embeded lung was used to measure the elastic tissue of the air spaces and alveolar wall area.

The results are as follows:

A. Type 2 cell and alveolar wall studies (frozen sections of the lung, i.e. no dehydration or fat extraction in tissue processing):

1) Increases in **Type 2 Cell number** and in **Mean Type 2 Cell area**, both increases becoming statistically significant at the 32 week postexposure period ($p<.001$ and $p<.04$, respectively);

2) Increases in **alveolar wall area, alveolar wall intercepts, and alveolar wall perimeter** throughout the three test periods, with borderline significance for intercepts ($p=.08$) and perimeter ($p=.09$) at the 0 week postexposure test period. Perimeters and linear intercepts reflect internal surface area of the air spaces. A large amount of internal surface area is needed for proper respiration. However, injury may lead to splitting and fragmentation of alveolar walls and this kind of increase in intercepts and perimeters impairs function.

3) The NO_2 animals showed an increase in the **ratio of Type 2 Cell ($\geq$12uM) number to alveolar wall area** ($p=.08$) at the 32 week postexposure test period). The increase in the cell number to alveolar wall area ratio indicates that the increase in cell number found cannot be attributed solely to collapse of airspaces (atelectasis), i.e. the smaller the airspaces the more lung tissue enters the measuring field.

B. Paraffin embeded lung tissue (dehydrated and cleared of fat).

1) The NO_2 exposed animals showed a decrease in the **perimeters of the alveolar wall** at the end of the exposure period ($p<.05$). This apparent decrease in internal surface area may be closer to the in vivo state than the frozen section appearance since shrinkage of the tissues by dehydration tends to minimize splitting and fragmentation (see Elastin discussion). However, a number of factors are interacting and no conclusions can be made at this time.

2) The NO_2 animals showed a fall in **alveolar wall area** at the 10 week postexposure period , with ($p<.07$) and without ($p=.1$) the exclusion of elastic fibers. The findings persisted at the 32 week postexposure period, with wall area minus elastic fiber area at a near significant level ($p<.06$).

3) The **mean number of elastic fibers** differed at all three test periods (p=.001, p=.01, and p=.09, respectively), with more numbers of elastic fibers for the exposed animals at the 0 week and 32 week postexposure periods, but a reversal at the 10 week postexposure period;

4) The **ratio of elastic fiber numbers to alveolar wall area (minus elastic fiber area)** was consistently greater for the exposed animals over the three test periods, but only the difference at the 32 weeks postexposure period was significant (p=.002). The ratio findings indicate a relative increase of elastic tissue regardless of air space collapse or hyperexpansion.

5) **Mean elastic fiber area (area/numbers)** was greater for the NO_2 exposed animals at the 0 week and 32 week postexposure periods respectively, but not at significant levels. At the 10 week postexposure period, the NO_2 exposed animals showed a significant decrease (p=.03);

The significance of the results is that NO_2 exposure has apparently affected three main components of the mouse lung, the epithelial cells, the alveolar wall in general, and the elastic tissue component of the alveolar wall. All of the foregoing alterations are well recognized signs of damage to both the lung parenchyma (breathing units) and the supporting tissues. In particular, Type 2 cell hyperplasia and increases in elastin (with splitting of fibers) are common findings in both human lung disease and experimental animal studies. Moreover, there is the further implication that the alterations may not be entirely reversible since the lungs of the NO_2 exposed animals at the final test period (32 weeks postexposure) showed statistically significant increases in the number of Type 2 Cells, the mean area of Type 2 cells, and several alveolar wall area measurements.

A tentative interpretation of the data is that the early effect of NO_2 exposure is congestion and edema of the alveolar walls. The edema is probably associated with fraying and fragmentation of the alveolar wall in view of the greater increase in intercept and perimeter measurements compared to alveolar wall area. While the 10 week postexposure test period showed similar trends without statistically significant differences, the persistence of the trend throughout all three test periods and a number of statistically significant differences at the 32 week postexposure period, implies an adverse health effect that may not be entirely reversible. Of particular concern is the likelihood that the NO_2 related increases in Type 2 Cell numbers (p<.002) and mean area (p<.04) at the final test period reflect alteration and/or loss of Type 1 Cells. Further, the increase in Type 2 cell size could be in part injury rather than a compensatory, work related change. Since the alveolar wall area increase seen in the frozen section preparations may in part be due to some atelectasis as well as congestion and edema, an alteration of surfactant production by the Type 2 Cell is suspect. Moreover, the Type 2 cell alteration may be understated since the proximal portion of the lung acinus tends to have the greatest degree of injury and we included the entire acinus in our measurements.

The results of elastic tissue and alveolar wall quantitation, using formalin fixed lung tissue, indicate that both elastic fiber number and fiber area change with time as a part of normal lung development. However, the findings suggest that NO_2 exposure may cause a fragmentation of elastic fibers. Immediately after NO_2 exposure, elastic fiber numbers are increased (p=.001), but elastic area is unchanged. At the 10 week postexposure period, both numbers and area of elastic fibers failed to reach the levels for the controls (p=.01 and p=.03, respectively), suggesting faulty development as well as fragmentation. Finally, at the 32 week postexposure period, elastic fiber areas no longer differ, but the suggestion of fragmentation (more pieces of elastin for the same area) persists (p=.09). Further, all three test periods showed an increase in the ratio of elastic fiber numbers to alveolar wall area, with the increase statistically significant at the final test period, with or without elastic tissue excluded from the wall measurement (p<.002).

Statistically significant differences in body and spleen weights occurred at the 10 week postexposure period, but there were no differences at the final 32 week postexposure test period.

7. RECOMMENDATIONS

A. Background and rationale for recommendations

The data analysis on the majority of experiments completed to date indicates that ambient levels of NO_2 as low as the California one hour average standard (0.25ppm) alter the cells and tissues of the mouse lung, and that the alterations persist as long as 32 weeks after NO_2 exposure. The alterations are well recognized as common denominators and early lesions in diverse kinds of human lung disease (17-20,30). Whether or not the alterations observed are ultimately reversible has not to date been answered. More pertinently, the question raised by the repeated finding of these alterations is whether or not the rate of depletion of lung reserves has been inordinately accelerated. Lung function studies of the human well population have uniformly reported a decremental loss with time, and the rate of loss in the Los Angeles area has been reported to be twice that of all other areas tested (21). The Tashkin et al report (21) states that they have not excluded the possibility that the higher rate of lung function loss in Los Angeles County may be due to the poorer air quality of this region.

The decremental loss of lung function in humans has a well known and important pathologic correlate, a progressive disruption and loss of lung cells and tissues (27). More specifically, pathologists recognize that all adults have more than trace amounts of emphysema, and this type of destruction is often referred to as "vanishing lung disease" because of its largely covert nature. The frequency of clinically manifested emphysema is rapidly increasing for reasons that are poorly understood, notwithstanding the well recognized role of cigarette smoking (25,26). Furthermore, the various emphysematous diseases represent only a small part of the spectrum of destructive lung diseases and the list of agents causing destructive lung disease is rapidly expanding. The listing now includes an extraordinary number of sensitizing agents (see Table 46, page 74; 22) and therapeutic drugs (see Tables 47&48; pages 75&76; 23,24). Thus, a most important public health concern is minimizing the role that air pollutants play in causing, promoting, facilitating, and exacerbating lung disease. In short, reducing or eliminating one or more of the links in the chain of lung destruction can substantially reduce not only the rate of depletion of structural and functional reserves of the lung, but also the magnitude of overtly destructive disease processes.

B. Specific recommendations: Cumulative results based on present and prior experiments.

We have carried out tests of NO_2, O_3, and combinations of NO_2 and O_3 The results point to two major needs shared in common, determination of the minimal level of exposure that causes an effect and extension of the postexposure period to evaluate reversibility. While some information has been derived in both areas of concern, the data do not afford definitive answers. A few of the specific needs are the following:

1. Specific pollutant studies; Quantitative image analysis of Type 2 Cell populations and components of the alveolar wall

a. While the 0.1ppm O_3 exposure did not show statistically significant effects, the experiment did not include postexposure test periods. Pertinently, the 0.3ppm O_3 experiment did result in statistically significant differences, but these became evident **only** at the 4 week postexposure test, namely increases in Type 2 Cell numbers ($p=.03$), Type 2 Cell mean area ($p=.05$), and mean alveolar wall area minus Type 2 Cell area ($p=.02$). The 0.1ppm O_3 experiment should be expanded to include 4 week, 10 week and 32 week postexposure periods.

b. One experiment where newborn mice were exposed to combined 0.2ppm NO_2 and 0.3ppm O_3 was unsuccessful (high mortality and no statistically significant differences) due to an infectious disease outbreak. The same levels of combined pollutants applied to adult mice has demonstrated alterations of cells and interstices at significant levels. The specific recommendation is to test combined 0.15ppm NO_2-0.1ppm O_3 exposure, the former representating the 24 hour average air quality standard and the latter the one hour average California Air Quality Standard. Postexposure testing would be a part of the investigation.

c. With respect to the elastin aspect of the study presently being reported, this is the first investigation of its kind and should be extended to an experiment that will both confirm and extend the findings. Pertinently, elastic tissue is a critical factor in lung compliance, and the enzyme elastase has been shown to induce emphysematous change.

2. Filtered air - developing lung studies

Image analysis measurements of the cell populations and infrastructure of the lungs of mice that have been maintained in a clean air environment in order to establish baselines on lung development and "normal" loss of lung reserves.

We now have preliminary type data only on the cell populations and structural changes that occur in control animal groups with time. A major deficiency is the wide gap between study periods, notably between the ages of 16 and 44 weeks. Closer test periods are needed to answer the questions of when do major developmental changes take place in the mouse lung and when is full maturity reached. Further, there are the two broader and very critical questions of what is a "normal" (or "acceptable") rate of structural damage and loss, and what are the roles of the various pollutants in modulating the "normal" rate of decremental loss. The use of a large colony of animals (400) with examination of just four pairs (control and ambient exposed) at gradually increasing intervals over a period of 18 months (50 test periods) would provide a "curve of best fit" type of long term baseline. The specific question to be answered is "What is the "normal" decrement in lung tissue for animals in a clean air environment?"

3. Ambient atmosphere studies; Inventories of cells and respiratory units of the human and animal lung.

This is an extension of the question of "normal" lung tissue decrement. There are two main objectives, the study of the animal model and an investigation of the human "well population" using Coroner cases. The specific goal is to obtain inventories of Type 1 & 2 Cells, endothelial cells, subpopulations of lymphocytes, and basic structural units of the lung, e.g. alveoli and terminal bronchioles. The frequency of bronchioles exhibiting inflammatory changes would be a part of the quantitation in view of the importance of covert bronchiolitis and also our experience to date with coroner cases showing that chronic bronchiolitis is commonly found in the lungs of teenagers.

Studies of the human well population can be carried out in cooperation with the Los Angeles Coroner's Office. We have discussed our recommendations for future investigations with the Acting Chief Coroner, Dr. Ronald Kornblum and he has agreed to join with us in a cooperative study, especially in view of his own personal interest in environmental factors influencing the involution of the thymic gland.

An experimental animal study is recommended to answer the question of decremental loss of mouse tissues as influenced by environments having different levels of air quality. The studies would be an extension in part of an ongoing project where alterations were found but had not reversed at the final test period.

The mouse model offers an excellent opportunity for correlative inventory studies since the alveoli of the mouse lung share in common a great many responses of the human lung and the approximately 50,000 alveoli in the mouse lung are more amenable to large proportion sampling. We have recently carried out a pilot study with an advanced image analyzer and believe the data now being collated will demonstrate a potential for obtaining airspace inventories for the purposes intended. The analyzer presently available is potentially capable of inventories of airspaces, but at a much less sophisticated level and only with a heavy investment in supporting (manual) methodologies. On the other hand, the present analyzer is adequate for cell population inventories (epithelial, endothelial, connective tissue, and host defense cells of the alveolus).

A final consideration is the exploration of new areas of study. We have carried out a pilot study that demonstrated altered levels of serotonin and its major metabolite in the brains of mice exposed to NO_2 (31). The latter study warrants further exploration since alterations of the brain undoubtedly influence the lung and shock lung from diverse initiating events is thought to be mediated primarily through a central nervous system mechanism. We are presently exploring other hormonal and metabolic activities of lung and brain.

8. BODY OF REPORT

A. Scope, Purpose, and General Background of the Project

The overall scope of this project has been to determine the adverse effects on the mouse lung of an ambient level (0.25 ppm) of NO_2. To accomplish this, objective measurements were made of the kinds of cellular and tissue alterations that occur as early lesions in diverse human lung diseases. Specifically, Type 2 cells of the alveolar lining were measured because they are the progenitors of the ultrathin Type 1 cells of the lining, and the finding of an increase in Type 2 cell numbers reflects injury to the Type 1 cell (1-6). If the increase in numbers of Type 2 cells (hyperplasia) persists, especially in association with an increase in cell size (hypertrophy), the implication is a continuing injury and/or incomplete recovery.

Further, we have in this study extended our work with Type 2 cell measurements to include measurements of the elastin component of the lung scaffolding. The main reason for this is that an alteration of elastin tends to implicate some degree of irreversible lung damage. Elastin is not only a critical factor in the contraction and expansion of the lung (compliance), but is essential for a complete regeneration of the epithelial lining of the associated alveoli. As a part of the elastin measurements, we included measurements of the alveolar wall as a whole, i.e. encompassing lining cells, capillaries, and connective tissues. The reason for this is that noxious insults to the human lung commonly cause congestion and edema of the alveolar walls. A number of kinds of measurements were made for insight into different kinds of alveolar wall alterations. An area measurement was used since it tends to reflect the thickness of the alveolar walls. Alveolar wall perimeter and linear intercept measurements were in large part reflections of internal surface area. Elastin fiber number was used to evaluate the presence of fragmentation, clumping, and regeneration.

The level of NO_2 selected was based on two considerations. First, that 0.25 ppm NO_2 is the one hour average established as the California State standard, and secondly that intermittent and variable lengths of exposure to NO_2 would tend to mimic the ambient experience.

The background for the proposed study comes from a number of our prior investigations where we exposed adult and newborn mice to ambient levels of nitrogen dioxide (NO_2) and demonstrated cell and tissue alterations, i.e. increases in the number and size of Type 2 Cells of the alveoli, and also increases in alveolar wall area. The alterations were observed after 6 weeks of NO_2 exposure and were statistically significant (3,4). A subsequent experiment with NO_2 exposure of newborn mice showed that the alterations had not reversed to control animal levels despite a 32 week postexposure period (cf. Experiment 156 below). Moreover, the Type 2 Cell itself appeared to be damaged following NO_2 exposure since there was an apparent increase in size that may represent edematous change. Additional support for edematous change and some irreversible damage was obtained from data on the alveolar wall measurements. We demonstrated in a number of related studies that an increase in alveolar wall area accompanied the cellular alterations, and we suspected that the alteration was at least in part edema and congestion since several earlier studies had shown that ambient levels of NO_2 increase lung capillary permeability (1,2,7,8). Further, the persistence of an increase in alveolar wall area at 10 week and 32 week postexposure periods suggested that alterations of the connective tissue itself may be involved. The very important question of possible elastin change was raised by the findings since, as mentioned above, the integrity of the lung scaffolding is a critical factor in reversibility of cellular damage. In lieu of available data on NO_2 effects on lung elastin, and in view of the importance of obtaining such data, we proposed and carried out the investigation presently being reported.

With respect to potential meaning of the investigation insofar as human health is concerned, we have commented in publications emanating from the prior work that all of the cellular and tissue alterations demonstrated as NO_2 effects on the mouse lung represent early kinds of lesions commonly seen in diverse human lung diseases and, most importantly, in all adults of the well population. In effect, the cellular and tissue alterations are a part of the decremental loss of lung function and structure ubiquitous in all adults. The key question is the magnitude of the alterations attributable to air pollution as well as to other environmental noxious agents. With respect to one major category of lung disease alone, the chronic obstructive respiratory diseases (CORD), there is reason to be concerned that air pollution may be covertly but substantially contributing to the rapidly rising frequency. CORD is fundamentally characterized by an irreversible progression of the cellular and tissue alterations we measured in the present project. Moreover, all degrees of cellular alteration constitute some diminution of functional and structural reserves. Even the potentially reversible increase in numbers and size of Type 2 cells impacts on the immediate availability of reserves, e.g. the replacement of ultrathin Type 1 cells by very thick Type 2 cells impedes the exchange of oxygen and carbon dioxide in the lung. In addition, injury to the Type 2 cell itself may alter surfactant production and impair the regeneration of Type 1 cells.

B. Rational and Objectives

This investigation is based on image analysis quantitation of cells and tissues in the lungs of mice exposed to 0.25 ppm NO_2 Type 2 cells of the alveolar lining were measured because they are the progenitors of Type 1 cells and an increase in these cells is a very early sign of lung injury (3-6). They proliferate (Type 2 cell hyperplasia) when Type 1 cells of the alveolar lining are injured or lost, and they gradually transform into Type 1 cells to regenerate the epithelial lining. Persistent Type 2 cell hyperplasia in the human as well as animal lung indicates continuing injury or incomplete recovery. An important pathophysiological consequence of a Type 1/Type 2 cell population shift is a thickening of the diffusion barrier between air spaces and alveolar capillaries. The presence of very thick Type 2 cells instead of the ultrathin Type 1 cells impedes the exchange of oxygen and carbon dioxide in the lung. In addition, injury to the Type 2 cell itself may alter surfactant production and impair regeneration of the Type 1 cell. Some degree of irreversible loss of lung reserves results from the cellular injury, and creates a decrement in lung function in all mammalian lungs that progressively increases with time. We believe that the major health problem of air pollution is that it may be adding subtly but significantly, over the long term, to an inordinate depletion of lung reserves. With each incremental loss of reserves there is a corresponding increase in susceptibility to disease, and an incremental exacerbation of disease already pressent. The ultimate measure of reserve depletion is an inventory of cell population and tissues. The Type 2 cell measurements are a first step in that direction. We have extended this investigation to include measurements of the elastin component of the lung scaffolding. Damage to the underlying scaffolding of the lung is especially likely to lead to irreversible loss of lung reserves since an intact scaffolding is essential for proper regeneration of the epithelial cell lining. Moreover, the elastin portion of the scaffolding not only contributes to the structural integrity of the alveolus, but plays a major role in the contraction and expansion of the lung (lung compliance). One other tissue included in the image analysis quantitation was the alveolar wall as a whole, i.e. cells, capillaries, and connective tissues. Pollutants and noxious agents in general characteristically alter capillary permeability in the early stages of injury. The two main consequences are edema and congestion of the cells and tissues involved. While edema and congestion are largely reversible, e.g. the "red eyes" of pollutant irritated conjunctivae, there is an increased susceptibility to disease from the alterations. Pulmonary congestion and edema, under circumstances that are presently poorly understood for the most part, can progress to serious complications, e.g. air space collapse (atelectasis), decreased compliance ("stiff lung"), and "congestive-atelectasis" (shock lung,

Adult Respiratory Distress Syndrome). One of the poorly understood factors is the status of the health reserves of the lung and the body in general, in particular the questions how much lung function has been lost, how much is committed to repair, and how much is needed to cover exigencies.

While our long range goal is an inventory of human and animal lung reserves and correlations with air pollution effects, the immediate goal of this study was determining the effect of 0.25 ppm NO_2 exposure on the lungs of weanling mice. The level of NO_2 selected was based on two considerations. First, that 0.25 ppm NO_2 is the one hour average established as the California State standard, and secondly that a variable dosage of NO_2 would more closely mimic NO_2 exposure as it occurs in ambient community air over a six week period of relative high air pollution. The specific measurements obtained afford insight into what is essentially measurements of an ecology at the cellular and tissue levels. An increase in Type 2 cell numbers reflects a cell population shift from the delicate Type 1 cells of the alveolar lining to the relatively huge progenitor Type 2 cell. An increase in Type 2 cell size reflects compensatory hypertrophy (work related) and/or adverse effects (e.g. edema). Alveolar wall area increases reflect probable congestion and edema for the most part; alveolar wall perimeter and linear intercepts are in large part measures of internal surface area; elastin fiber number is an indication of amount and possible fragmentation (as is also linear intercepts of elastin); elastin area measures amount (altered and unaltered); and the size of elastin fibers, combined with other meassurements, affords insight into adverse effects such as fragmentation, clumping, regeneration.

C. Materials and Methods

1. Experimental Design

The major objectives for the contractual period completed during this project period were:

a. Measurements of lung cell populations and alveolar walls following 210 hour exposure of young adult Swiss Webster male mice to 0.25 ppm nitrogen dioxide over a 6 weeks period, and 10 weeks and 32 weeks after exposure was terminated (M171); and

b. Measurements of elastic fibers and alveolar walls following 210 hour exposure of young adult Swiss Webster male mice to 0.25 ppm of nitrogen dioxide, and 10 weeks and 32 weeks after exposure was terminated (M171).

2. Methodology

Nitrogen Dioxide Exposure

The duration of nitrogen dioxide exposure for this initial study was varied to simulate the Los Angeles atmosphere. Weanling male mice were used to test for the effects of ambient levels of NO_2 on the developing lung immediately after exposure and following long term 10 week and 32 week postexposure periods.

Details of the chambers and the NO_2 method have been reported earlier (8,9). In brief, room air was filtered by a combination of particle and NO_2 absorbing (Purafil) filters. NO_2 was introduced into the air intake mixing unit of the exposure chamber through a silicone drip method. The silicone used was of medical grade and a high viscosity (500 centistokes) with an essentially negligible vapor pressure. The delivery of the NO_2-silicone mixture was regulated by the caliber of the teflon delivery tube, the height of the column, and air flow. The following means of NO_2 monitoring were employed:

a. Fritted bubbler readings of approximately 4 liter air samples;
b. Beckman liquid analyzer continuous flow recording; and
c. Chemiluminescent continous recordings (Teco).

Measurements were obtained of control chamber, exposure chamber, and ambient room atmosphere. Periodic calibration of the monitoring equipment was carried out through the assistance of the California Air Resources Board, El Monte Division.

Environmental Chambers

The mice were housed in two identical laminar air flow stainless steel environmental chambers in an air conditioned vivarium exclusively used for the pollution studies. Each chamber has a 40 ft^3 volume. The air flow rate resulted in a change of chamber air approximately every other minute. The air passed through a Purafil filter system, with the latter replaced on a routine schedule or earlier if indicated by a color change of the pellets. The room temperature, chamber temperatures, and humidity were recorded daily. The cages were cleaned three times per week.

The chambers were steam cleaned and the hoses washed after termination of each experiment.

Animal Colonies

Half of the colony, i.e. 200 weanling mice (12 gms; 21-24 days old, and purchased from Charles River, was exposed to ambient levels of NO_2 according to the proposed protocol (cf Flow Chart 17). Following the 6 week exposure period, 60 pairs of mice, 60 controls and 60 experimental, were killed and their lungs processed. The remaining animals were housed in special holding chambers. At the end of 10 week and 32 week postexposure 120 animals (60 control and 60 experimental) and 152 animals (76 control and 76 experimental) were killed and their tissues processed.

At each test period, the animals were weighed and their weights recorded. In addition, the spleen weights and left lung volumes were also recorded.

The animals were fed a standard pellet chow and received water ad libitum. Animal weights were recorded prior to placing them into exposure chambers and at the time of testing. The animals were placed in cages according to weights, generally five animals per cage. Two separate exposure chambers, with laminar air flow and stainless steel fabrication, were available for the study. For experiments with postexposure periods the animals were housed in special holding chambers.

Lactate Dehydrogenase Methodology

For image analysis quantitation of LDH stained Type 2 Cells, the right lower lobe was inflated with 6% gelatin (pH 7.0) at 20 cm H_2O height with a 22 gauge cannula inserted into the trachea. The gelatin-inflated lungs were then placed in a refrigerator at 4^oC to solidify the gelatin, placed with hilar area down on a marked Mylar sheet identifying the experiment, experimental group and lung lobe, wrapped in aluminum foil and stored at -75^oC. For sectioning, the lobes were oriented to provide topographical lung tissue areas consistent for all lobes. Sections 15 micrometers thick were cut on a Cryostat, with 8-10 sections per lobe. The details of the LDH reaction, carried out on lyophilyzed frozen sections, has been published (10). In brief, a tetrazolium salt is used as an electron acceptor and is reduced to an insoluble formazan in the presence of the LDH enzyme, with lactate as a substrate.

Aldehyde Fuchsin-Metanil Yellow Staining of Elastin

For image analysis quantitation studies of elastin fibers, the left lung was inflated with 2% glutaraldehyde buffered with 0.1M sodium cacodylate and fixed for 48 hours. Following fixation, the lungs were washed in 0.1M cacodylate buffer, and their lung volumes measured. Lungs were processed, embedded in paraffin, sectioned and stained with metanil yellow. A total of 381 mouse lungs with 4 sections per lung were used to quantitate elastic fibers and alveolar walls.

Quantitative Image Analysis

A Cambridge 720 Image Analyzer with a shading corrector and recording terminal (cassette tape and print out) was used to obtain measurements of lung elastin, Type 2 Cells and alveolar wall. The gray values for detection of elastin fibers or Type 2 Cells were set with one of the two discriminators at a level which resulted in no change between the area of elastic fibers or the Type 2 Cells as seen on the in the video display of the microscopic image, and that observed in the positive electron image displayed by the detector system. The numbers of elastin fibers or Type 2 Cells detected were "fine-tuned" by comparing by manual counting how well the electronic detection matched the "flagging" signals in a selected area of the video display. The detected image was also compared to the field observed through the microscope. The second discriminator was used for measurement of wall area, its perimeters and linear intercepts. Comparisons between video display, electronic image and microscopic fields were made to effect the best representation of the alveolar wall area with minimal background "noise." With the two detector settings set for elastin (Type 2 Cells) and wall area, an automatic program was used for measurement recordings. The quantitative measurements were recorded on casette tape using a Texas Instrument terminal, were then "captured" on floppy disks through a computer program, and finally formatted and corrected in preparation for statistical analysis. A 10X ocular and either 6.3x (Type 2 Cell) or 10x (elastin) objective were used for all experiments carried out during the previous contractural periods. For the present contractual period, 10x ocular and 16x objective were used for the quantitation of both the Type 2 cells and elastin fibers. One technician carried out each individual experiment to ensure consistency in the image analysis quantitation.

Image Analysis Quantitation of Elastin and Alveolar Wall

The two detectors of the image analyzer were set for elastin and alveolar wall area on the basis of the specific aldehyde fuchsin stain for elastin (the delicate and coarse elastic strands in the alveolar walls, alveolar sacs, bronchioles and other supportive tissues of the lung) and the nonspecific, light background metanil yellow stain for the alveolar wall. Four lung sections were used per each animal. Each lung section was divided into three sectors which represented apical, middle and basilar portions of the right lower lobe. The first field in each sector that contained only alveoli, alveolar sacs, small bronchioles and blood vessels and was free of technical imperfections was used to obtain measurements from control and exposed animals in an alternating sequence. Fields containing medium, large blood vessels and conducting airways in which lining cells could be clearly detected were considered technically unacceptable. The measurements included both the edited fields (in which the blood vessels and their supporting elastic structures were excluded) and unedited fields. The measurements of elastin included number of fibers and elastin area, with and without sizing factors, and also elastin linear intercepts and perimeters. The quantitative measurements of the alveolar wall included area and perimeters.

Image Analysis Quantitation of Type 2 Pneumocytes and Alveolar Walls

The deposits of formazan reaction product at the sites of LDH activity are essentially selective for the Type 2 pneumocyte under the conditions of the tissue section preparation: the cell is exceptionally large and round, uniquely occupies alveolar corners, and is the only cell in the alveolar wall with a heavy perinuclear cytoplasmic deposit. Other alveolar wall cells, Type 1 pneumocytes, endothelial cells, fibroblasts and macrophages have greatly attenuated cytoplasm, and the finely granular, diffuse LDH reaction products in their cytoplasm are not detected by the Image Analyzer at the grey level value of the detector set for the Type 2 cell. Further, desquamated and rounded macrophages in the alveolar lumina are essentially excluded from the quantitation by the gelatin washout. The bronchial mucosa cells in the conducting airways are also strongly positive for LDH. However, these structures are readily identifiable and, when present, are excluded from the lung field by an image editor. A weak background staining of the alveolar wall serves to delineate the walls for measurement by a second detector grey value setting of the image analyzer.

For image analysis quantitation, four lung sections from each animal were used. Each lung section was divided into apical, middle and basilar sectors. The first lung field within each quadrant meeting set criteria was selected for analysis. The criteria for acceptability were absence of pathological lesions, relative absence of bronchovascular structures, and technically satisfactory tissue areas for the most part. The measurements of the Type 2 Cells and wall area included edited and unedited fields. A total of 18 measurements, nine edited and nine unedited, were recorded per each field. The unedited field measurements were used to check the integrity of the lung structure and to assure that the editing of the fields was not biased.

Data Processing

The Biomedical Data Program of UCLA (BMDP STATISTICAL PACKAGE) programmed for the IBM-XT was made available to us as a part of a "Beta Tester" program, and has since been expanded to new and upgraded statistical programs. We also expanded the computer's memory to 640K RAM and added an 8087 coprocessor. The statistical analysis programs used (e.g. t-test, 2-way analysis of variance, Mann-Whitney non-parametric) have been worked out and tested for the processing of our image analysis data. Programs for capturing data from casettes directly onto floppy disks, and for formatting data were provided to us by our computer consultant.

Basic Statistical Analysis of the Data.

The statistical analyses included Student's t-test, two factor analysis of variance, and appropriate non-parametric tests and were used for comparisons within and between test periods, i.e group, time, and group x time.

D. Results

There were 196 mice in the control group and 196 mice in the NO_2 exposed group (382 total), with a minimum of 60 animal pairs for each of three test periods (Table 1). Frozen sections were prepared for lactate dehydrogenase staining (Type 2 Cell and alveolar wall measurements) and paraffin sections for elastic tissue stains and alveolar wall measurements (Tables 2, 3 & 4).

Image Analysis Quantitation of Lung Tissue for Type 2 Cell and Alveolar Wall Measurements (Lactate Dehydrogenase Stain)

A total of 390 left lungs, 195 control and 195 experimental, were technically suitable for image analysis. For the control group, 2,340 fields, representing 780 lung sections, gave 619,380 data points for statistical evaluation. For the experimental group, the corresponding values were 2,340 fields, 780 sections, 60,344 data points, Table 3.

The Type 2 Cell measurements included numbers, field area (total area of all cells in one field), linear intercepts, and mean area (field area of cells/field divided by numbers of cells/field). The alveolar wall area measurements included total wall area (including Type 2 cells included in the wall), wall area minus Type 2 Cell area, wall perimeters, and wall linear intercepts.

Type 2 cell numbers were greater for the NO_2 exposed animals at the end of the six weeks of exposure (3.6% difference for the $\geq$12uM cells) but the difference was not statistically significant (0 week postexposure; Table 5). There was essentially no difference at the 10 week postexposure period although a greater number of Type 2 cells was again noted for the exposed animals (Table 7). At the 32 week postexposure period (Table 9), the difference became statistically significant and was greatest for the largest of the Type 2 cell categories ($\geq$12uM; 8.8%; $p<.001$).

Type 2 cell mean area was consistently greater for the exposed animals throughout all three test periods, 4.7%, 2,4%, and 7.7% respectively (Tables 6, 8, & 10). The difference became statistical significant at the 32 week postexposure period ($p<.04$; Table 10).

The alveolar wall area was consistently greater for the NO_2 exposed animals throughout the three test periods, 3.7%, 2.1%, and 3.9% respectively (Tables 5, 7, & 9). However, none of the differences were statistically significant. Both alveolar wall linear intercept and perimeter reached a borderline level of statistical significance at the end of the six weeks of exposure ($p=.08$ and .09, respectively; Table 5).

The ratio of Type 2 Cell mean area to alveolar wall area (minus Type 2 cell field area) was also consistently greater for the NO_2 exposed animals for all three test periods (2.0%, 0.3%, and 6.0%, respectively; Tables 6, 8 & 10), but none of the differences were statistically significant.

The ratio of Type 2 Cell ($\geq$12uM) number to alveolar wall area (minus Type 2 cell field area) was also greater for the NO_2 exposed animals at the 32 week postexposure period, but at a borderline level of statistical significance ($p=.08$; Table 10).

Image Analysis Quantitation of Lung Tissue for Elastin and Alveolar Wall Measurements (Aldehyde Fuchsin Stain)

A total of 381 left lungs, 191 control and 190 experimental, were technically suitable for image analysis. For the control group, 2,084 fields, representing 753 lung sections, gave 31,260 data points for statistical evaluation. For the experimental group, the corresponding values were 2,101 fields, 740 sections, and 31,515 data points (Table 4).

At the 0 week test period, all mean elastic fiber measurements, i.e. mean number, areas (including computed areas), intercepts, and perimeters, were greater for the experimental group, all at statistically significant levels (Tables 11, 12, & 13). The alveolar wall area (with and without elastic fiber area) was higher for the experimental group but not at a statistically significant level (Table 11). The alveolar wall perimeter was however greater for the control group, and the difference was statistically significant (p=0.05; Table 11). The ratios of **elastic fiber numbers to alveolar wall area**, and elastic fiber area to alveolar wall area were also higher for the experimental group but failed to reach significance (Table 12). Alveolar wall area showed no differences (Table 11).

The mean values of all elastic fiber measurements at the 10 week postexposure period were significantly higher for the control group (Tables 14, 15 & 16). For the alveolar wall at this test period, the mean values were higher for the control group at a border level of significance, and the increase in alveolar wall perimeter for the control group was not statistically significant (Table 14). The ratios of elastic fiber/alveolar wall area were not statistically significant (Table 15). Alveolar wall area was greater for the control animals at a borderline level of significance (with or without elastic tissue excluded (p=.1, p<.07, respectively; Table 14).

At the 32 week postexposure test period, the mean elastic fiber measurements were greater for the experimental animal group (Tables 17, 18, & 19). The increase was highly significant for linear intercepts of elastic fibers (outliers excluded; p<0.006; all data, p=.09), and at a borderline level for number of elastic fibers (with or without outliers, p=0.09). In addition, the mean field area of elastic fibers >8 um increased at a borderline level of significance (p=0.1003, with or without outliers excluded). Elastic fiber perimeter was greater for the experimental group at a borderline level of significance (p=0.0908, with or without outliers excluded). The ratios of elastic fiber/alveolar wall area were also significantly higher for the experimental group (Table 18). The elastic fiber areas segregated according to size groups (Computed Mean Field Elastic Fiber Area) were also higher for the experimental group, but there were no statistically significant differences (Table 19). Mean alveolar wall area was greater for the control animals at a near statistically significant level when elastic fiber area was excluded (p <.06; Table 17).

Animal Body and Spleen Weights

The mean animal body and spleen weights, their ratios, and lung volumes are summarized in Table 1.

The mean body weights were significantly greater for the exposed animals at the end of the 10 week postexposure period (p=0.015). For the 0 week and the 32 week postexposure periods, mean body weights between groups showed no differences, but by the 32 week test period the control animals weighed essentially the same as the exposed animals.

The mean spleen weight of the experimental group for the 10 week postexposure period was also significantly greater (p=0.0069) and, despite a greater increase in body weight, the mean spleen/body weight ratio for the exposed animals was also greater that that of the controls, although at a borderline level of significance, p=0.0882. The mean value of

spleen weights and spleen/body weight ratios were consistently less for the exposed animals at the 0 week and 32 week test periods but the differences were not statistically significant.

The lung volumes of experimental animals were consistently larger throughout the entire experiment, but the differences were not statistically significant by t test analysis.

E. DISCUSSION

1. CONTROL ANIMAL GROUP FINDINGS

The results of this experiment (M171) are most profitably discussed in conjunction with two prior experiments since data on the developing mouse lung are available for comparison. One of the two prior studies (M156) involved four test periods where newborn animals were exposed to 0.35ppm NO_2 for 12 weeks and then followed through three postexposure test periods of when the animals were 14 weeks, 22 weeks, and 44 weeks of age (see Appendix). The latter test periods compare with the present study (M171) of animals 9, 19, and 41 weeks of age. The second prior experiment (M117) was a study of newborn animals (4) exposed to 0.3ppm NO_2 and tested at 6 weeks, 10 weeks, and 16 weeks of age. While differences in age and other factors obviate a strict comparison of the results obtained from the three independent studies, a number of trends were found that appear to be valid reflections of chronologic events.

The data provided in the charts demonstrate percentage (delta) changes throughout the three or four test periods. More specifically, a zero reference value was set up for all charts using the measurement observed for the control animal group immediately following the NO_2 exposure period, and this reference point is referred to as the "0 week postexposure period".

Ratio determinations were calculated partly to control for hyperinflation which accompanies normal lung expansion (development), abnormal expansion (emphysematous change), and abnormal deflation (atelectasis). For example, the ratio of numbers of Type 2 cells to alveolar wall area would not change with either hyperinflation or deflation alone, but if both numerator and denominator increased and the ratio remained constant, it would indicate some degree of atelectasis. Decreases in each would indicate alveolar expansion above the control or prior levels. With a measured decrease in Type 2 cell numbers and an increase in alveolar wall area, as occurred at 19 wks of age for the control animals, a decrease in the ratio would indicate a decrease for Type 2 cell numbers that cannot be attributed to air space change alone.

In all three experiments (M117, M156, & M171), the ratio of Type 2 Cell Number to Mean Alveolar Wall Area (minus Type 2 Cell Area) progressively fell, except for a 16 week-44 week plateau for the control animals of M156 (Charts 5&10). Since a fall in cell number/wall area ratio was both clearly and consistently present in the three experiments, it is apparent (as explained above) that a change in lung volume (numbers of alveoli per lung field) cannot by itself explain the altered relationship between Type 2 Cell number and alveolar wall area. However, there is the likelihood that a combination of increased lung expansion and increased alveolar wall thickness has played some role in both the cell number and wall area changes. An increase in alveolar airspace does occur in the maturing rat lung up to two months of age (11), and capillary blood volume up to 4 months of age (12). The significance of continuing development of the mouse lung at 44 weeks of age is the implication that the animals are at increased risk for adverse health effects. The findings of the present study support and extend the earlier M117 experimental finding (4) that lung development seems to be longer than the two months reported for the rat lung. More specifically, the data imply that the mouse lung does not fully mature until a

time between 22 and 44 weeks of age (M156), or between 16 weeks and 38 weeks of age (M171). Note the lack of plateaus for Mean Type 2 Cell Numbers (Charts 1&6), Mean Type 2 Cell Area (Charts 2&7), Mean Elastic Fiber Area (Chart 12), and two ratio transforms (Charts 10 & 16). It should be emphasized that complete consistency within the control animal group is not to be expected since they are by no means completely sheltered from noxious environmental influences and some indigenous organisms persist in all mouse colonies.

One finding was particularly consistent in all three studies (M117, M156, & M171) and warrants special attention, namely a decrease in Type 2 Cell number with time. Since mean Type 2 Cell Area was in all instances greater than the baseline value at the final test period (Charts 2&7), and an almost straight line increase in M156 (Chart 2) and M117 (4), it seems likely that the fall in number of cells is in large part due to cell aggregation. When one Type 2 Cell abuts against the border of another, it will be detected by the image analyzer as a single cell. Pertinently, some Type 2 Cell aggregates are normally present in alveoli, although most Type 2 Cells are individually situated in alveolar "corners". Moreover, there is a tendency for Type 2 Cell aggregates to concentrate in alveoli of the proximal portion of the acinus (28). At this time, we have not determined cell abutment/hypertrophy ratios. There is a special meaning for proximal acinus localization with respect to noxious agents, as is further discussed below.

2. EXPERIMENTAL ANIMAL DATA COMPATED TO THOSE OF CONTROL GROUPS

A. NO_2 EXPOSURE, TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS

A brief summary of the findings is the following. In both of the long term experiments, the present M171 study and the earlier M156, data from the 32 week postexposure period showed a number of significant and consistent differences between the control and experimental animal groups. **Type 2 Cell numbers** per lung field, while decreasing relative to the start of the experiment, were greater than those of the control group at statistically significant levels, i.e. for the NO_2 exposed young adult animals of M171 ($p=.002$; Table 9), and for the NO_2 exposed newborn animals of M156 ($p=.0001$; Table 27). In addition, the **ratios of Type 2 Cell numbers to alveolar wall area (minus Type 2 Cell area)** were also greater ($p=.08$; M171, and $p=.008$; M156). Further, **Mean Type 2 Cell area** was statistically greater for the exposed animals of both experiments, $p=.04$ (M171; Table 10) and $p=.05$ (M156; Table 28). The findings of the latter two experiments add strong support to the conclusions drawn from the earlier three test period experiment (M117) where very similar statistically significant differences and trends were found (4).

The cumulative findings from the three experiments (M171, M156, and M117) strongly imply that NO_2 exposure at an ambient level has altered both the epithelial cell populations of the mouse alveolus and the supporting tissues of the lung parenchyma (lung infrastructure or scaffolding). The finding that the control and experimental animal groups still had disparate measurements of cells and walls at the 32 week postexposure period further implies that the effects of NO_2 exposure have not been reversed despite a relatively long time for recovery. While there is clearly a need for a much longer follow up period, and at the same time a more definitive investigation of the nature of the lung alterations, it is nevertheless important to recognize that even a temporary alteration of cells and alveolar walls has biological significance. Pertinently, all of the alterations noted (changes in cell number and size, and an increase in alveolar wall area) are early events and common denominators for a great variety of human lung diseases.

Increases in Type 2 Cell number are now well recognized as a response to damage and/or loss of Type 1 alveolar cells, and an increased turnover can be expected to produce both more small cells and large cells, the latter through cell aggregation, work hypertrophy,

and cellular edema (with and without other alterations such as fatty change). With respect to the alveolar wall area, capillary congestion with cell and tissue edema are to be expected as early and multifocal pathobiologic events. Since the lung bidirectional transport mechanism and circulation in general have considerable reserve capacity, the magnitude of alteration often must be high before compensatory mechanisms are overcome and disease becomes clinically manifest. A heavier than normal loading of the bidirectional protein transport system with clinically covert interstitial fluid retention could of course readily account for increases in alveolar wall area that are detectable only by large volume quantitation.

That NO_2 exposure at ambient levels does in fact adversely affect the capillary bed and the bidirectional fluid transport system has the consistent support of several experiments investigating capillary permeability. Our own reports have been based on a number of tracers used to detect protein leakage, including radioactively labeled albumin (13), in situ plasma protein labeling (1), and horseradish peroxidase (2).

The increase in ratio of Type 2 Cells to alveolar wall area (minus Type 2 Cell area), attesting to replacement of Type 1 Cells by Type 2 Cells, is believed to be understated since alveolar wall area as well as Type 2 Cell number was greater for the exposed animals. Also, some cell crowding occurs as Type 2 Cells increase in number and two cells with abutting margins are read as one cell by the analyzer. Manual counting of Type 2 Cells in samples of the lung acinus (especially alveoli of the proximal acinus) is needed to determine abutment/hypertrophy ratios and thereby clarify the magnitude of the abutment phenomenon. Of relevance to human health, the replacement of Type 1 Cells in the human alveolus by Type 2 and bronchiolar cells ("bronchiolization") is an early finding and common denominator for essentially all destructive lung diseases (14).

A tentative interpretation of the data is that the early effect of NO_2 exposure is congestion and edema of the alveolar walls. The edema is probably associated with fraying and fragmentation of the alveolar wall in view of the greater increase in intercept and perimeter measurements compared to alveolar wall area. While the 10 week postexposure test period showed similar trends without statistically significant differences, the persistence of the trend throughout all three test periods and a number of statistically significant differences at the 32 week postexposure period, implies an adverse health effect that may not be entirely reversible. Of particular concern is the likelihood that the NO_2 related increases in Type 2 Cell numbers ($p<.002$) and mean area ($p<.04$) at the final test period reflect alteration and/or loss of Type 1 Cells. Further, the increase in Type 2 cell size could be in part injury rather than a compensatory, work related change. Since the alveolar wall area increase seen in the frozen section preparations may in part be due to some atelectasis as well as congestion and edema, an alteration of surfactant production by the Type 2 Cell is suspect. Moreover, the Type 2 cell alteration may be understated since the proximal portion of the lung acinus tends to have the greatest degree of injury and we included the entire acinus in our measurements.

When the second part of the study was completed, i.e. the use of formalin fixed lung tissue for the quantitation of alveolar wall area and elastic tissue, alterations were again found. For alveolar wall area, there was a trend essentially the reverse of that seen with the frozen section lung tissue. While wall area initially increased for the exposed animals (1.2% at the 0 week postexposure test), the second test period showed a 14.8% decrease, and the third and final test a 4.8% decrease ($p<.06$). The reversal is readily explained by differences in tissue processing. The frozen section preparation essentially maintains the in vivo state whereas the processing of formalin fixed tissue fails to preserve some abnormalities maintained by freezing the lung. Specifically, the organic solvents used in the paraffin preparation not only remove the normal water content of the alveolar wall, but also the greater part of watery, proteinaceous, and fatty substances associated with pulmonary edema. Even with the processing of normal tissue, paraffin embedment ordinarily

results in a 50% tissue shrinkage. Edematous tissue would be expected to shrink relatively more in view of cell and tissue degradory products that accompany the edema, as for example fatty change. With the foregoing in mind, the finding with the paraffin sections of statistically significant or near significant differences in various alveolar wall measurements throughout the three test periods is supporting evidence for an NO_2 effected pulmonary edema. Presumably, congestion of alveolar capillaries accompanies the putative edema, and so does alteration of the elastica as discussed below. Alteration of the alveolar wall, with evidence that the damage may not be entirely reversible, has special significance for recovery of the respiratory units. Architectural integrity of alveolar walls (i.e. of the lung scaffolding) is essential for regeneration of the epithelial and endothelial cell linings, and for proper ventilation-perfusion (blood) relationships of those units.

With respect to the study of elastic tissue in the paraffin sections, the results of the control animal study imply that both elastic fiber number and fiber area change with time as a part of normal lung development. NO_2 exposure appears to alter elastic fiber development, but the nature of the alterations is not clear at this time. A consistent finding throughout the three test periods was an increase in the ratio of elastic fiber numbers to alveolar wall area, and this became statistically significant at the final test period ($p<.003$). The latter consistency occurred despite a reversal in the numbers of fibers at the 10 week test period, i.e. a greater number of fibers for the control animals ($p=.01$), and in association with a marked decrease in alveolar wall area for the exposed animals ($p=.1$). As mentioned earlier, the trend towards a decrease in alveolar wall area for the exposed animals was accentuated at the final test period (wall area without the inclusion of elastic area; $p<.06$).

The differences observed in elastic fiber numbers and the reversal at 10 weeks postexposure is probably the result of an interplay of factors, in particular fragmentation, fraying, and edematous change superimposed on developmental changes. Thus, noxious effects may result in either an increase or a decrease in elastic fiber number and size depending on the balance between reactive proliferation and developmental factors on one hand and degenerative swelling and scarring processes on the other hand. In the human experience, tissue damage is often associated with an increase in elastic tissue ("elastosis"), but the elastica is often abnormal in both composition and function ("pseudoelastosis") and may include altered collagen (15,16). The finding of an increased ratio of elastic fiber numbers to alveolar wall area throughout the study and with statistical significance at the final test period ($p<.003$) implicates alterations of both elastic tissue and non-elastic components of the alveolar wall. In the latter respect, an increase in elastic fiber number, area, and linear intercepts at near statistical levels ($p=.09$ to $P<.1$) occurred at a time when alveolar wall tissue other than elastica was significantly decreased ($p<.06$). Pertinently, degenerative change in general, e.g. skin and breast (15,16) commonly leads to an increase rather than decrease in elastic tissue, although the elastica may be abnormal in nature. The combined results of the Type 2 Cell and elastic studies imply a possible acceleration in the depletion of structural and functional reserves of the lung. The relevance to human health is that the well population in general now experiences a chronologic decrement in both structure and function of the lung due to diverse noxious agents, the majority of which remain to be identified. Thus, the conservation of human lung reserves is of paramount importance in the setting of air quality standards. It seems reasonable to conclude from our total experimental and human pathology experience to date, and the literature in general, that NO_2 at levels from 0.25ppm to 0.5ppm may presently be playing some role in the causation, promotion, facilitation, and/or exacerbation of lung reserve depletion. Aside from the well population concern, it should be noted that reserve depletion is a common denominator for all destructive lung diseases.

A final consideration with respect to the lung is the implication from the control animal data that the lung was in a developmental process during the greater part of the investigation. Both control and exposed animal lungs had an absolute fall in Type 2 Cell num-

bers with time, and there was an accompanying absolute increase in cell area. An expanding alveolus and an enlarging Type 2 Cell population have probably played some role in these measurement changes.

B. ELASTIN STUDIES

With respect to the control mouse lung study itself, there are no reported morphometric studies on elastin fibers for comparison or confirmation of the present findings. From the data on hand, we believe that developmental remodeling of elastin has occurred throughout the experimental period in view of the changing values of both numbers of elastin fibers and alveolar wall area (Charts 11-13). In support of developmental change is the almost 200% rise in elastin at 16 weeks of age (**elastic fiber field area**; Chart 13). Moreover, elastin appeared to increase disproportionately to **alveolar wall area** (52.3% increase, excluding elastin content; Chart 15). At 41 weeks of age, elastin and wall area values had fallen and seemed to be approaching baseline values were (Charts 13 & 15). Since **Mean Elastic Fiber Area** (area per lung field/numbers per lung field) increased by 120% at 16 weeks of age (Chart 12) whereas fiber number increased by only a third as much (41%; Chart 11), it would appear that the increase in elastin fiber **area** may in large part represent a thickening and/or fusion of elastin fibers.

With respect to elastic tissue measurements of the NO_2 exposed animals, the data suggest that both elastin and the alveolar wall in general have been adversely affected. The basis for this is the following:

1) the numbers of elastic fibers increased by 12.1% after 6 weeks of exposure (Chart 11), and so did mean area of the fibers (by 6.7%; Chart 12). Both fibers and area then fell short of control values at 16 weeks of age (Charts 11&12), implying a lag in development, and then, as control levels fell, remained higher (38 weeks of age; Charts 11 & 12)., suggesting a slower elastin remodeling. The differences observed at the 0 week and 10 week postexposure periods were statistically significant, p=.001 and p=.01, respectively, and of borderline significance at 32 weeks postexposure (Tables 11 & 14). Possible explanations in terms of the fibers themselves are an initial fragmentation followed by suboptimal growth of both fragmented and newly developed small fibers, i.e. the coalescence, remodeling, and/or growth of the fibers are slowed by the NO_2 exposure.

2) the mean area of the elastic fibers showed NO_2 related differences similar in direction to those for numbers of fibers. (Table 15). Presumably, the trend again reflects an initial increase in the thickness of elastic fibers due to edematous and/or other change, and also a subsequent lag in the development of the elastic network.

3) alveolar wall area (with and without inclusion of elastic tissue area) essentially paralleled elastic tissue differences between groups. There was a lagging of NO_2 related findings behind those of the control group at both postexposure periods, i.e. at a borderline level of statistical significance at the 10 weeks postexposure test period (alveolar wall area minus elastic tissue; p=.1; Table 14), and at the 94 percentile level (p=.06) for the 32 week postexposure period (Table 17). The wall area changes are, as mentioned above, suggestive of edema of connective tissue and congestion of lung capillaries, with both impacting adversely on developmental growth of the alveolar wall.

A consistent finding throughout the three test periods was an increase in the **ratio of elastic fiber numbers to alveolar wall area**. The ratio defines a relationship that is independent of lung volume. At the final test period, the ratio became statistically significant (Chart ; p<.003). The latter consistency occurred despite a reversal in the numbers of fibers at the 10 week test period, i.e. a greater number of fibers for the control animals (p=.01), and in association with a marked decrease in alveolar wall area for the exposed

animals ($p = .1$). As mentioned earlier, the trend towards a decrease in alveolar wall area for the exposed animals was accentuated at the final test period (wall area without the inclusion of elastic area; $p < .06$).

The differences observed in elastic fiber numbers and the reversal at 10 weeks postexposure is probably the result of an interplay of factors, in particular fragmentation, fraying, and edematous change superimposed on developmental changes. Thus, noxious effects may result in either an increase or a decrease in elastic fiber number and size depending on the balance between reactive proliferation and developmental factors on one hand and degenerative change, edema, and scarring processes on the other hand.

An NO_2 induced edematous change of connective tissue is strongly suggested by the disparate findings using paraffin embeded lung tissues and tissue sections processed via frozen section. Specifically, an NO_2 induced injury to the alveolar wall may be associated with a shift towards a more labile and soluble tissue protein. Thus, a tissue processing method that includes water extraction by alcohol, fat extraction by xylene, and embedment in heated paraffin will extract the more labile and soluble tissue components along with the water content. It should be noted that paraffin embedment ordinarily shrinks tissue to approximately 50% of in vivo volume. Removing edema fluid that includes tissue proteins will reduce the amount of alveolar wall tissue remaining below control animal levels. Conversely, frozen sections of the lung, preparations that are close to the in vivo state, will preserve the fluid excess in cells, tissues, and vascular channels of the lung, and will exhibit alveolar walls that are increased in thickness.

With respect to edema, several of our prior studies using molecular probes have demonstrated an NO_2 induced protein leakage and overload of the bidirectional fluid transport system of the lung (capillary leakage). The findings at the final test period (32 weeks postexposure) point to both alterations of the alveolar wall in general and a lack of reversal of the alterations, i.e. NO_2 induced edema of the wall with increased shrinkage (alveolar wall area minus elastic tissue; $p < .06$), increased elastic fiber numbers ($p < .1$) and perimeter ($p < .1$), and increases in the ratios of fiber **numbers** and fiber **areas** to **alveolar wall area minus elastic area**, $p = .002$ and $p < .02$, respectively). The finding that elastic fiber number was greater for the exposed animals at the 32 week test and alveolar wall area was less than that of the control animals tends to implicate damage to the non-elastic portions of the alveolar wall. In the latter respect, it is pertinent to note that a consistent finding throughout the three test periods was a greater ratio of elastic fiber numbers to the alveolar wall area with or without deletion of the elastic component of the alveolar wall.

Experimental Design

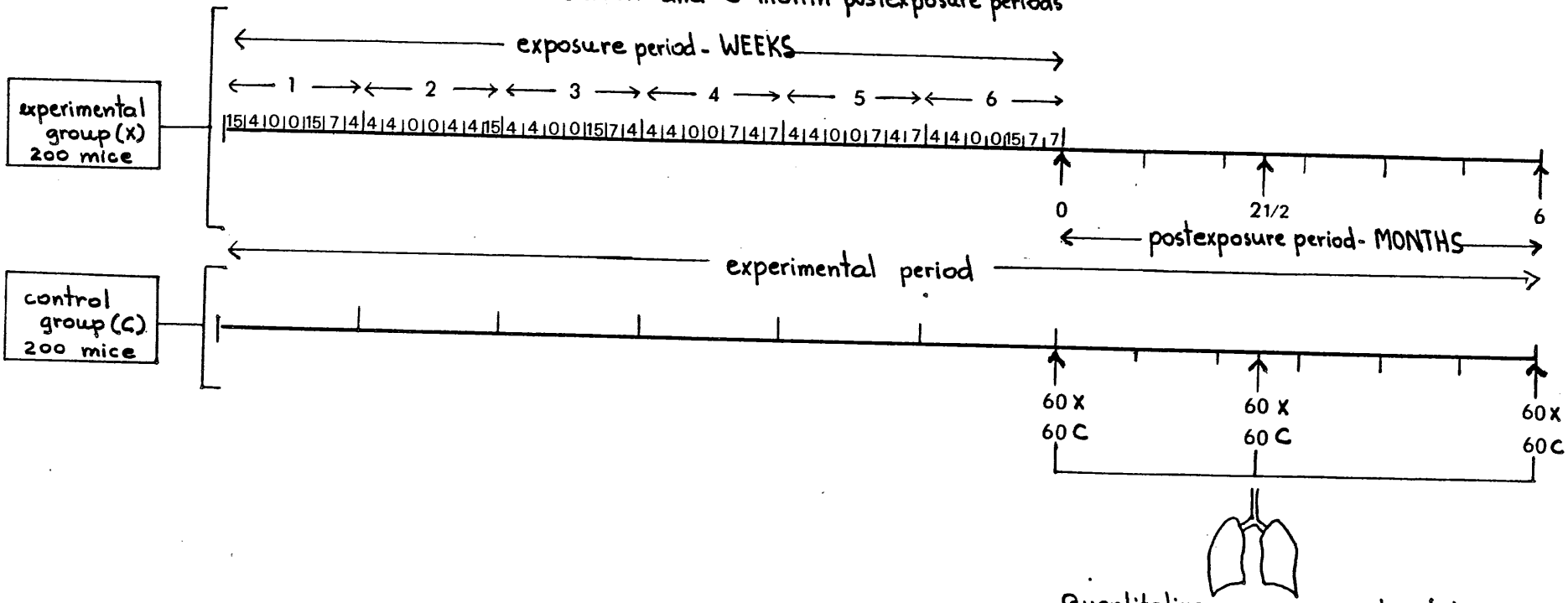
Swiss Webster Weanling Male Mice (≥12gms)

0.25 ± 0.05 ppm NO_2 exposure

15 hrs, 7 hrs or 4 hrs/day*, 5 days/week for 6 weeks

and

10 week and 6 month postexposure periods



Quantitative measurements of lungs:

A. Left - 1. connective tissue
   a. collagen
   b. elastin
   2. lung volume

B. Right - 1. upper lobe - alveolar wall, 1 μM
   2. lower lobe - LDH / Type 2 cell and alveolar wall
   3. cardiac lobe - ultrastructure

* HOURS/DAY= 210 ; ASSIGNMENT BY RANDOM GENERATION

TABLE 1
Mean Value of Body and Spleen Weights, and Lung Volume
Swiss Webster Young Adult Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide; 210 Hours Total Exposure
15, 7 or 4 hours ± 1/2 hrs/day, 5 days/week, and 6 week exposure period
with
10 week and 32 week postexposure periods
(M171)

Measurements	Control Group		Experimental Group		DF	t-statistic	p value two tail
	Number Animals	M ± SD	Number Animals	M ± SD			
0 Week Postexposure Period							
Body weight (gms)	60	27.102 ± 2.387	60	27.123 ± 2.379	118	0.0498	NS
Spleen weight (gms)	60	0.099 ± 0.027	60	0.092 ± 0.016	118	1.5986	NS
Spleen/body weight ratio (%)	60	0.368 ± 0.116	60	0.341 ± 0.052	118	1.6438	NS
Left lung volume (uL)	58	127.362 ±24.973	60	130.950 ±16.897	116	0.9168	NS
10 Week Postexposure							
Body weight (gms)	60	32.77 ± 3.51	60	34.58 ± 2.32	118	3.3365	0.015
Spleen weight (gms)	60	0.102 ± 0.027	60	0.114 ± 0.023	118	2.8029	0.006
Spleen/body weight ratio (%)	60	0.310 ± 0.071	60	0.331 ± 0.064	118	1.7131	0.085
Left lung volume (ul)	59	184.70 ± 24.93	59	187.05 ± 26.24	116	0.4999	NS
32 Week Postexposure							
Body weight (gms)	76	38.11 ± 4.39	76	38.54 ± 2.38	150	0.6154	NS
Spleen weight (gms)	76	0.1131± 0.0238	75	0.128 ± 0.041	149	0.4612	NS
Spleen/body weight ratio (%)	76	0.344 ± 0.098	75	0.332 ± 0.104	149	0.7067	NS
Left lung volume (ul)	59	240.58 ± 37.76	76	244.25 ± 26.35	150	0.6164	NS

M - mean
SD - standard deviation
DF - degrees of freedom

Table 2

Frozen Section Cutting (Right Lower Lobe)
and
Lactate Dehydrogenase Staining Workload

0.25 ± 0.05 ppm Nitrogen Dioxide
7 ± 1/2hr/day, 5days/week and 6 weeks with total 210 hour exposure
with
10 and 32 Week Postexposure Periods
(M171)

Postexposure period (weeks)	Control Group		Experimental Group	
	Number Animals	Number Slides	Number Animals	Number Slides
0	60	360	60	360
10	60	360	60	360
32	76	456	76	456
TOTAL	196	1,176	196	1,176

Table 3

IMAGE ANALYSIS WORK LOAD OF TYPE 2 CELLS AND ALVEOLAR WALLS
Swiss Webster Young Adult Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week and and for 6 weeks with total 210 hour exposure
with
10 and 32 Week Postexposure Period
(M171)

Postexposure Period (Weeks)	Animal Group							
	Control				Experimental			
	Number				Number			
	Lungs (Animals)	Lung Sections	Fields	Data Points	Lungs (Animals)	Lung Sections	Fields	Data Points
0	59	236	708	18,285	60	240	720	18,000
10	60	240	720	19,440	59	236	708	18,701
32	76	304	912	23,670	76	304	912	23,643

Table 4

Image Analysis Work Load of Elastin and Alveolar Walls
Swiss Webster Young Adult Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week and and for 6 weeks with total 210 hour exposure
with
10 and 32 Week Postexposure Period
(M171)

Postexposure Period (Weeks)	Animal Group							
	Control				Experimental			
	Number				Number			
	Lungs (Animals)	Lung Sections	Fields	Data Points	Lungs (Animals)	Lung Sections	Fields	Data Points
0	57	228	663	9,945	59	228	650	9,750
10	60	228	628	9,420	60	232	634	9,510
32	74	286	793	11,895	71	280	817	12,255

Table 5

TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total of 210 hours exposure
Swiss Webster young adult male mice
(171)

0 WEEK POSTEXPOSURE PERIOD

Measurements	Control Group N=58 Mean ± SD	Experimental Group N=59 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS					
1) ≥ 8 uM	171.01 ± 29.72	175.51 ± 27.20	+ 2.63	0.86	NS
2) ≥10 uM	133.44 ± 54.02	131.48 ± 18.71	- 1.47	−0.26	NS
3) ≥12 uM	111.16 ± 19.73	115.18 ± 16.12	+ 3.62	1.21	0.2304
4) ≥ 8 um - <12 uM	59.85 ± 12.35	60.33 ± 13.25	+ 0.80	0.21	NS
II. TYPE 2 CELL (≥8 uM) FIELD AREA	8679.15 ± 2466.47	9253.65 ± 2176.17	+ 6.62	1.34	0.1845
IV. ALVEOLAR WALL					
1) Area	97579.62 ± 22934.42	101185.13 ± 22869.51	+ 3.69	0.85	NS
2) Area - Type 2 cells area (≥8 uM)	88900.46 ± 21516.37	91931.47 ± 22286.86	+ 3.41	0.75	NS
3) Linear Intercept	33947.22 ± 4772.81	36119.84 ± 8397.46	+ 6.40	1.72	0.0895
4) Perimeter	10831.60 ± 1627.17	11610.05 ± 2945.16	+ 7.19	1.77	0.0809

area in pixel units
SD - standard deviation

Table 6

RATIOS OF TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week and 6 weeks with a total 210 exposure hours
Swiss Webster young adult male mice
(171)

0 WEEKS POSTEXPOSURE PERIOD

Measurements Edited Data	Control Group N=58 Mean ± SD	Experimental Group N=58 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS (≥8uM)* / ALVEOLAR WALL AREA	1.81 ± 0.36	1.88 ± 0.46	0	-0.02	NS
II. TYPE 2 CELL (≥ 8 uM) FIELD AREA / TYPE 2 CELL (≥ 8 uM) NUMBER	50.63 ± 11.10	53.01 ± 11.21	+ 4.70	1.15	NS
III. TYPE 2 CELL MEAN AREA* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	5.96 ± 1.78	6.08 ± 1.85	+ 2.01	0.35	NS
IV. TYPE 2 CELL NUMBER (≥12 uM)* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	0.13 ± 0.03	0.13 ± 0.03	0	0.37	NS

* ratio x 100

Table 7

TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total of 210 hours exposure
Swiss Webster young adult male mice
(171)

10 WEEK POSTEXPOSURE PERIOD

Measurements	Control Group N=58 Mean ± SD	Experimental Group N=58 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS					
1) ≥ 8 uM	164.63 ± 25.79	165.53 ± 24.99	+ 0.55	0.19	NS
2) ≥10 uM	125.57 ± 17.50	126.08 ± 16.73	+ 0.41	0.16	NS
3) ≥12 uM	112.00 ± 15.13	112.73 ± 14.22	+ 0.65	0.27	NS
4) ≥ 8 um - <12 uM	52.63 ± 12.83	52.80 ± 12.52	+ 0.32	0.07	NS
II. TYPE 2 CELL (≥8 uM) FIELD AREA	11116.70 ± 2530.20	11438.46 ± 2462.23	+ 2.89	0.69	NS
IV. ALVEOLAR WALL					
1) Area	111819.73 ± 19579.37	114118.69 ± 16753.14	+ 2.06	0.68	NS
2) Area - Type 2 cells area (≥8 uM)	100703.01 ± 18936.13	102680.22 ± 16510.44	+ 1.96	0.60	NS
3) Linear intercept	36847.89 ± 6865.79	37678.23 ± 5866.50	+ 2.25	0.70	NS
4) Perimeter	11833.20 ± 2196.91	12135.00 ± 1944.71	+ 2.55	0.78	NS

area in pixel units
SD - standard deviation

Table 8

RATIOS OF TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
Swiss Webster Young Adult Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week and 6 weeks with a total 210 exposure hours
(171)

10 WEEKS POSTEXPOSURE PERIOD

Measurements Edited Data	Control Group N=58 Mean ± SD	Experimental Group N=58 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS (>8uM)* / ALVEOLAR WALL AREA	1.51 ± 0.36	1.48 ± 0.30	- 1.99	-0.55	NS
II. TYPE 2 CELL (≥ 8 uM) FIELD AREA / TYPE 2 CELL (≥ 8 uM) NUMBER	68.09 ± 14.15	69.73 ± 13.57	+ 2.41	0.63	NS
III. TYPE 2 CELL MEAN AREA* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	6.99 ± 1.92	7.01 ± 2.09	+ 0.29	0.06	NS
IV. TYPE 2 CELL NUMBER (≥12 uM)* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	0.11 ± 0.03	0.11 ± 0.02	0	-0.46	NS

* ratio x 100

Table 9

TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total of 210 hours exposure
Swiss Webster young adult male mice
(171)

32 WEEK POSTEXPOSURE PERIOD

Measurements	Control Group N=74 Mean ± SD	Experimental Group N=76 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS					
1) ≥ 8 uM	146.34 ± 20.85	158.69 ± 27.01	+ 8.44	3.14	0.0021
2) ≥10 uM	109.59 ± 15.12	119.06 ± 19.31	+ 8.64	3.35	0.0010
3) ≥12 uM	97.82 ± 13.56	106.44 ± 17.52	+ 8.81	3.38	0.0009
4) ≥ 8 um - <12 uM	48.52 ± 10.16	52.24 ± 12.94	+ 7.67	1.96	0.0517
II. TYPE 2 CELL (≥8 uM) FIELD AREA	10368.80 ± 2188.39	12106.83 ± 3309.43	+ 16.76	3.80	0.0002
IV. ALVEOLAR WALL					
1) Area	107364.13 ± 19350.75	111525.39 ± 20578.41	+ 3.88	1.28	0.2043
2) Area - Type 2 cells area (≥8 uM)	96995.30 ± 19734.20	99414.55 ± 19766.37	+ 2.49	0.77	NS
3) Linear intercept	36641.96 ± 5921.38	37565.63 ± 5518.75	+ 2.52	0.99	NS
4) Perimeter	11882.21 ± 1969.98	12181.61 ± 1838.11	+ 2.52	0.96	NS

area in pixel units
SD - standard deviation

Table 10
RATIOS OF TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
Swiss Webster Young Adult Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
5 days/week and 6 weeks with a total of 210 hours exposure
(171)

32 WEEKS POSTEXPOSURE PERIOD

Measurements Edited Data	Control Group N=67 Mean ± SD	Experimental Group N=60 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS (≥8uM)* / ALVEOLAR WALL AREA	1.39 ± 0.25	1.45 ± 0.28	+ 4.32	1.43	0.1548
II. TYPE 2 CELL (≥ 8 uM) FIELD AREA / TYPE 2 CELL (≥ 8 uM) NUMBER	71.27 ± 13.08	76.73 ± 18.01	+ 7.66	2.13	0.0351
III. TYPE 2 CELL MEAN AREA* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	7.65 ± 2.07	8.11 ± 2.82	+ 6.01	1.14	NS
IV. TYPE 2 CELL NUMBER (≥2 uM)* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	0.10 ± 0.02	0.11 ± 0.03	+ 10.00	1.75	0.0814

* ratio x 100

Table 11
MEAN ELASTIC FIBER AND ALVEOLAR WALL MEASUREMENTS
0.25 ± ppm Nitrogen Dioxide
5/days/week for 6 weeks with a total 210 exposure hours
(171)

0 WEEK POSTEXPOSURE PERIOD

Measurements	Experimental Group N=58 Mean ± SD	Control Group N=57 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
ELASTIC FIBERS					
Number	605.27 ± 149.20	718.47 ± 125.96	+12.08	3.37	0.0010
Area > 0um	12483.64 ± 3303.65	10976.60 ± 2559.77	+13.73	2.73	0.0073
Area > 8um	8724.19 ± 2505.36	7576.87 ± 1942.86	+15.14	2.74	0.0071
Area > 9um	6263.25 ± 2002.76	5392.94 ± 1555.83	+16.14	2.60	0.0106
Area >12um	3180.91 ± 1228.84	2684.92 ± 927.88	+18.47	2.44	0.0163
Area >14um	2312.97 ± 941.48	1943.98 ± 712.03	+18.98	2.37	0.0196
Area >16um	215.43 ± 123.83	204.24 ± 87.25	+25.06	2.56	0.0118
Linear Intercept	12669.16 ± 2788.76	11349.02 ± 2135.98	+11.63	2.85	0.0053
Perimeter	3737.12 ± 832.88	3353.57 ± 651.03	+11.44	2.75	0.0077
ALVEOLAR WALL					
Area	106207.72 ±21786.42	103639.63 ±29252.60	+ 2.48	0.53	NS
Area - Elastic Fiber Area	93724.09 ±21570.77	92663.05 ±29064.78	+ 1.15	0.22	NS
Perimeter	53471.62 ± 7539.74	56827.92 ±10463.13	- 5.91	-1.97	-0.0512

Measurements in pixel units
SD = standard deviation

Table 12
MEAN RATIOS OF ELASTIC FIBERS AND ALVEOLAR WALL
0.25 ± ppm Nitrogen Dioxide
5/days/week for 6 weeks with a total 210 exposure hours
(171)

0 WEEK POSTEXPOSURE PERIOD

RATIOS	Experimental Group N=58 Mean ± SD	Control Group N=57 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
Number of Elastic Fibers* / Wall Area	0.79 ± 0.22	0.75 ± 0.25	+ 5.33	0.94	NS
Number of Elastic Fibers* / Wall Area- Elastic Fiber Area	0.91 ± 0.29	0.86 ± 0.31	+ 5.81	0.96	NS
Elastic Fiber Area > 0um / Number of Elastic Fibers	15.57 ± 3.35	15.38 ± 2.91	+ 1.24	0.32	NS
Elastic Fiber Area >0um / Wall Area	0.13 ± 0.04	0.11 ± 0.04	+ 7.69	1.20	NS
Elastic Fiber Area >0um / Wall Area-Elastic Fiber Area	0.14 ± 0.05	0.13 ± 0.05	+ 8.86	1.17	NS

* x 100
SD = standard deviation

Table 13
COMPUTED MEAN ELASTIC FIBER AREAS
0.25 ± ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total 210 exposure hours
(171)

0 WEEK POSTEXPOSURE PERIOD

ELASTIC FIBER AREA MEASUREMENTS	Experimental Group N=58 Mean ± SD	Control Group N=57 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
Area 0um - < 8um	3759.45 ± 916.74	3399.72 ± 754.79	+10.58	2.74	0.0235
Area 8um - < 9um	2460.95 ± 652.49	2183.93 ± 517.97	+12 68	2.52	0.0132
Area 9um - <12um	3082.34 ± 854.06	2708.02 ± 674.65	+13.82	2.61	0.0104
Area 12um - <14um	867.94 ± 323.45	740.93 ± 251.02	+17.14	2.35	0.0205
Area 14um - <16um	2057.54 ± 850.29	1739.74 ± 650.16	+18.27	2.25	0.0265
Area 12um - <16um	2925.47 ± 1145.03	2480.675 ± 869.49	+17.93	2.34	0.0209
Area 0um - <16um	12228.21 ± 3223.19	10772.35 ± 2505.92	+13.51	2.70	0.0080

Measurements in pixel units
SD = standard deviation

Table 14
MEAN ELASTIC FIBER AND ALVEOLAR WALL MEASUREMENTS
0.25 ± ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total 210 exposure hours
(171)

10 WEEK POSTEXPOSURE PERIOD

Measurements	Experimental Group N=57 Mean ± SD	Control Group N=57 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
ELASTIC FIBERS					
Number	931.88 ± 143.16	1011.67 ± 162.20	- 8.56	-2.52	-0.0130
Area > 0um	27861.05 ± 8361.33	32865.19 ±10031.55	-15.22	-2.89	-0.0046
Area > 8um	21704.45 ± 7074.02	25974.92 ± 8675.89	-16.44	-2.88	-0.0048
Area > 9um	16675.12 ± 5900.34	20281.01 ± 7449.48	-17.78	-2.86	-0.0050
Area >12um	9799.51 ± 4071.99	12384.31 ± 5408.27	-20.87	-2.88	-0.0048
Area >14um	7693.85 ± 3440.93	9819.96 ± 4634.03	-21.65	-2.78	-0.0064
Area >16um	1076.85 ± 846.32	1535.86 ± 1182.10	-29.89	-2.38	-0.0188
Linear Intercept	20757.18 ± 4787.43	23346.11 ± 5112.30	-11.09	-2.79	-0.0062
Perimeter	6114.40 ± 1510.88	6835.83 ± 1539.66	-10.55	-2.52	-0.0130
ALVEOALAR WALL					
Area	148460.66 ±62796.49	174380.41 ±85494.48	-14.85	-1.84	-0.0682
Area - Elastic Fiber Area	120619.56 ±59223.35	141515.20 ±79829.17	-14.77	-1.59	-0.1153
Perimeter	48215.10 ± 4536.35	48694.19 ± 4299.05	- 1.00	-0.58	NS

Measurements in pixel units
SD = standard deviation

Table 15
RATIOS OF MEAN ELASTIC FIBER AND ALVEOLAR WALL MEASUREMENTS
0.25 ± ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total 210 exposure hours
(171)

10 WEEK POSTEXPOSURE PERIOD

Measurements	Experimental Group N=57 Mean ± SD	Control Group N=57 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
Number of Elastic Fibers* / Wall Area	0.72 ± 0.27	0.70 ± 0.30	+ 2.86	0.44	NS
Number of Elastic Fibers* / Wall Area-Elastic Fiber Area	0.95 ± 0.44	0.93 ± 0.18	+ 2.15	0.25	NS
Elastic Fiber Area > 0um / Number of Elastic Fibers	29.52 ± 6.01	32.76 ± 9.51	- 9.89	-2.17	-0.0325
Elastic Fiber Area >0um / Wall Area	0.21 ± 0.08	0.21 ± 0.08	0	-0.44	NS
Elastic Fiber Area >0um / Wall Area-Elastic Fiber Area	0.27 ± 0.15	0.29 ± 0.18	- 7.41	-0.50	NS

* x 100
SD = standard deviation

Table 16
COMPUTED MEAN ELASTIC FIBER AREAS
0.25 ± ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total 210 exposure hours
(171)

10 WEEK POSTEXPSOURE PERIOD

ELASTIC FIBER AREA MEASUREMENTS	Experimental Group N=57 Mean ± SD	Control Group N=57 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
Area 0um - < 8um	6156.60 ± 1565.15	6890.27 ± 1631.78	-10.65	-2.45	-0.0158
Area 8um - < 9um	5029.33 ± 1331.14	5693.91 ± 1388.10	-11.68	-2.61	-0.0103
Area 9um - <12um	6875.61 ± 2003.06	7896.70 ± 2236.06	-12.93	-2.57	-0.0115
Area 12um - <14um	2105.66 ± 700.70	2564.35 ± 818.19	-17.86	-3.21	-0.0017
Area 14um - <16um	6616.99 ± 2515.98	8284.09 ± 3607.71	-20.12	-2.79	-0.0062
Area 12um - <16um	8722.66 ± 3376.42	10848.45 ± 4399.75	-19.59	-2.88	-0.0046
Area 0um - <16um	26784.20 ± 7847.45	31329.33 ± 9201.98	-14.51	-2.84	-0.0054

Measurements in pixel units
SD = standard deviation

Table 17
MEAN ELASTIC FIBER AND ALVEOLAR WALL MEASUREMENTS
0.25 ± ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total 210 exposure hours
(171)

32 WEEK POSTEXPOSURE PERIOD

Measurements	Experimental Group N=73 Mean ± SD	Control Group N=74 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
ELASTIC FIBERS					
Number	844.55 ± 160.23	796.527 ± 180.65	+ 6.02	1.70	0.0902
Area > 0um	17056.61 ± 6142.50	15491.18 ± 6360.81	+10.11	1.52	NS
Area > 8um	12488.76 ± 4779.49	11172.70 ± 4865.94	+11.78	1.65	0.1003
Area > 9um	12086.27 ± 5304.28	10958.21 ± 5628.06	+10.29	1.25	NS
Area >12um	7012.25 ± 3532.75	6319.31 ± 3752.69	+10.97	1.15	NS
Area >14um	5466.36 ± 2900.53	4917.20 ± 3074.09	+11.16	1.11	NS
Area >16um	907.07 ± 666.35	796.49 ± 672.85	+13.94	1.00	NS
Linear Intercept	15496.41 ± 4390.21	14366.14 ± 4740.49	+ 7.87	1.50	NS
Perimeter	4707.81 ± 1365.47	4315.84 ± 1425.30	+ 9.08	1.70	0.0909
ALVEOLAR WALL					
Area	113321.36 ±16404.66	116567.95 ±17463.88	- 2.78	-1.13	NS
Area - Elastic Fiber Area	96264.75 ±15931.62	101076.77 ±14630.80	- 4.76	-1.91	-0.0584
Perimeter	48913.14 ± 4242.59	49549.75 ± 3890.54	- 1.28	-0.95	NS

Measurements in pixel units
SD = standard deviation

Table 18
RATIOS OF ELASTIC FIBER AND ALVEOLAR WALL MEASUREMENTS
0.25 ± ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total 210 exposure hours
(171)

32 WEEK POSTEXPOSURE PERIOD

Measurements	Experimental Group N=73 Mean ± SD	Control Group N=74 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
Number of Elastic Fibers* / Wall Area	0.76 ± 0.16	0.69 ± 0.13	+10.14	3.03	0.0029
Number of Elastic Fibers* / Wall Area-Elastic Fiber Area	0.90 ± 0.23	0.80 ± 0.18	+12.50	3.12	0.0022
Elastic Fiber Area > 0um / Number of Elastic Fibers	19.88 ± 4.43	19.03 ± 4.34	+ 4.47	1.17	NS
Elastic Fiber Area >0um / Wall Area	0.15 ± 0.05	0.13 ± 0.04	+15.38	2.59	0.0105
Elastic Fiber Area >0um / Wall Area-Elastic Fiber Area	0.18 ± 0.08	0.15 ± 0.06	+20.00	2.48	0.0145

* x 100
SD = standard deviation

Table 19
COMPUTED MEAN ELASTIC FIBER AREAS
0.25 ± ppm Nitrogen Dioxide
5 days/week for 6 weeks with a total 210 exposure hours
(171)

32 WEEK POSTEXPOSURE PERIOD

ELASTIC FIBER AREA MEASUREMENTS	Experimental Group N=73 Mean ± SD	Control Group N=74 Mean ± SD	Difference (%)	t-statistic	p value (2 tail)
Area 0um - < 8um	4567.86 ± 1448.48	4318.48 ± 1570.22	+ 5.77	1.00	NS
Area 8um - < 9um	402.49 ± 3093.78	214.50 ± 2566.18	+87.44	0.40	NS
Area 9um - <12um	5074.02 ± 1829.39	4638.89 ± 1928.75	+ 9.38	1.40	NS
Area 12um - <14um	1545.89 ± 676.69	1402.11 ± 733.76	+10.27	1.24	NS
Area 14um - <16um	4559.29 ± 2281.57	4120.71 ± 2438.54	+10.63	1.13	NS
Area 12um - <16um	6105.18 ± 2920.29	5522.82 ± 3127.70	+10.54	1.17	NS
Area 0um - <16um	16149.55 ± 5800.55	14694.68 ± 5884.92	+ 9.89	1.51	NS

Measurements in pixel units
SD = standard deviation

TABLE 20

Image Analysis Work Load of Type 2 Cells and Alveolar Wall Structures
Swiss Webster Newborn Male Mice
0.35 ± 0.05 ppm Nitrogen Dioxide
7 ± 1/2 hr/day, 5 days/week and 12 weeks
with
4, 10, and 32 week postexposure periods
(156)

Postexposure Period (Weeks)	Animal Group							
	Control				Experimental			
	Number				Number			
	Lungs (Animals)	Lung Sections	Fields	Data Points	Lungs (Animals)	Lung Sections	Fields	Data Points
0	34	136	544	9,792	34	136	544	9,972
4	34	136	544	9,792	34	136	544	9,972
10	33	132	528	9,504	35	140	560	10,044
32	65	257	1,021	18,378	56	224	896	16,128
TOTAL	166	661	2,637	47,466	159	636	2,544	45,756

Table 21

TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
0.35 + 0.05 ppm NO2
7±1/2 hrs/day, 5 days/week, 12 weeks
Swiss Webster Newborn Male Mice
(M156)

0 WEEK POSTEXPOSURE PERIOD

Measurements	Control Group N=26 Mean ± SD	Experimental Group N=26 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS					
1) ≥ 8 uM	611.41 ± 133.59	806.46 ± 126.04	+ 0.61	0.14	NS
2) ≥12 uM	447.74 ± 91.90	441.58 ± 76.93	+ 1.39	0.26	NS
3) ≥ 8 um - <12 uM	363.67 ± 49.00	364.88 ± 56.30	- 0.33	-0.08	NS
II. TYPE 2 CELL (≥8 uM) FIELD AREA	18415.52 ± 5186.94	18067.80 ± 4459.48	+ 1.92	0.26	NS
III. TYPE 2 CELL LINEAR INTERCEPT	5834.70 ± 1290.65	5772.67 ± 1139.28	+ 1.07	0.18	NS
IV. ALVEOLAR WALL					
1) Area	75110.53 ± 14954.66	73424.28 ± 13960.80	+ 2.30	0.42	NS
2) Area - Type 2 cells area (≥8 uM)	56695.01 ± 11316.33	55356.80 ± 10731.77	+ 2.42	0.44	NS
3) Linear intercept	47191.39 ± 6204.27	47652.88 ± 6299.76	- 0.97	-0.27	NS
4) Perimeter	14168.39 ± 1969.69	14178.86 ± 1981.27	- 0.74	-0.02	NS

*area in pixel units (1 unit = 3.176 square micra)
SD - standard deviation

Table 22

RATIOS OF TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
Swiss Webster Newborn Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
7 ± 1/2 hr/day, 5 days/week and and 12 weeks
(M156)

0 WEEKS POSTEXPOSURE PERIOD

Measurements Edited Data	Control Group N=26 Mean ± SD	Experimental Group N=26 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS (≥8uM)* / ALVEOLAR WALL AREA	10.97 ± 1.40	11.15 ± 1.96	- 1.61	-0.46	NS
II. TYPE 2 CELL (≥ 8 uM) FIELD AREA / TYPE 2 CELL (≥ 8 uM) NUMBER	22.45 ± 4.18	22.30 ± 3.96	+ 0.71	0.14	NS
III. TYPE 2 CELL MEAN AREA* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	4.07 ± 0.96	4.13 ± 0.88	- 1.45	-0.24	NS
IV. TYPE 2 CELL NUMBER (≥12 uM)* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	0.80 ± 0.13	0.81 ± 0.13	- 1.23	-0.24	NS

* ratio x 100

Table 23

TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
0.35 + 0.05 ppm NO2 .
7±1/2 hrs/day, 5 days/week, 12 weeks
Swiss Webster Newborn Male Mice
(M156)

4 WEEKS POSTEXPOSURE PERIOD

Measurements	Control Group N=34 Mean ± SD	Experimental Group N=33 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS					
1) ≥ 8 uM	768.18 ± 136.69	769.42 ± 122.36	- 0.16	-0.04	NS
2) ≥12 uM	406.38 ± 81.98	405.70 ± 82.61	+ 0.17	0.03	NS
3) ≥ 8 um - ≤12 uM	361.80 ± 67.00	363.73 ± 54.53	- 0.53	-0.13	NS
II. TYPE 2 CELL (≥8 uM) FIELD AREA	16804.96 ± 4842.60	16156.74 ± 5681.24	+ 4.01	0.50	NS
III. TYPE 2 CELL LINEAR INTERCEPT	5635.92 ± 1282.53	5416.53 ± 1361.85	+ 4.05	0.68	NS
IV. ALVEOLAR WALL					
1) Area	78374.46 ± 15728.55	73927.40 ± 14214.39	+ 6.01	1.21	NS
2) Area - Type 2 cells area (≥8 uM)	61569.49 ± 12033.78	57770.66 ± 10165.11	+ 6.57	1.39	NS
3) Linear intercept	52329.69 ± 7906.84	50628.36 ± 6483.53	+ 3.36	0.96	NS
4) Perimeter	15600.52 ± 2425.96	15010.09 ± 2032.56	+ 3.93	1.08	NS

*area in pixel units (1 unit = 3.176 square micra)
SD - standard deviation

Table 24

RATIOS OF TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
Swiss Webster Newborn Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
7 ± 1/2 hr/day, 5 days/week and and 12 weeks
(M156)

4 WEEKS POSTEXPOSURE PERIOD

Measurements Edited Data	Control Group N=34 Mean ± SD	Experimental Group N=33 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS (≥8uM)* / ALVEOLAR WALL AREA	9.93 ± 1.25	10.54 ± 1.19	- 5.79	-2.06	0.0437
II. TYPE 2 CELL (≥ 8 uM) FIELD AREA / TYPE 2 CELL (> 8 uM) NUMBER	21.72 ± 4.32	20.62 ± 4.98	+ 5.33	0.97	NS
III. TYPE 2 CELL MEAN AREA* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	3.74 ± 0.92	3.63 ± 0.93	+ 0.27	0.04	NS
IV. TYPE 2 CELL NUMBER (≥12 uM)* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	0.67 ± 0.09	0.72 ± 0.11	- 5.63	-1.75	0.0852

* ratio x 100

Table 25

Type 2 Cell and Alveolar Wall Measurements
0.35 + 0.05 ppm NO2
7±1/2 hrs/day, 5 days/week, 12 weeks
Swiss Webster Newborn Male Mice
(M156)

TEN WEEKS POSTEXPOSURE PERIOD

Measurements	Control Group N=34 Mean ± SD	Experimental Group N=35 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS					
1) ≥ 8 uM	741.51 ± 98.50	760.34 ± 92.99	+ 2.43	0.81	NS
2) ≥12 uM	365.23 ± 60.28	376.97 ± 54.96	+ 3.29	0.84	NS
3) ≥ 8 um - ≤12 uM	376.27 ± 45.09	383.36 ± 49.79	+ 2.66	0.62	NS
II. TYPE 2 CELL (>8 uM) FIELD AREA	12457.73 ± 3578.43	12682.49 ± 2805.93	+ 1.80	0.29	NS
III. TYPE 2 CELL LINEAR INTERCEPT	4645.78 ± 1039.02	4725.73 ± 765.61	+ 1.72	0.36	NS
IV. ALVEOLAR WALL					
1) Area	69281.19 ± 13447.07	73102.29 ± 14431.97	+ 5.52	1.13	NS
2) Area - Type 2 cells area (≥8 uM)	56823.47 ± 11071.57	60419.80 ± 12698.79	+ 6.33	1.24	NS
3) Linear intercept	46561.41 ± 6227.67	48952.59 ± 5827.09	+ 5.14	1.63	0.1069
4) Perimeter	13746.91 ± 1960.50	14475.87 ± 1941.62	+ 5.30	1.54	0.1282

*area in pixel units (1 unit = 3.176 square micra)
SD - standard deviation

Table 26

Ratios of Type 2 Cell and Alveolar Wall Measurements
Swiss Webster Newborn Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
7 ± 1/2 hr/day, 5 days/week and and 12 weeks
(M156)

TEN WEEKS POSTEXPOSURE PERIOD

Measurements Edited Data	Control Group N=34 Mean ± SD	Experimental Group N=35 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS (≥8uM)* / ALVEOLAR WALL AREA	10.91 ± 1.44	10.71 ± 1.96	- 1.83	-0.48	NS
II. TYPE 2 CELL (≥ 8 uM) FIELD AREA / TYPE 2 CELL (≥ 8 uM) NUMBER	16.62 ± 3.16	16.62 ± 2.69	0	0.01	NS
III. TYPE 2 CELL MEAN AREA* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	3.00 ± 0.68	2.85 ± 0.66	- 5.00	-0.97	NS
IV. TYPE 2 CELL NUMBER (≥12 uM)* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	0.65 ± 0.1	0.64 ± 0.12	- 1.54	-0.42	NS

* ratio x 100

Table 27

TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
Swiss Webster Newborn Male Mice
0.35 ± 0.05 ppm Nitrogen Dioxide
7 ± 1/2 hrs/day, 5 days/week, 12 weeks
(M156)

32 WEEKS POSTEXPOSURE PERIOD

Measurements	Control Group N=67 Mean ± SD	Experimental Group N=60 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS					
1) ≥ 8 uM	680.66 ± 66.10	735.23 ± 80.67	+ 8.02	4.13	0.0001
2) ≥12 uM	378.12 ± 45.48	420.22 ± 56.98	+11.13	4.55	0.0000
3) ≥ 8 um - ≤12 uM	302.54 ± 37.89	315.02 ± 42.09	+ 4.13	1.74	0.0840
II. TYPE 2 CELL (≥8 uM) FIELD AREA	20941.28 ± 6078.28	24688.65 ± 6754.59	+17.89	3.26	0.0014
III. TYPE 2 CELL LINEAR INTERCEPT	6011.23 ± 1106.87	6824.70 ± 1116.02	+13.53	4.00	0.0001
IV. ALVEOLAR WALL					
1) Area	78842.24 ± 15615.41	84041.92 ± 20416.79	+ 6.60	1.59	0.1138
2) Area - Type 2 cells area (≥8 uM)	57901.15 ± 12005.75	59353.27 ± 15370.09	+ 2.51	0.59	NS
3) Linear intercept	41507.06 ± 4879.67	43277.86 ± 5913.84	+ 4.27	1.82	0.0709
4) Perimeter	12772.13 ± 1513.43	13329.18 ± 1837.50	+ 4.36	1.85	0.0673

area in pixel units (1 unit = 3.176 square micra)
SD - standard deviation

Table 28

RATIOS OF TYPE 2 CELL AND ALVEOLAR WALL MEASUREMENTS
Swiss Webster Newborn Male Mice
0.25 ± 0.05 ppm Nitrogen Dioxide
7 ± 1/2 hr/day, 5 days/week and and 12 weeks
(M156)

32 WEEKS POSTEXPOSURE PERIOD

Measurements Edited Data	Control Group N=67 Mean ± SD	Experimental Group N=60 Mean ± SD	Differences (%)	t-statistic	p value (2 tail)
I. TYPE 2 CELL NUMBERS (≥8uM)* / ALVEOLAR WALL AREA	8.90 ± 1.63	9.12 ± 1.77	+ 2.47	0.69	NS
II. TYPE 2 CELL (≥ 8 uM) FIELD AREA / TYPE 2 CELL (≥ 8 uM) NUMBER	30.70 ± 8.13	33.61 ± 8.59	+ 9.48	1.95	0.0538
III. TYPE 2 CELL MEAN AREA* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	5.46 ± 1.55	5.86 ± 1.46	+ 7.33	1.53	0.1296
IV. TYPE 2 CELL NUMBER (≥12 uM)* / ALVEOLAR WALL AREA-TYPE 2 CELL AREA	0.67 ± 0.13	0.74 ± 0.13	+10.45	2.68	0.0084

* ratio x 100

CHART 1

MEAN TYPE 2 CELL (>8 um) NUMBERS

0.35 ppm NITROGEN DIOXIDE (M156)

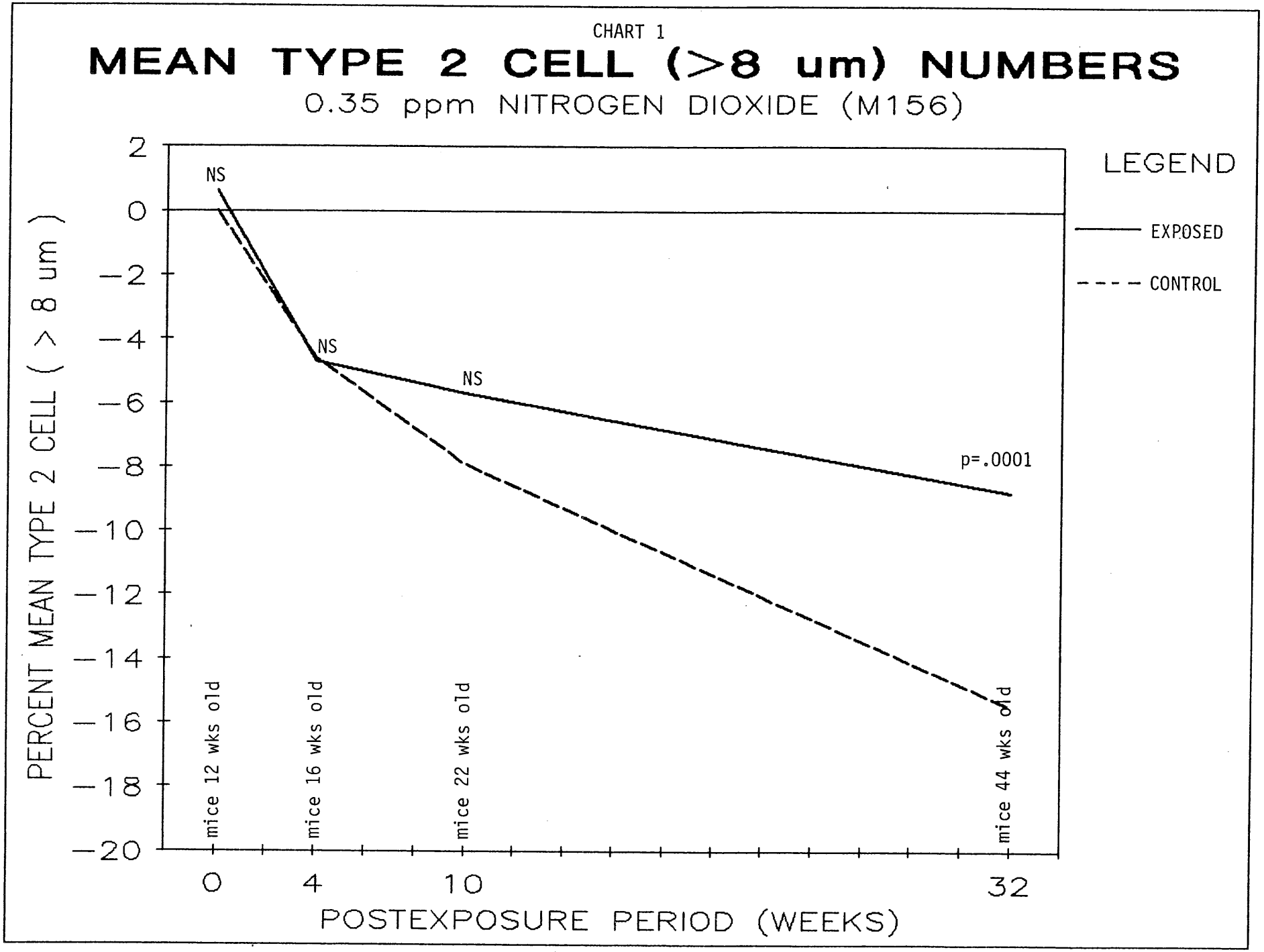


CHART 2

MEAN TYPE 2 CELL (>8 um) AREA

0.35 ppm NITROGEN DIOXIDE (M156)

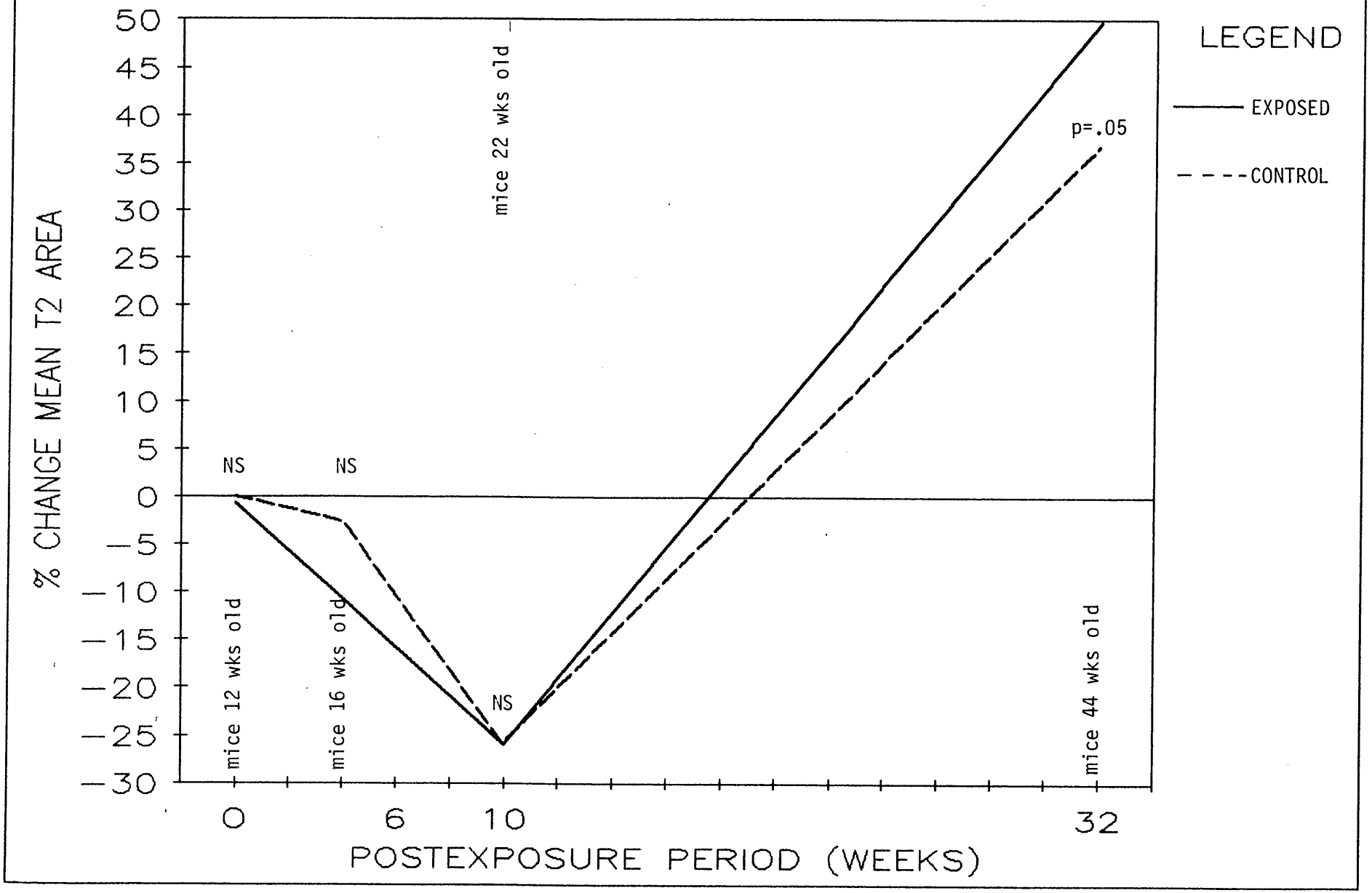


CHART 3

MEAN ALVEOLAR WALL AREA

0.35 ppm NITROGEN DIOXIDE (M156)

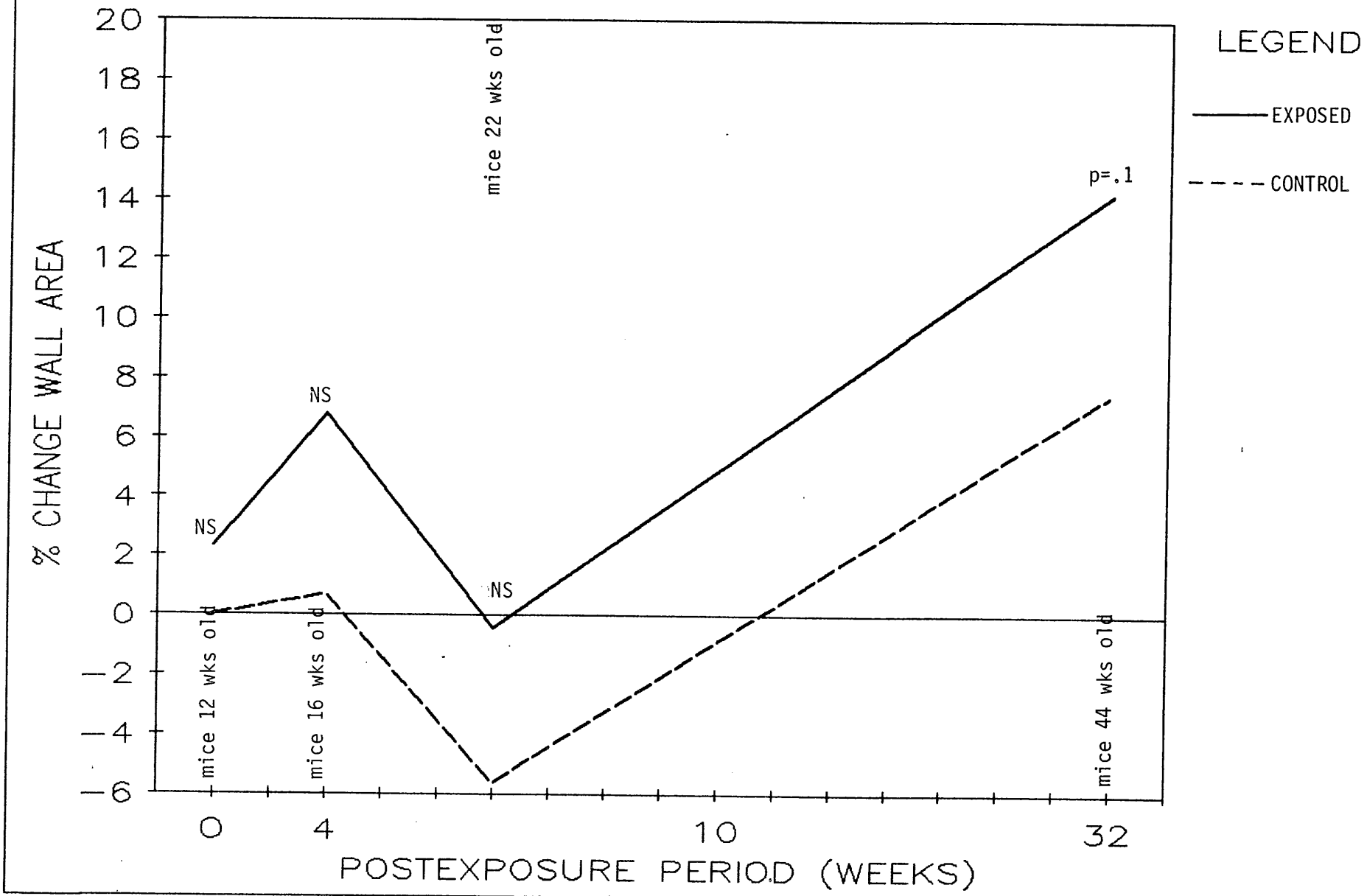


CHART 4

MEAN ALVEOLAR WALL AREA

MINUS

TYPE 2 CELL (>8 um) AREA

0.35 ppm NITROGEN DIOXIDE (M156)

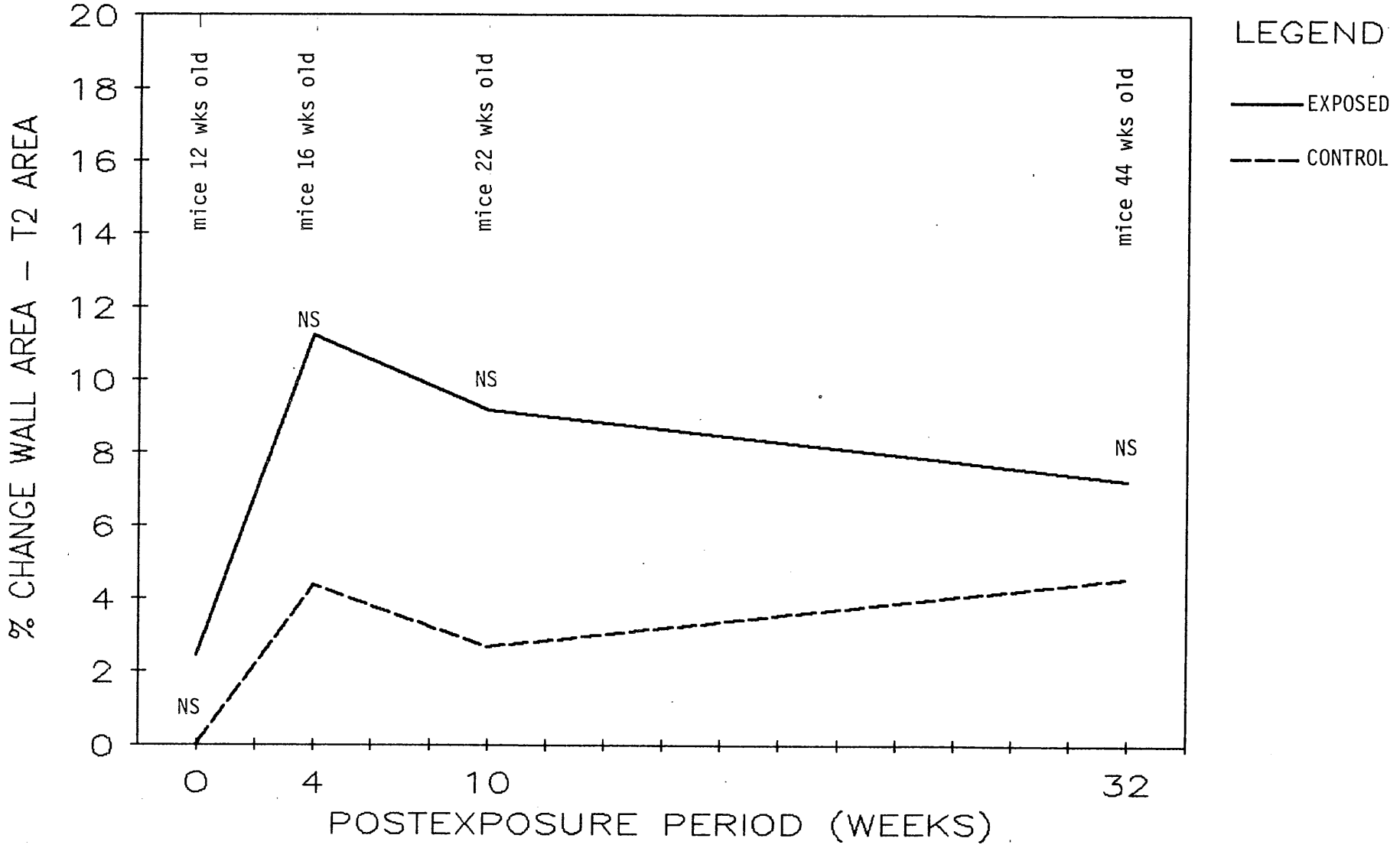


CHART 5

RATIO OF TYPE 2 CELL (>12 um) NUMBERS TO WALL AREA MINUS TYPE 2 CELL AREA

0.35 ppm NITROGEN DIOXIDE (M156)

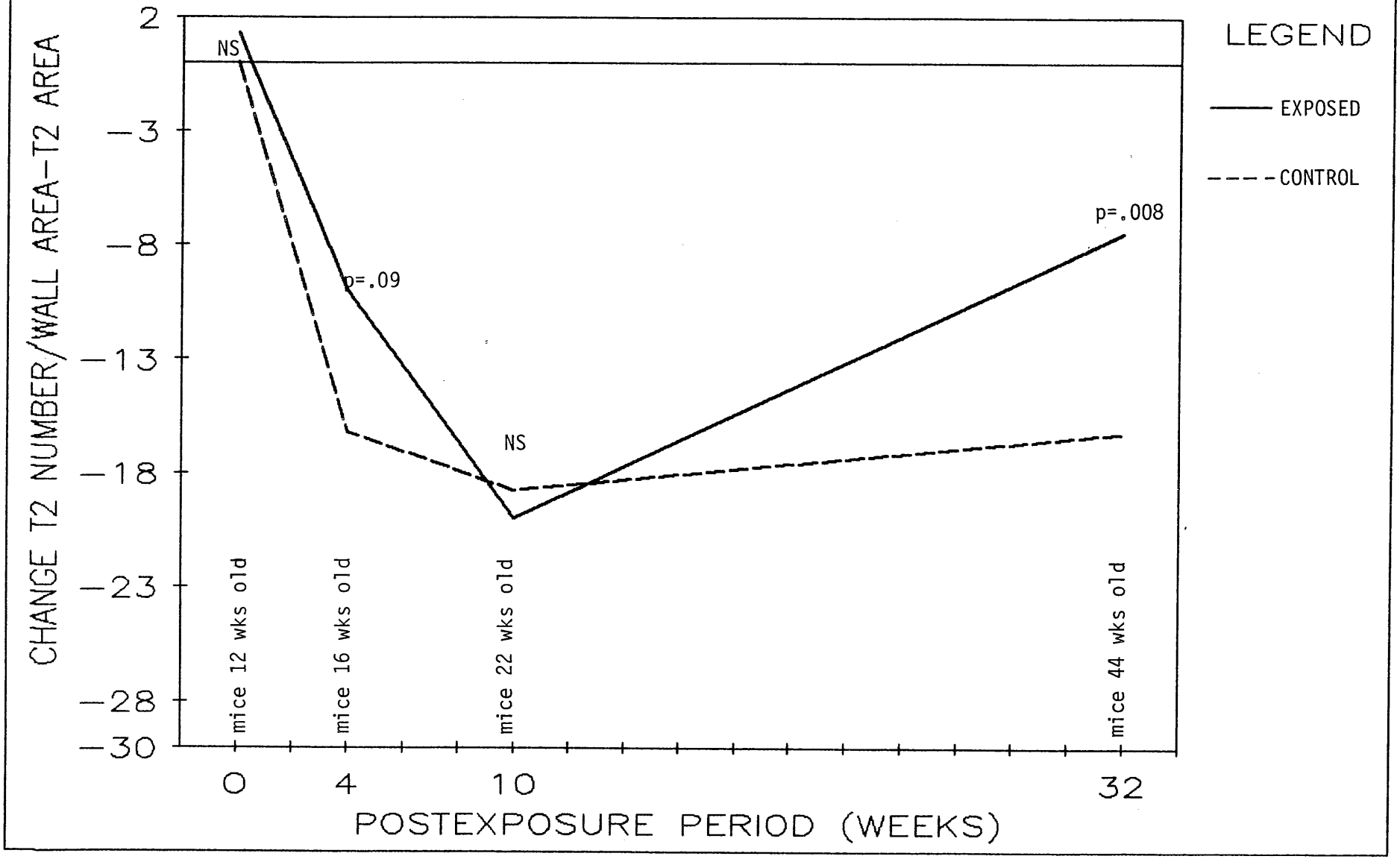


CHART 6

MEAN TYPE 2 CELL NUMBERS

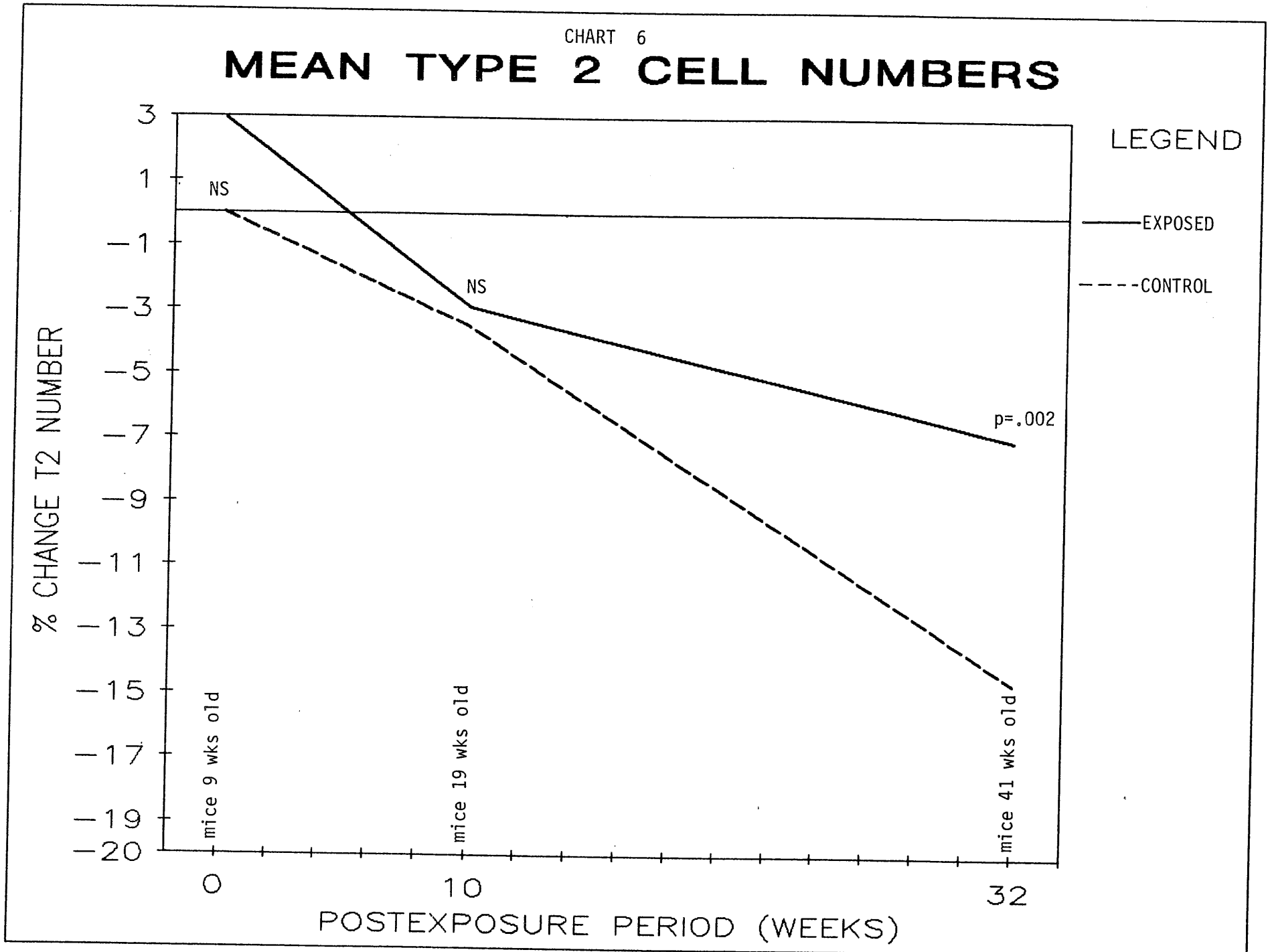


CHART 7

MEAN TYPE 2 CELL (>8um) AREA

0.25 ppm NITROGEN DIOXIDE (M171)

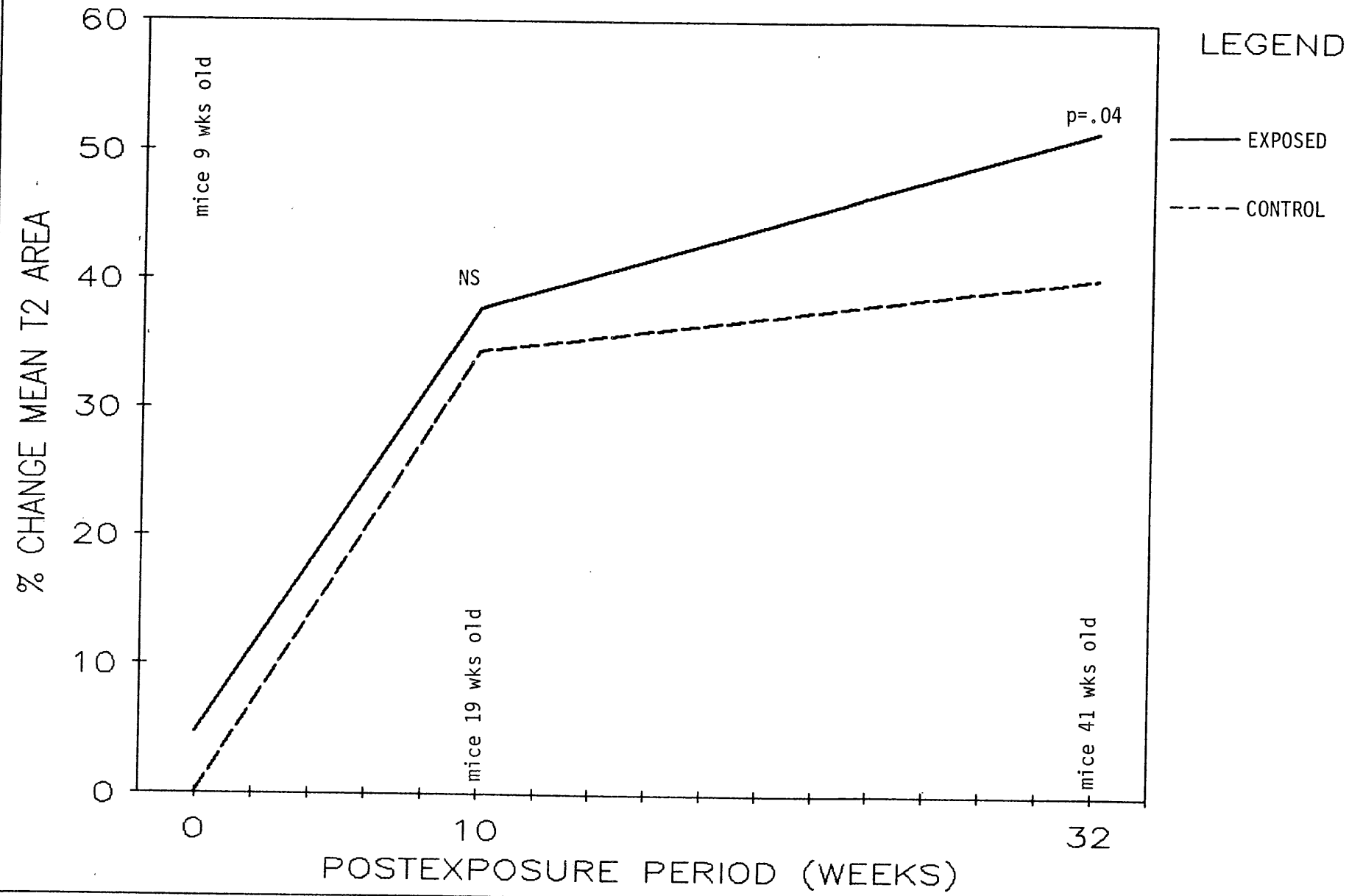


CHART 8

MEAN ALVEOLAR WALL AREA

0.25 ppm NITROGEN DIOXIDE (M171)

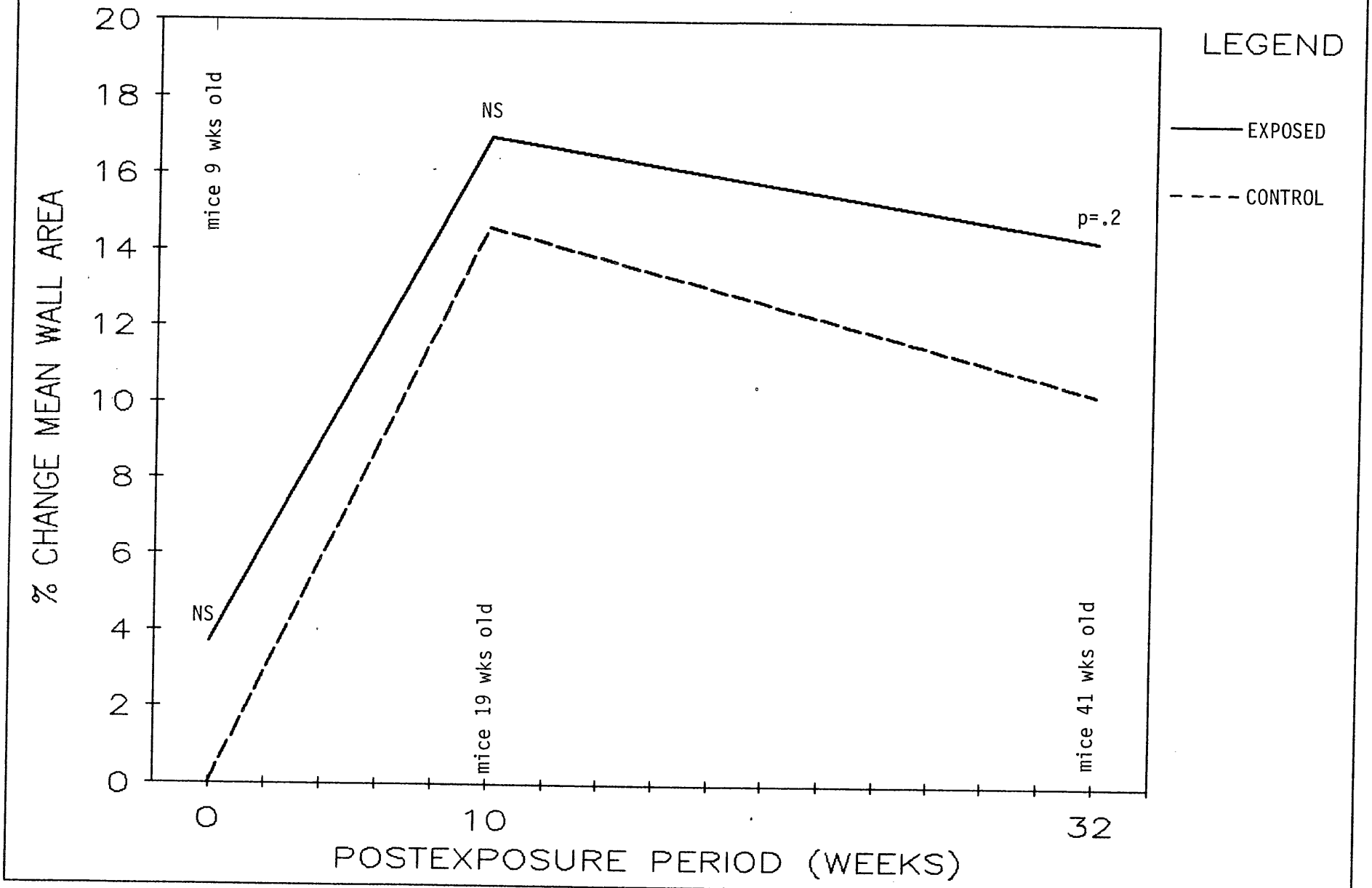


CHART 9

MEAN ALVEOLAR WALL AREA
MINUS
TYPE 2 CELL (> 8 um) AREA
0.25 ppm NITROGEN DIOXIDE (M171)

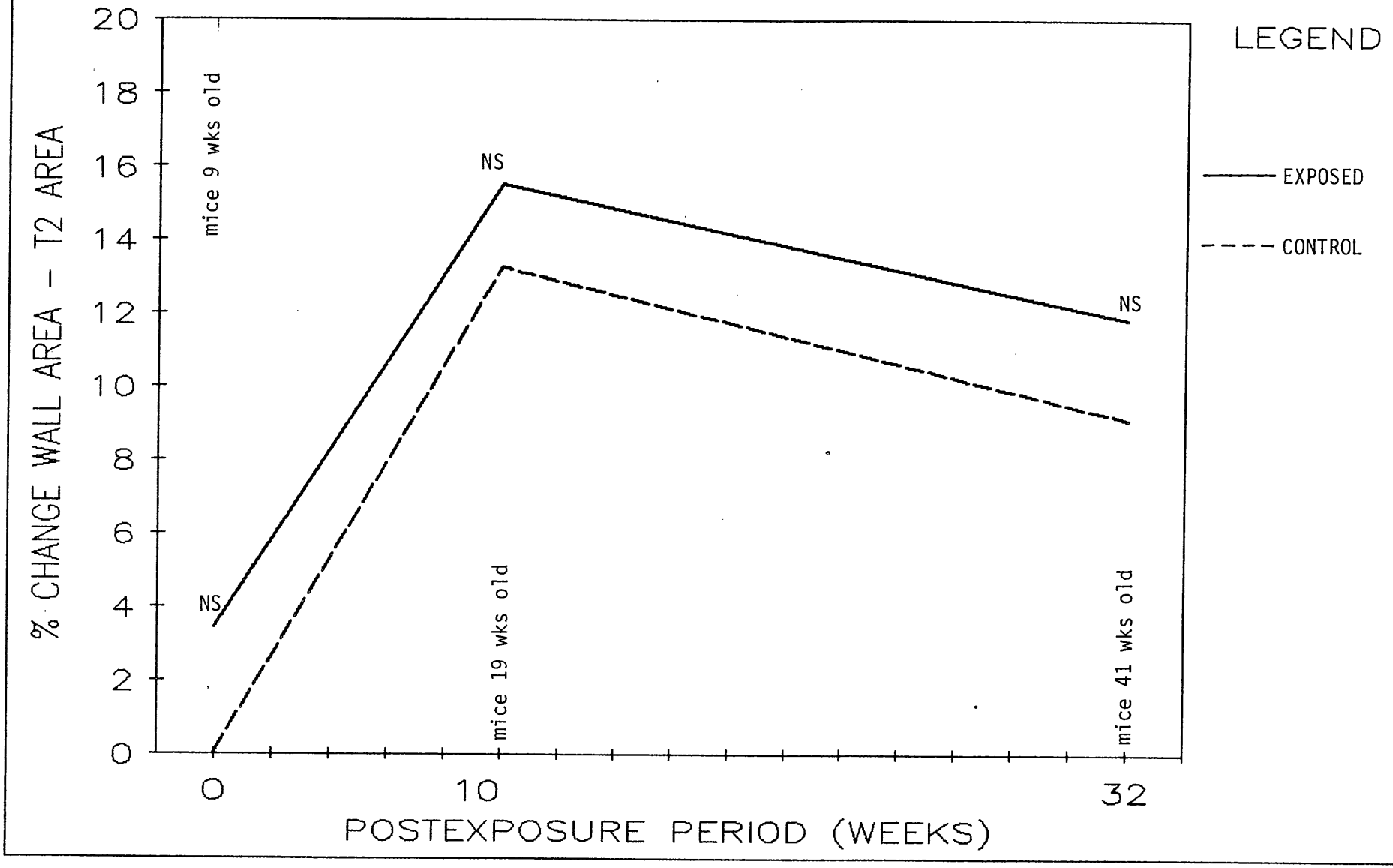


CHART 10

RATIO OF TYPE 2 CELL (>12 um) NUMBERS
TO
WALL AREA MINUS TYPE 2 CELL AREA
0.25 ppm NITROGEN DIOXIDE (M171)

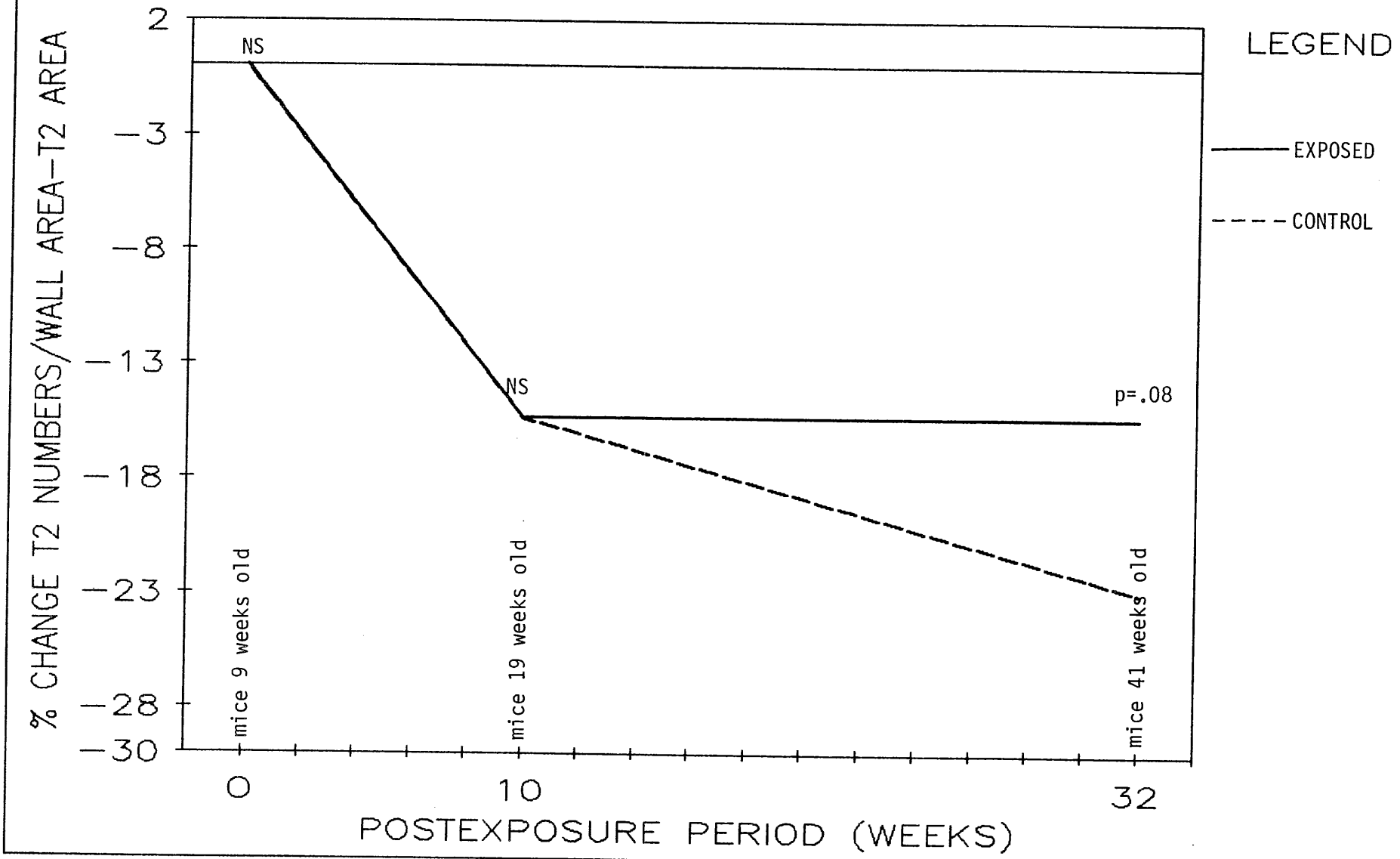


CHART 11

MEAN NUMBER OF ELASTIC FIBERS

0.25 ppm NITROGEN DIOXIDE (M171)

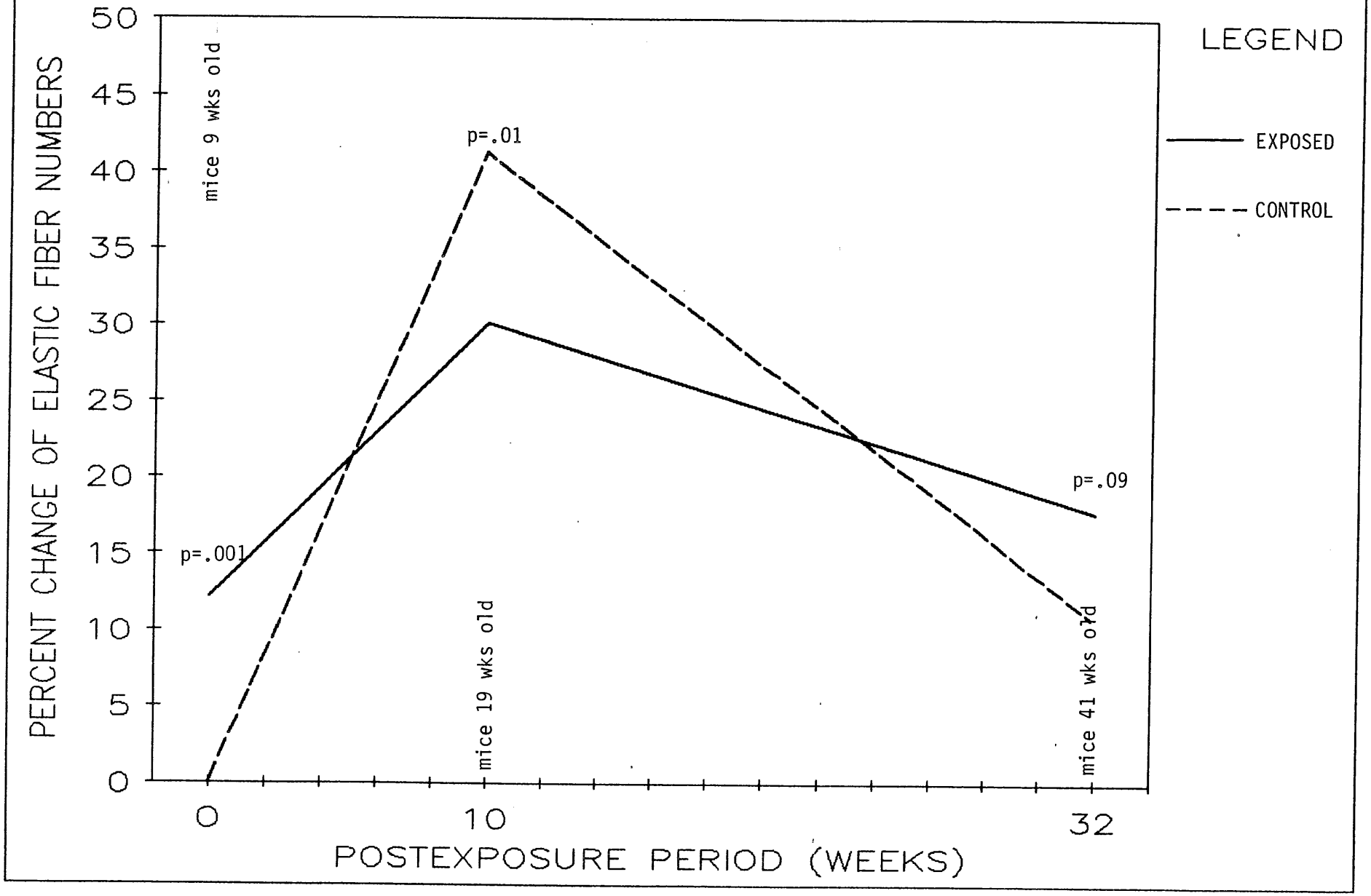


CHART 12

MEAN ELASTIC FIBER AREA

0.25 ppm NITROGEN DIOXIDE (M171)

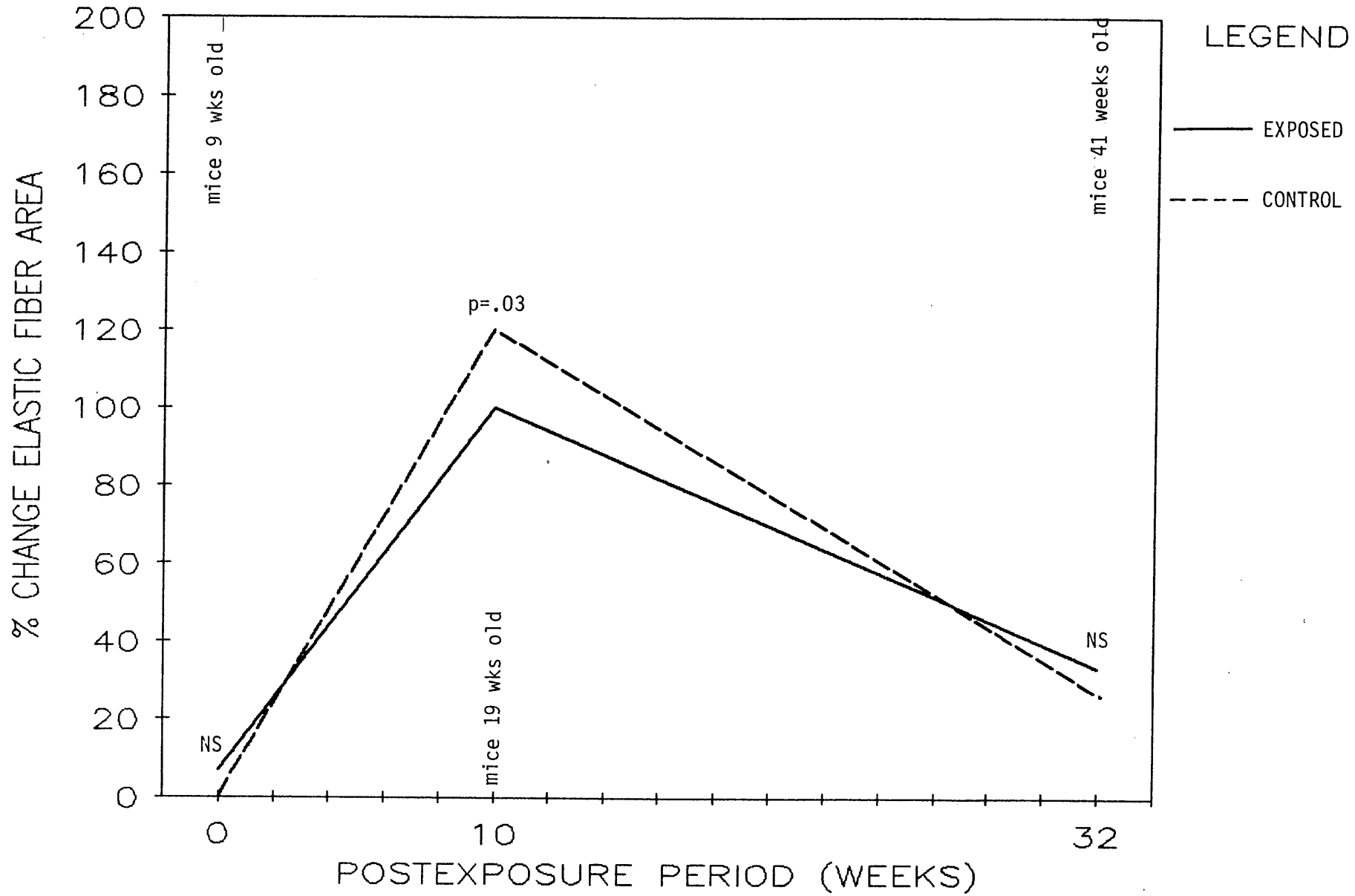


CHART 13

MEAN ELASTIC FIBER FIELD AREA

0.25 ppm NITROGEN DIOXIDE (M171)

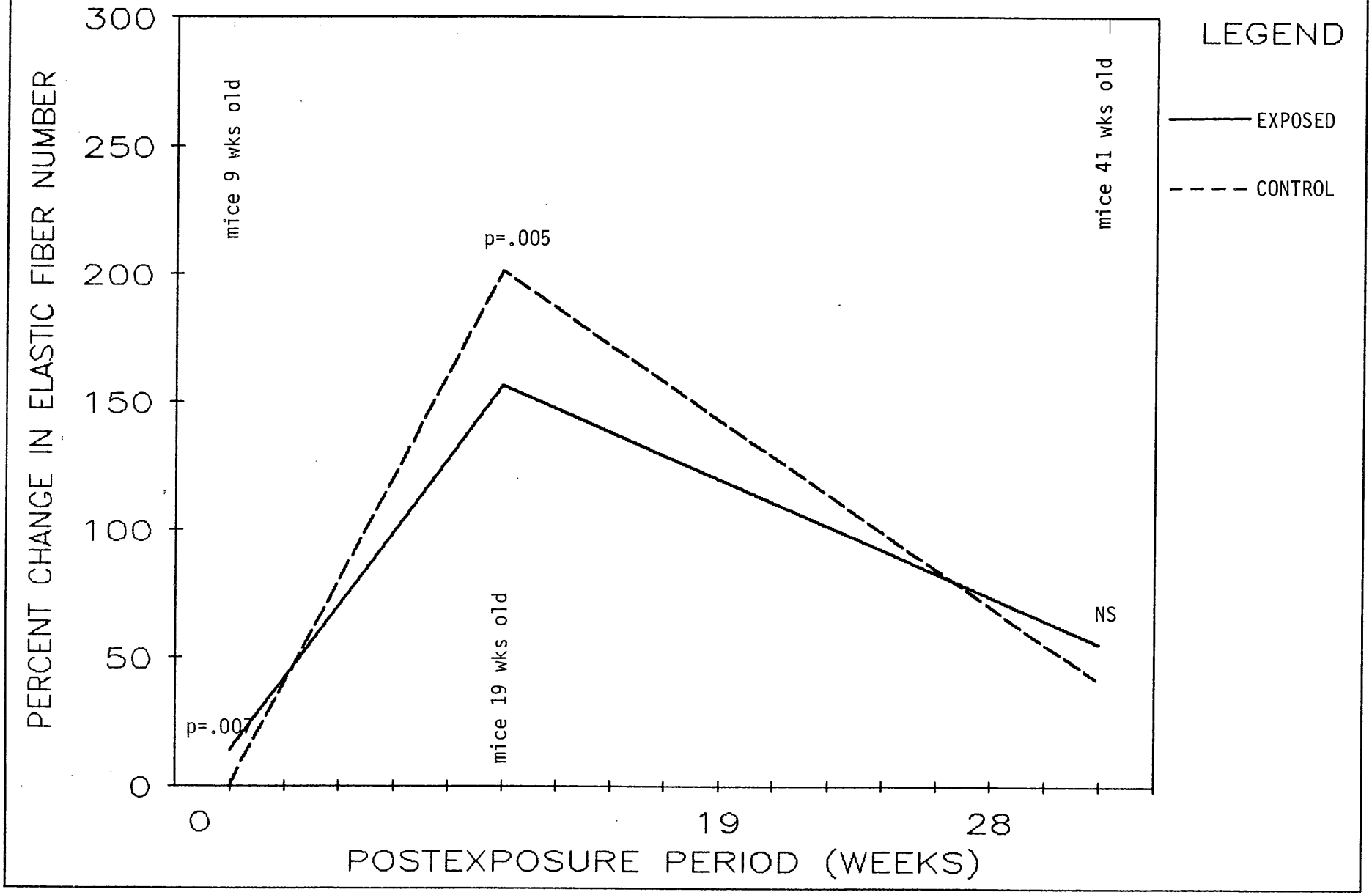


CHART 14

MEAN ALVEOLAR WALL AREA

0.25 ppm NITROGEN DIOXIDE (M171)

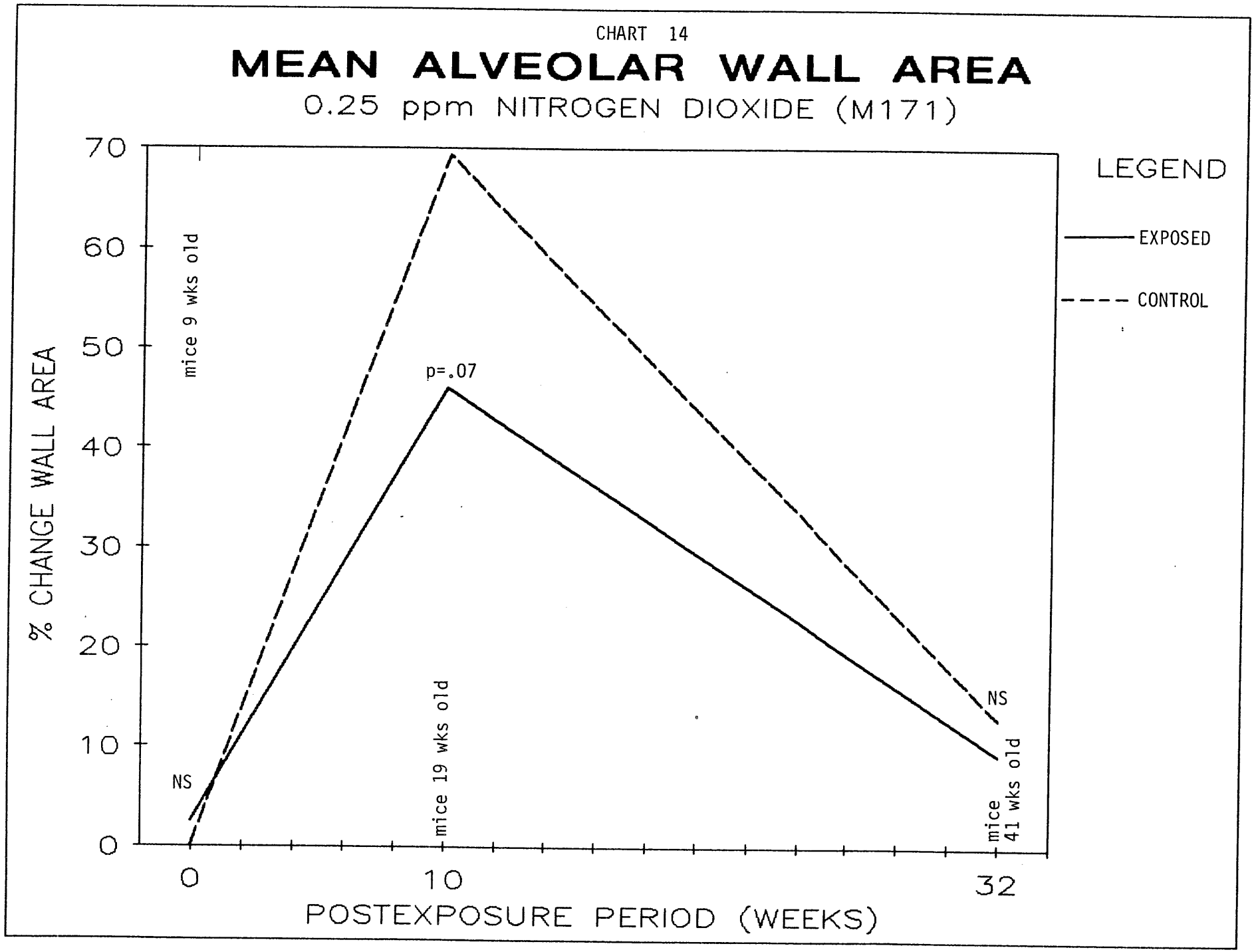


CHART 15

MEAN ALVEOLAR WALL AREA

MINUS

ELASTIC FIBER AREA

0.25 ppm NITROGEN DIOXIDE (M171)

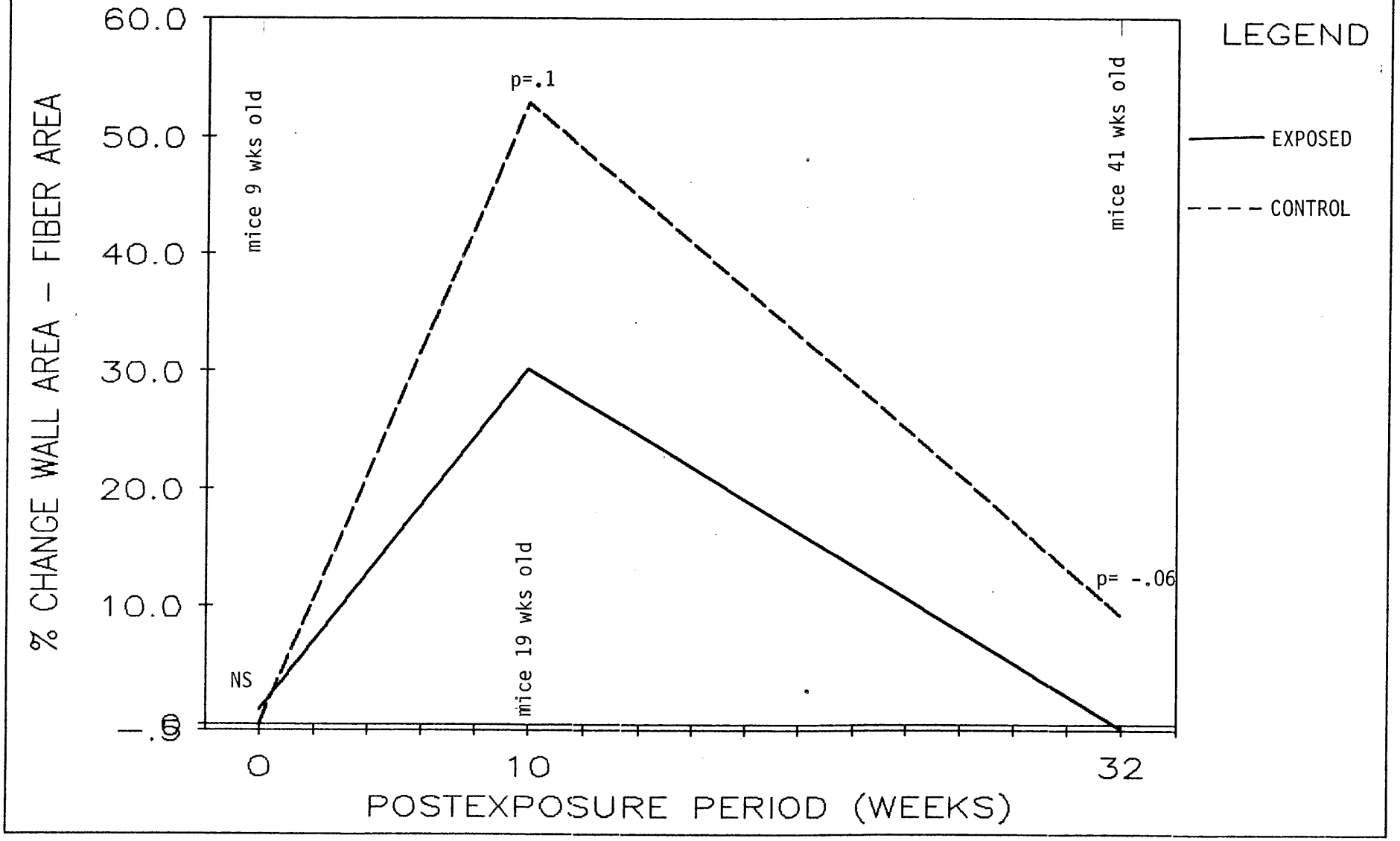


CHART 16

RATIO OF ELASTIC FIBER NUMBERS

TO

WALL AREA–ELASTIC FIBER AREA

0.25 ppm NITROGEN DIOXODE (M171)

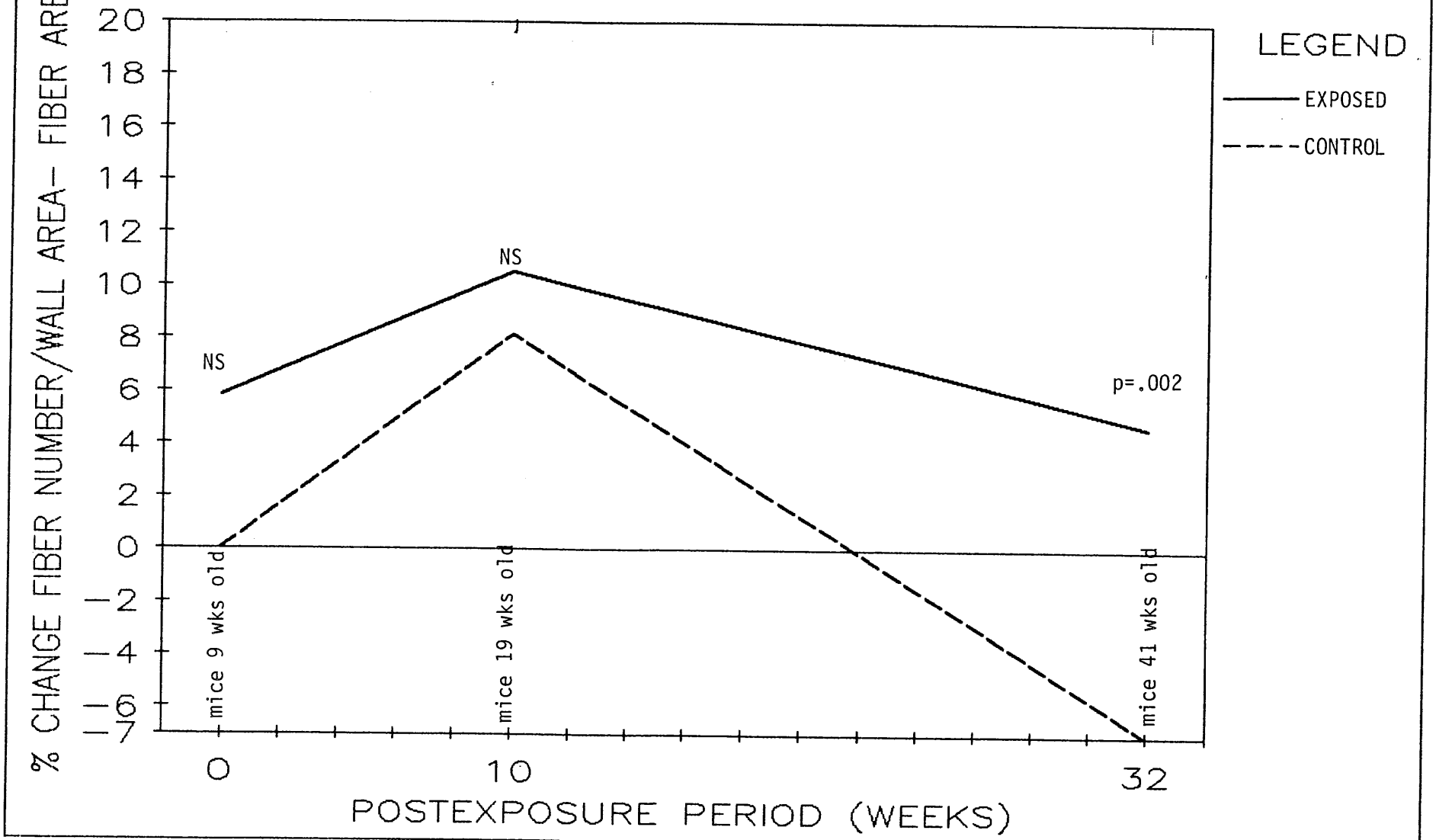


TABLE 46

CIBA Collection of Map Illustrations
RESPIRATORY SYSTEM
Vol.7
Netter, F.H.
1979

Hypersensitivity Pneumonitis Due to Inhalation of Organic Dusts *(Continued)*

that the fiber is not used for manufacturing or is processed by methods other than the baling and storing technique.

The onset of the disease may be acute or insidious, and is characterized by dyspnea, cough, fever, chills, weakness, chest pain, anorexia and rapid loss of weight. Illness may result from contact with bagasse dust for only a few days or after many months; it may be critical in nature or consist of mild transient symptoms after short periods of intermittent exposure.

When bagassosis is acute, the chest roentgenogram shows a variety of patterns, including changes typical of extensive pulmonary edema, diffuse bilateral and symmetrical small nodular deposits suggestive of miliary tuberculosis, and upper or lower lobe lesions, which are most often bilateral but which may be unilateral and simulate bacterial or viral pneumonia. In its chronic form the disease may show x-ray changes of a fine reticular "honeycomb" appearance, or those of intense fibrosis and bullous emphysema.

Pulmonary function testing characteristically shows a restrictive defect manifested by reduced vital capacity and total lung capacity. Gas transfer at the alveolar-capillary level is also impaired, and [illegible] present. A

On pathologic examination, the acute disease presents as a granulomatous pneumonia. Accumulations of plasma cells, lymphocytes and histiocytes are seen in the interstitial tissues, while the alveolar spaces contain edema fluid and collection of macrophages with vacuolated cytoplasm known as foam cells. When the illness becomes chronic, the granulomatous changes are replaced by interstitial fibrosis.

Treatment. Treatment involves simply removing the patient from contact with bagasse dust; gradual and eventually complete recovery then usually takes place in a matter of weeks or months.

If contact with the offending antigen is reest: lished, the illness almost invariably recurs and t danger of permanent disability increases acco ingly. When the illness is severe, recovery may hastened and symptoms relieved by ster therapy. Prednisone is usually administered doses of 60 mg daily for about one week, a which the dosage is gradually decreased ove period of a month or more.

Prevention of bagassosis, and of all other fo of hypersensitivity pneumonitis, can be acc plished by eliminating the offending anti from the individual's environment.

Etiologic Agents in Hypersensitivity Pneumonitis

Disease	Exposure	Antigen
Farmer's lung	Moldy hay or grain	*Micropolyspora faeni, Thermoactinomyces vulgaris*
Bagassosis	Stored sugar cane fiber (bagasse)	*T. saccharii* and possibly other organisms
Mushroom picker's disease	Moldy vegetable compost	*M. faeni, T. vulgaris*
Humidifier, air-conditioner or heating system disease	Contaminated forced air system	Thermophilic actinomycetes and other organisms
Fog fever (cattle)	Moldy hay	Same as farmer's lung
Maple bark stripper's disease	Maple tree logs or bark	*Cryptostroma corticale*
Sequoiosis	Redwood sawdust	*Graphium, Pullularia, Aureobasidiu pullulans* and other fungi
Suberosis	Moldy cork dust	*Penicillium* species
Paper mill worker's disease	Moldy wood pulp	*Alternaria*
Pulpwood handler's disease	Moldy wood pulp	Same as above
Brewer's or malt worker's lung	Malt or barley dust	*Aspergillus clavatus, A. fumigatus*
Cheese washer's lung	Cheese mold	*P. casei*
Paprika slicer's disease	Moldy paprika pods	*Mucor stolonifer*
Wheat thresher's lung or grain measurer's lung	Wheat flour containing weevils	*Sitophilus granarius*
Pigeon breeder's disease	Pigeon serum and droppings	Avian proteins
Budgerigar fancier's disease	Contact with parakeets	Parakeet proteins
Chicken handler's or feather plucker's disease	Contact with chickens	Chicken proteins
Turkey handler's disease	Contact with turkeys	Turkey proteins
Pituitary snuff disease	Porcine, bovine pituitary gland (Pitressin snuff)	Porcine, bovine proteins
Smallpox handler's lung	Smallpox scabs	Unknown
Thatched roof disease (Papuan or New Guinea lung)	Dried grass and leaves	Unknown
Tobacco grower's disease	Tobacco plants	Unknown
Joiner's disease	Sawdust	Unknown
Tea grower's disease	Tea plants	Unknown
Bible printer's disease	Moldy typesetting water	Unknown
Coptic or mummy disease	Cloth wrappings of mummies	Unknown
Detergent disease (asthmalike symptoms—true pneumonitis not identified)	Enzyme detergents	*Bacillus subtilis*
Furrier's lung	Animal hairs	Unknown
Coffee worker's lung	Coffee beans	Coffee bean dust
Doghouse disease	Moldy straw	*Aspergillus versicolor*
Lycoperdonosis	Puffball spores [illegible]	Unknown

TABLE 1

ADVERSE EFFECTS OF NONCYTOTOXIC DRUGS ON LUNG HOMEOSTASIS

Target Cell or System	Factors Released by Target Cells or Direct Effects of Agent	Potential Adverse Effects	Implicated Noncytoxic Drug
Macrophages	Neutral proteases	Increased epithelial permeability	Colchicine* (260)†
Polymorphonuclear leukocytes (PMN)	Enhanced PMN aggregation	Endothelial damage	Amphotericin* (247)
Lymphocytes	Autoantibodies to albumin	Pulmonary immune complex deposition	Nitrofurantoin* (313)
	Autoantibodies to glomerular basement membrane	Pulmonary and renal immune complex deposition	Penicillamine‡ (257)
	Enhanced lymphocyte blastogenesis	Autologous cytotoxicity	Amiodarone* (242) Nitrofurantoin* (240, 241) Gold salts* (236, 237)
	Lymphokine release (monocyte chemotactic factor, macrophage inhibitory factor)	Parenchymal lung damage	Gold salts* (238)
Fibroblast	Direct alteration of collagen metabolism	Abnormal matrix repair	Penicillamine* (258) Gold salts* (259)
Medullary or hypothalmic neurons	Endothelial permeability modulating nervous signals	Enhanced endothelial permeability	Opiates§ (262) Aspirin§ (250) Antipsychotics§ (263)
Complement system	Direct complement activation	PMN aggregation endothelial damage	Heroin* (244) Terbutaline* (243)
Acellular pulmonary environment	Endogenous oxidant production	Endothelial membrane injury	Nitrofurantoin§ (233)

* Demonstrated in pulmonary system.
† Numbers in parentheses are references.
‡ Demonstrated in nonpulmonary system.
§ Proposed.

TABLE 47

(Am. Rev. Resp. Dis. 133:488–505, 1986)

Immunologic System

Noncytotoxic drugs produce pulmonary histologic changes, including an inflammatory cell influx, which suggest an immune pathogenesis. In addition, several of these drugs cause hypersensitivity lung disease (table 6). The marked peripheral or pulmonary eosinophilia noted in this syndrome implies either a hyperacute reaction to some precipitating antigen, such as the specific drug, or an alteration in the normal immune balance of the lung, which is discussed in Part 1 of this review.

There is also good experimental evidence for immune alteration as a mechanism for pulmonary toxicity associated with noncytotoxic drugs (236–257). Lymphocytes may play a role in pulmonary damage associated with these agents (236–241). Enhanced lymphocyte blastogenesis in the presence of gold salts (236, 237) and nitrofurantoin (240, 241) has been noted in patients with pulmonary injury caused by these agents; release of lymphokines after exposure to the gold salts has also been observed (238). In addition, findings consistent with a T lymphocyte alveolitis have been documented in a patient with gold-induced pneumonitis (239). All of these findings suggest that gold salts and nitrofurantoin may exert some toxic effects through activation and sequestration of pulmonary lymphocytes.

Another noncytotoxic drug that appears to cause pulmonary damage through immune mechanisms is amiodarone (242), an increasingly used antiarrhythmic agent. In 1 patient (242), evidence of amiodarone hypersensitivity developed in conjunction with onset of pulmonary disease. Bronchoalveolar lavage in this patient showed signs of a lymphocyte alveolitis. Analysis of T lymphocyte subsets in both studies showed a reduction in the normal helper/suppressor ratio, suggesting an alteration in normal pulmonary immune parameters. These findings parallel the histologic picture of amiodarone pulmonary toxicity, which consists of a diffuse mononuclear inflammatory cell infiltrate (230).

TABLE 2

DRUGS THAT CAUSE HYPERSENSITIVITY LUNG DISEASE

Cytotoxic Drugs	Noncytotoxic Drugs
Bleomycin (27)*	Nitrofurantoin (276)
Methotrexate (28)	Sulfasalazine (278)
Procarbazine (58)	Sulfadimethoxine (279)
	Diphenylhydantoin (277)
	Carbamazepine (280)
	Chlorpropamide (281)
	Imipramine (282)
	Isoniazid (283)
	Para-aminosalicylic acid (284)
	Penicillin (285)
	Cromolyn (286)
	Dantrolene (287)
	Methylphenidate (287)
	Mephenesin carbamate (288)
	Hydralazine (287)
	Mecamylamine (289)
	Ampicillin (290)

* Numbers in parentheses are references.

In contrast to cytotoxic drugs, some noncytotoxic agents cause changes that may result in pulmonary leukocyte sequestration as a mechanism for pulmonary damage. Patients treated with beta-sympathomimetics (243) and opiates (244) have shown signs of serum complement activation with development of noncardiogenic pulmonary edema. Complement activation results in the generation of immunoreactive fragments that cause leukocyte chemotaxis and aggregation. Evidence exists that noncardiogenic pulmonary edema, such as that occurring after dialysis or during septicemia (245) may be due to complement activation. Amphotericin B, another drug that may cause acute pulmonary disease (246), injures polymorphonuclear leukocytes (PMN) directly, possibly through binding to the cellular membrane (247). Amphotericin-induced pulmonary toxicity occurs after leukocyte transfusions, which are also known to result in circulation of damaged PMN (248). These 2 modes of therapy may cause increased pulmonary sequestration of PMN and subsequent alveolar capillary damage.

Several noncytotoxic drugs that cause pulmonary injury have immunoregulatory properties in other systems, and these alterations may be relevant to the mechanism of pulmonary toxicity by

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cytotoxic drug-induced pulmonary injury, and (*3*) specific aspects of pulmonary damage caused by individual cytotoxic agents. Cytotoxic antibiotics, alkylating agents, antimetabolites, and other chemotherapeutic agents are reviewed separately. The second part of the review covers noncytotoxic drugs in a similar manner. These nonchemotherapeutic drugs are divided into 10 categories, (*1*) antibacterial agents, (*2*) analgesics, (*3*) anticonvulsants, (*4*) antiarrhythmics, (*5*) diuretics, (*6*) sympathomimetics, (*7*) opiates, (*8*) antipsychotics, (*9*) antirheumatic agents, and (*10*) other noncytotoxic drugs.

Because of space constraints, this review is limited to pulmonary parenchymal damaging effects of pharmacologic agents. Effects of drugs on respiratory muscles or airway reactivity are not addressed. Moreover, damage caused by therapeutic modalities such as radiation and oxygen therapy are not reviewed, except when interactions with the included drugs are important. The reader is referred to several reviews written on this subject (4–6). Finally, indirect effects of drugs, such as immunosuppression resulting in pulmonary infections, are not considered except when pertinent to the differential diagnosis.

The goals of this article are to provide a comprehensive review of the pathogenesis of drug-induced pulmonary disorders, to summarize common clinical and pathogenic aspects, and to detail any clinical or pathologic features that are characteristic of individual agents or categories of drugs. It is our hope that this will facilitate diagnosis of the specific disorders.

TABLE 1

PHARMACOLOGIC AGENTS THAT CAUSE PULMONARY PARENCHYMAL INJURY

Cytotoxic Drugs	Noncytotoxic Drugs
Antibiotics	Antibacterial Agents
Bleomycin	Nitrofurantoin
Mitomycin	Amphotericin
Neocarzinostatin	Other antibiotics
Alkylating agents	Sulfasalazine
Busulfan	Analgesics
Cyclophosphamide	Acetylsalicylic acid (aspirin)
Chlorambucil	Opiates
Melphalan	Heroin
Nitrosoureas	Propoxyphene
Carmustine (BCNU)	Methadone
Semustine (methyl CCNU)	Sedatives
Lomostine (CCNU)	Ethchlorvynol
Chlorozotocin	Chlordiazepoxide
Antimetabolites	Anticonvulsants
Methotrexate	Diphenylhydantoin
Azathioprine	Carbamazepine
Mercaptopurine	Diuretics
Cytosine arabinoside	Hydrochlorthiazide
Miscellaneous	Major Tranquilizers
Procarbazine	Haloperidol
VM-26	Fluphenazine
Vinblastine	Antiarrhythmics
Vindesine	Amiodarone
	Lidocaine
	Tocainide
	Miscellaneous
	Gold salts
	Penicillamine
	Colchicine

Cytotoxic Drugs

Mechanisms of Pulmonary Injury

The lung has a number of homeostatic mechanisms for maintaining a fine balance between damaging inflammatory reactions and protective detoxification pathways. Several of these systems may be affected by exogenous agents including cytotoxic drugs (table 2). These systems and effects of particular drugs are summarized here and more specific details are presented under each drug heading.

Oxidant/Antioxidant System

Reactive oxygen metabolites have been implicated in a number of diseases including the adult respiratory distress syndrome, emphysema, pulmonary oxygen toxicity, and radiation-induced pulmonary damage (7). Oxidant species include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH\cdot$), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl) (8). These molecules are formed within phagocytic cells including polymorphonuclear leukocytes (PMN), macrophages, monocytes, and eosinophils or spontaneously in the atmosphere, especially in areas of high combustion (7). One mechanism of pulmonary damage by cigarette smoke may be through oxidant production (9). Toxic effects appear to occur through participation of these molecules in redox reactions and subsequent fatty acid oxidation, which may lead to membrane instability (7). In addition, oxidants may be involved in triggering other inflammatory reactions. For example, oxidation of arachidonic acid is an initial step in a metabolic cascade that produces several immunoreactive substances including prostaglandins and leukotrienes (10).

Because these oxidant molecules not only are toxic to invading organisms but also cause autologous cytotoxicity, antioxidant defense systems are required to avoid tissue damage. These antioxidants include: superoxide dismutase (SOD), which metabolizes O_2^- to H_2O_2; catalase, which catabolizes H_2O_2; glutathione peroxidase, which allows glutathione to accept an electron and subsequently detoxify a reactive molecule; ceruloplasmin, a scavenging agent that employs copper to accept an electron and thus reduce one of these molecules; and alpha-tocopherol (vitamin E), which is also able to accept an electron as a method for detoxification (8). These antioxidant systems are present in plasma and in virtually every cell in the body to variable degrees.

Certain cytotoxic drugs may induce pulmonary injury through alteration of the normal balance between oxidants and antioxidants. There is evidence that bleomycin functions as a "minienzyme" system, producing superoxide radicals when incubated with oxygen and iron *in vitro* (11–16).

Cyclophosphamide also has been shown to independently generate reactive oxygen species (17) and the drug produces lipid peroxides *in vitro* (18), which may lead to membrane instability (7). A reactive metabolite of cyclophosphamide, acrolein, is a powerful oxidant, which may be an important component in cig-

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10. APPENDIX

A. PRIOR CONTRACTUAL WORK: ADDITIONAL FINDINGS AND COMPLETION OF STUDIES

Experiment M156; Newborn mice exposed to 0.35ppm NO_2, five days/week for 12 weeks (454 hours total exposure), and with 4, 10, & 32 week postexposure periods.

1. Abstract-Summary

See Main Report ABSTRACT (pages 1-16) and SUMMARY-CONCLUSIONS (page)

2. Scope, Purpose, and General Background of the Project

The investigations carried out up to the time of this experiment have shown that ambient levels of nitrogen dioxide (NO_2) cause Type 2 Cell hyperplasia in the lungs of young adult and newborn Swiss Webster male mice following intermittent six weeks NO_2 exposure (8,9). A recent study of newborn mice provided data on reversibility by including two postexposure periods of four and ten weeks after exposure to NO_2 for six weeks. The data obtained showed that NO_2 exposure influenced the size and numbers of Type 2 Cells at all three test periods (9). The persistence of statistically significant differences at the ten weeks postexposure period indicated that Type 2 Cell hyperplasia the associated (putative) Type 1 Cell damage and loss was not reversible insofar as the last postexposure time period (10 weeks) was concerned. The significance of the Type 2 hyperplasia is that it is believed to reflect damage to the gas exchange cells of the alveolar lining (the Type 1 Cells). Moreover, persistence at the final test period implies a certain degree of functional impairment due to the partial replacement of alveolar linings by large cells that impair gas exchange. Whether the impairment is or is not permanent has not yet been established. However, since the mammalian lung in general shows a decremental and irreversible loss of structure and function with time, there is a real concern that the detection of Type 2 Cell hyperplasia is associated with some degree of increase in the decremental loss, i.e. a facilitation of lung reserve depletion.

Since no comparable studies of reversibility were available, the main objective of this study was to confirm and to expand the reversibility study beyond the ten week postexposure period, and to answer the questions: Does the Type 2 hyperplasia persist beyond 10 weeks postexposure and are there other alterations (e.g. alveolar wall connective tissue and capillaries) that may be a sign of irreversibility? To answer these questions, a long term experiment was designed to study the effect of NO_2 on Type 2 Cells using a longer exposure period (12 weeks vs 6 weeks) and three (vs the prior two) postexposure test periods, i.e. at 4, 10 and 32 week postexposure.

3. Rationale

The replacement of damaged or lost Type 1 Cells of the alveolar lining by the Type 2 Cell is now well recognized as a common denominator for diverse kinds of lung damage (5). The Type 2 Cell hyperplasia seen in human diseases and its occurrence as an early lesion in many experimentally produced lung diseases is also well known and has been reviewed by a number of investigators (2-4,25,26). The concept of cell population shift in which the Type 2 cells replacement of Type 1 cells follows damage and loss of Type 1 Cell has had additional strong support by an ultrastructural study which showed correlation between thymidine uptake of Type 2 Cells and measurements of Type 1 Cell alterations (6).

Less well appreciated is the vulnerability of the type 2 Cell itself to noxious agents. While many noxious agents may primarily affect the Type 1 Cell, the sparing of other cells (Type 2 Cells, endothelial, and connective tissue) is relative. Irreversible damage to the Type 1 Cell, initally manifest as a Type 2 hyperplasia, is an early event and common denominator for many human lung diseases, including diffuse interstitial fibrosis and emphysema. In the latter disease, the loss of the Type 1 and Type 2 Cells leads to the disappearance of alveoli, alveolar sacs, or the entire basic functional gas exchange unit, i.e. alveoli, sacs, alveolar ducts and respiratory bronchioles. Also, the disappearance of alveoli in interstitial fibrosis is a major part of the disease process in which the fibrosis in large part represents a condensation of lung scaffolding after the more susceptible cell populations have been lost.

4. Experimental Design, Materials and Methods

The methodology for this study involved the following procedures: 1) NO_2 exposure and monitoring, using and environmental chamber with duplicate facilities for the control and exposed animals; 2) processing of lungs for frozen sectioning and lactate dehydrogenase staining of Type 2 pulmonary alveolar cell; and 3) computer assisted image analysis of the lung for quantitative measurements of Type 2 Cell numbers, Type 2 Cell size (area), and alveolar wall.

Environmental Chambers

The mice were housed in two identical laminar air flow stainless steel environmental chambers in an air conditioned vivarium exclusively used for the pollution studies. Each chamber has a 40 ft^3 volume. The air flow rate resulted in a change of chamber air approximately every other minute. The air passed through a Purafil filter system, with the latter replaced on a routine schedule or earlier if indicated by a color change of the pellets. The room temperature, chamber temperatures, and humidity were recorded daily. The cages were cleaned three times per week. For experiments with postexposure periods, the animals were housed in special holding chambers, equipped with special filtering system. The chambers were steam cleaned and the hoses washed after termination of each experiment.

Nitrogen Dioxide Exposure

Newborn Swiss Webster male mice were exposed for 12 weeks to 0.35 ± 0.005 ppm NO_2 7 $\pm 1/2$ hr/day and 5 days/week, with a total of 454 exposure hours.The 12 weeks exposure period was followed by 4, 10 and 32 weeks postexposure test periods.

Details of the NO_2 method have been reported published (13). In brief, room air was filtered by a combination of particle and NO_2 absorbing (Purafil) filters. NO_2 was introduced into the air intake mixing unit of the exposure chamber through a silicone drip method. The silicone used was of medical grade and a high viscosity (500 centistokes) with an essentially negligible vapor pressure. The delivery of the NO_2-silicone mixture was regulated by the caliber of the teflon delivery tube, the height of the column, and air flow. The following means of NO_2 monitoring were employed:

a. Fritted bubbler readings of approximately 4 liter air samples;
b. Beckman liquid analyzer continuous flow recording; and
c. Chemiluminescent continous recordings (Teco).

Measurements of NO_2 concentrations were obtained of control chamber, exposure chamber, and ambient room atmosphere. Periodic calibration of the monitoring equipment was carried out through the assistance of the California Air Resources Board, El Monte Division.

Animal Colonies

Swiss Webster newborn animals were obtained by placing "timed pregnant" mice (7 to 10 days prior to expected delivery) in the environmental chambers, providing them with appropriate bedding (nestlets), and exposing them to NO_2 immediately upon completion of cage arrangements. There was daily exposure up to the time all animals had delivered, i.e. the weekends were included. Subsequently, a five day per week schedule was in operation. The newborn animals were weaned, weighed and sexed at 3 weeks of age (21 ± 2 days of age). A total of 352 male mice, 175 control and 177 experimental, were matched by weight and placed in cages, generally five animals per cage. The animals were fed a standard pellet chow and received water ad libitum.

Lactate Dehydrogenase Methodology

The lungs were inflated with 6% gelatin (pH 7.0) at 20 cm H_2O height with a 22 gauge cannula inserted into the trachea. The gelatin-inflated lungs were then placed in a refrigerator at 4^oC to solidify the gelatin. The lobes then were separated, placed with hilar area down on a marked Mylar sheet identifying the experiment, experimental group and lung lobe, wrapped in aluminum foil and stored at -75^oC. For sectioning, the lung lobes were oriented to provide topographical lung tissue areas consistent for all lobes. Sections 15 micrometers thick were cut on a Cryostat, with 8-10 sections per lobe. The details of the LDH reaction, carried out on lyophilyzed frozen sections, have been published (8). In brief, a tetrazolium salt is used as an electron acceptor and is reduced to an insoluble formazan in the presence of the LDH enzyme, with lactate as a substrate. The insoluble formazan salt is deposited in the cell's cytoplasm at the site of the lactate dehydrogenase activity and serves as a marker for cell identification.

Quantitative Image Analysis

A Cambridge 720 Image Analyzer with a shading corrector and recording terminal (cassette tape and print out) was used to obtain measurements of Type 2 cells and alveolar wall. The gray values for detection of Type 2 cells were set with one of the two discriminators at a level which resulted in no change between the Type 2 cells as seen on the in the video display of the microscopic image, and that observed in the positive electron image displayed by the detector system. The numbers Type 2 cells detected were "fine-tuned" by comparing by manual counting how well the electronic detection matched the "flagging" signals in a selected area of the video display. The detected image was also compared to the field observed through the microscope. The second discriminator was used for measurement of wall area, its perimeters and linear intercepts. Similarly, the upper limit set for wall area was a maximum grey level value that corresponded to the image in the microscope and fell just short of producing electronic background "noise".

Image Analysis Quantitation of Type 2 Pneumocytes and Alveolar Walls

The deposits of formazan reaction product at the sites of LDH activity are essentially selective for the Type 2 pneumocyte under the conditions of the tissue section preparation: the cell is exceptionally large and round, uniquely occupies alveolar corners, and is the only cell in the alveolar wall with a heavy perinuclear cytoplasmic deposit. Other alveolar wall cells, Type 1 pneumocytes, endothelial cells, fibroblasts and macrophages have greatly attenuated cytoplasm, and the finely granular, diffuse LDH reaction products in their cytoplasm are not detected by the Image Analyzer at the grey level value of the detector set for the Type 2 cell. Further, desquamated and rounded macrophages in the alveolar lumina are essentially excluded from the quantitation by the gelatin washout. The bronchial mucosa cells in the conducting airways are also strongly positive for LDH. However, these structures are readily identifiable and, when present, are excluded from

the lung field by an image editor. A weak background staining of the alveolar wall serves to delineate the walls for measurement by a second detector grey value setting of the image analyzer.

Four left lung sections from each animal were divided into four quadrants in a specified sequence: the posterior apical, anterior apical, anterior basal and posterior basal. The first field in each quadrant of approximately 2mm x 2 mm (10X ocular and 6.3x objective) showing uniform staining for LDH, intact lung structure, and less than 50% bronchial and vascular structures was used for image analysis. The bronchial tree branches and vascular structures were deleted from the field with an image editor. The measurements of the Type 2 cells and wall area included edited and unedited fields. A total of 18 measurements, nine edited and nine unedited, were recorded per each field. The unedited field measurements were used to check the integrity of the lung structure and to assure that the editing of the fields was not biased.

An automated program was used to count Type 2 Cells according to a sizing factor, i.e 8um, 10um and 12 um. In addition, in each field the total area of the Type 2 cell was measured. Other measurements included wall area, perimeter of the alveoli and alveolar wall liner intercepts.

A single command activated the automatic programmer to provide nine measurements of the edited field and nine measurements of the unedited field. The data were recorded on a cassette tape and were also printed out as permanent record.

Data Processing

The Biomedical Data Program of UCLA (BMDP STATISTICAL PACKAGE) programmed for the IBM-XT was made available to us as a part of a "Beta Tester" program, and has since been expanded to new and upgraded statistical programs. We also expanded the computer's memory to 640K RAM and added an 8087 coprocessor. The statistical analysis programs used (e.g. t-test, 2-way analysis of variance, Mann-Whitney non-parametric) have been worked out and tested for the processing of our image analysis data. Programs for capturing data from casettes directly onto floppy disks, and for formatting data were provided to us by our computer consultant.

Basic Statistical Analysis of the Data.

The statistical analyses included Student's t-test, two factor analysis of variance, and appropriate non-parametric tests and were used for comparisons within and between test periods, i.e group, time, and group x time.

Statistical analysis was carried out on the measurements of Type 2 Cells, alveolar wall and their transforms, i.e. Type 2 Cell numbers, their field area (total area of all cells in one field), linear intercepts, mean cell area (field area of cells/field divided by numbers of cells/field),total wall area (including Type 2 cells included in the wall), wall area minus Type 2 cell area, wall perimeters, and wall linear intercepts,
number of Type 2 Cells/alveolar wall area and ratio of Type 2 Cell area/ alveolar wall area.

5. Results

A total of 325 male mice, 166 control and 159 mice NO_2 exposed, group were available for the study: 34 pairs each for the 12 weeks exposure period (0 week postexposure) and the 4 week postexposureperiod. For the 10 weeks and 32 weeks postexposure periods 33 pairs and 56 animal were examined, respectively. (Table 20).

Type 2 cell numbers were essentially equal for the control and experimental animals groups at the end of the NO_2 exposure period (0 weeks postexposure) and also at 4 weeks postexposure period (Tables 21,23). At 10 weeks postexposure,there was slight increase in Type 2 cell numbers in the exposed animals (Table 25). At the end of 32 weeks postexposure, the 8% increase in the number of Type 2 Cells in the experimental group had become significant. ($p<.0001$; Table 27).

Mean Type 2 cell Area (field area of cells/number of Type 2 Cells) was essentially equal immediately following exposure (Table 22), decreased by 5.3% for the exposed animals at 4 weeks postexposure (Table 24) and at 10 weeks postexposure, was again essentially identical to that of the control group (Table 26). However, a substantial and statistically significant rise (9.5%, $p=.05$) for the exposed animals occurred at the 32 week postexposure (Table 28).

The alveolar wall area was consistently higher for the exposed animals throughout all four time periods, with increases of 2.3%, 6.0%, 5.5%, and 6.6% respectively (Tables 21,23,25,27). The difference at the 32 weeks postexposure period was at a borderline level of statistical significance ($p=.1$; Table 27). When the measurements of alveolar wall area were modified to exclude Type 2 Cell Area, the trend was comparable to the unmodified findings for all four test periods (Tables 21,23,25,27). Alveolar wall perimeters tended to parallel the alveolar wall area findings, with the 10 week and 32 week differences showing borderline statistical significance ($p=.1$ and $p=.07$, respectively). The alveolar wall linear intercept differences were closely aligned with those of the perimeter, including statistically significant differences at the $p=.1$ and $p=.07$ levels, respectively (Tables 25&27).

A number of ratio transforms were carried out to relate Type 2 cell numbers and area to the modified and unmodified alveolar wall area measurements (Tables 22,24,26&28). The ratios of Type 2 Cell numbers/alveolar area were increased at statistical or borderline levels for the exposed animals at four weeks postexposure ($p<.05$ unmodified, and $p<.09$ modified; Table 24). The difference at 32 weeks postexposure was also statistically significant ($p<.01$ modified; NS unmodified; Table 28).

The 32 week postexposure period was in general remarkable for the consistently greater differences in all measurement categories shown by the exposed animal group (Tables 27&28). Of 13 measurements and transforms, only 3 failed to reach a statistically significant level (Tables 27&28).

Significant differences due to <u>treatment</u> (NO_2) were observed for Type 2 cells ($p=0.047$), alveolar wall minus Type 2 cell area ($p=0.5$), wall area ($p=0.02$), Type 2 cell field area ($p=0.019$), and Type 2 cell linear intercepts ($p=0.01$). Mean Type 2 cell area was only of borderline significance ($p=0.09$).

<u>Interaction</u> (time x treatment) was significant for Type 2 cells ($p=0.03$), Type 2 cell field area ($p=0.006$) and Type 2 cell linear intercepts ($p=0.006$). The ratio of Type 2 cells (>8 um) to alveolar wall area minus Type 2 cell area was of borderline significance ($p=0.1$).

<u>Time</u> (exposure and postexposure periods) significantly affected wall area ($p=0.0003$).

6. Discussion

1. Control Animal Group Findings

Reported data include measurements of Type 2 Cells and alveolar wall obtained at various ages of newborn mice at 12 weeks, 16 weeks, 22 weeks, and 44 weeks of age. While several factors obviate a strictly controlled comparison between measurements taken at

the different age periods (e.g. variations in the staining process, technician differences, and image analyzer variations), a number of consistent trends were found that tend to validate the changes as chronologic events. In addition, an earlier experiment (M117;13) where the lungs of newborn control group animals were examined at 6 weeks, 10 weeks, and 16 weeks of age serves as a useful reference point. The zero reference value for all charts is the measurement observed for the control animal group immediately following the 6 week or 12 week NO_2 exposure period, a time referred to as the "0 postexposure period".

Type 2 Cell numbers fell progressively, almost in straight line fashion and reached maximal level of -14.6% at 44 weeks of age (Chart 1) as compared to the earlier findings (4), where the initial fall at 10 weeks of age was subsequently followed by a 6.3% rise above the baseline level at 16 weeks of age (13).

Mean Type 2 Cell area, following a decrease at 16 and 22 weeks of age, showed a sharp increaseabove the baseline at 44 weeks of age 39.2% (Charts). A progressive rise in Mean Type 2 Cell area for M117 resulted in a final increase, at 16 weeks of age, of 21.8% (4).

Mean Alveolar Wall Area, with and without Type 2 Cell area included, with one exception was increased and above the baseline level for all ages of mice tested (Chart 3) However, changes in Type 2 Cell area resulted in a fall in modified alveolar wall area between 16 and 22 weeks of age (Chart 4). The earlier (M117) newborn animal study showed little change in wall area at 10 weeks of age but a 22.5% increase at 16 weeks of age (4,13).

The ratio of Type 2 Cell Number to Modified Alveolar Wall Area (minus Type 2 Cell Area) progressively fell and then plateaued at 16 weeks (Chart 5). This ratio serves in partas a control for both hyperexpansion (normal development or emphysematous change) and atelectasis. Since a fall in cell number/wall area ratio was both clearly and consistently present in the present and previous experiments, it is apparent that a change in lung volume (i.e. either an increase or a decrease in the numbers of alveoli per lung field) cannot by itself explain the altered relationship between Type 2 Cell number and alveolar wall area. However, there is the possibility if not likelihood that a combination of increased lung expansion and increased alveolar wall thickness could be a contributing factor. An increase in alveolar airspace does occur in the maturing rat lung up to two months of age (11), and capillary blood volume up 4 months of age (12). If there is in fact a continuing development of the mouse lung at 44 weeks of age, there is the implication of a longer time period of increased susceptibility of the animals to adverse effects. The findings of the present study support and extend the earlier M117 experimental finding (13) that lung development is at least longer than the two months reported for the rat lung (11). It would appear from our findings that the mouse lung fully matures sometimes after 16 weeks of age, based on the lack of a plateau for the majority of the measurements and transforms. Note that Mean Type 2 Cell Numbers produced almost straight line plots (Chart 1), and see data on Mean Type 2 Cell Area (Chart 2). However, the evidence is tempered by the fact that the charts of modified Mean Alveolar Wall Area and of the cell/wall area ratio suggest a trend towards a plateau after 16 and 22 weeks of age. One other consideration is the possibility that a delay in development of the control animal lungs could be in part reflecting the adverse effects of some covert noxious agent. The control animals are by no means completely sheltered from noxious environmental influences, and indigenous organisms are ubiquitous in mouse colonies.

One finding that warrants special attention, since it was particularly consistent for the present and previous experiments, is a decrease in Type 2 Cell number with time. Since mean Type 2 Cell Area was greater than the baseline value at the final test period (Chart), and an almost straight line (Chart 2), it is possible that the fall in number of cells is partly due to cell aggregation. When one Type 2 Cell abuts against the border of another, it will be detected by the image analyzer as a single cell. Some Type 2 Cell aggregates are

normally present in alveoli, although most Type 2 Cells are individually situated in alveolar "corners". Moreover, there is a tendency for Type 2 Cell aggregates to concentrate in alveoli of the proximal portion of the acinus (17,18), and the special meaning for this localization with respect to noxious agents will be further discussed below. No doubt other factors are involved in the lower density of the Type 2 Cell population, especially expansion of the alveoli as the lung "unfolds" and otherwise matures.

2. **Experimental Animal Data Compared to Those of Control Groups**

a. NO_2 Exposure, Type 2 Cell and Alveolar Wall Area Measurements

The 32 week postexposure period data showed a number of significant and consistent differences between the control and experimental animal groups. **Type 2 Cell numbers**, while decreased relative to the start of the experiment, were greater than those of the control group at 10 week postexposure and were at statistically significant levels at the 32 week postexposure period ($p=.0001$; Table 27). In addition, the ratio of Type 2 Cell numbers to alveolar wall area (minus Type 2 Cell area) was also greater $p=.008$ at the 32 week postexposure period (Chart 5, Table 28). Further, at the 32 week postexposure period the Mean Type 2 Cell area was again statistically greater for the exposed animals $p=.05$ (Table 28). The findings not only confirm the significant differences seen in the earlier experiment with postexposure periods but add support to the conclusions drawn and trends that were found.

The findings from the two experiments strongly imply that NO_2 exposure at an ambient level has altered the epithelial cell populations of the mouse alveolus and alveolar wall itself. The disparate measurements of cells and alveolar walls at the 32 week postexposure period between the control and experimental animal groups further implies that the effects of NO_2 exposure have not been reversed despite a relatively long 32 week postexposure period for recovery. While there is clearly a need for a much longer follow up period, and at the same time a more definitive investigation of the nature of the lung alterations, it is nevertheless important to recognize that even a temporary alteration of cells has biological significance. Pertinently, all of the alterations noted (changes in cell number and size, and an increase in alveolar wall area) are early events and common denominators for a great variety of human lung diseases (7-20,29).

Increases in Type 2 Cell number are now well recognized as a response to damage and/or loss of Type 1 alveolar cells (5), and an increased turnover can be expected to produce both more small cells and large cells, the latter through cell aggregation, work hypertrophy, and cellular edema (with and without other alterations.) With respect to the alveolar wall area, capillary congestion with cell and tissue edema are to be expected as early and multifocal pathobiologic events. Since the lung bidirectional transport mechanism and circulation in general have considerable reserve capacity, the magnitude of alteration often must be high before compensatory mechanisms are overcome and disease becomes clinically manifest. A heavier than normal loading of the bidirectional protein transport system with clinically covert interstitial fluid retention could of course readily account for increases in alveolar wall area that are detectable only by large volume quantitation.

That NO_2 exposure at ambient levels does in fact adversely affect the capillary bed and the bidirectional fluid transport system has the consistent support of several experiments investigating capillary permeability. Our own reports have been based on a number of tracers used to detect protein leakage, including radioactively labeled albumin (8), in situ plasma protein labeling (1), and horseradish peroxidase (2).

B. RELATED WORK

Two related areas of investigation have been reported, one a study of serotonin content of the lungs, brains, and blood of mice exposed to 0.45 ppm NO_2 (31), and the other an ozone exposure at simulated high altitude (32). The measurements of brain serotonin content were done in view of the major role the central nervous system is believed to play in the control of lung function, and in particular the control of the pulmonary vasculature. In the latter respect, the Adult Respiratory Distress Syndrome (including "shock lung") is characterized by marked congestion and atelectasis, and this appears to be largely mediated through disturbances of the central nervous system. Serotonin is also a major hormonal product of the lung itself, but we have relatively little knowledge about its function in the lung and body in general, and essentially no information on perturbations by air pollution. The finding in our preliminary study that NO_2 exposure of mice increases brain serotonin and its metabolic product suggests that inhaled NO_2 has directly or indirectly reached and disturbed the brain, and is an indication for further exploration.

The report on ozone exposure at high altitude is the first to provide data in this research area. The increase in lung elastin is considered an adverse health effect on the basis of a number of experimental animal studies and reports of elastin increase and abnormalities in a variety of destructive human lung diseases.

C. PUBLICATIONS

a. Published

1. Sherwin, R.P. and Richters, V.
   Image analysis quantitation of Type 2 cells and alveolar walls.
   Part I. Influence of time on the developing mouse lung.
   J. Am. Coll. Toxicol. 4:17-26, 1985

2. Sherwin, R.P., Richters, V. and Richters, A.
   Image analysis quantitation of Type 2 cells and alveolar walls.
   Part II. Influence of 0.3 ppm nitrogen dioxide on the developing mouse lung.
   J. Am. Coll. Toxicol. 4:27-43, 1985

3. Sherwin, R.P. and Richters, V.
   The effect of 0.3 ppm ozone exposure to Type 2 cells and alveolar walls of newborn mice: An image analysis quantitation.
   J. Toxicol. Environ. Health 16:535-546, 1986

4. Richters, A., Richters, V. and Alley, W.P.
   The mortality rate from lung metastases in animals inhaling nitrogen dioxide (NO_2).
   J. Surg. Oncol. 28:63-66, 1985

5. Sherwin, R.P., Shih, J.C., Ransom, R. and Le, J.D.
   Serotonin content of the lungs, brains and blood of mice exposed to 0.45 ppm nitrogen dioxide.
   J. Am. Coll. Toxicol. 5(6), 1986

6. Richters, A., Richters, V. and Sherwin, R.P. Influence of ambient level NO_2 exposure on newborn and adult mice body weights.
   J. Environ. Path. Toxicol. Oncol. 7:65-72, 1987.

7. Damji, K.S. and Sherwin, R.P. The effect of ozone and simulated high altitude on murine lung elastin: Quantitation by image analysis
   Adv. Mod. Environ. Toxicol. in Press

b.In Preparation

1. Richters, V. and Sherwin, R.P.

   The effects of 0.25 ppm Nitrogen Dioxide on the developing mouse lung
   Part I. Animal growth and quantitation of Type 2 cells and alveolar wall area.

2. Richters, V. and Sherwin, R.P.

   The effects of 0.25 ppm Nitrogen Dioxide on the developing mouse lung
   Part II. Animal growth, and elastic tissue and alveolar wall quantitation

3. Sherwin, R.P. and Richters, V.
   Alveolar wall thickening of the mouse lung following ozone and/or nitrogen dioxide exposures at ambient levels. Part I. Experience with several studies and lack of reversibility postexposure.

4. Sherwin, R.P. and Richters, V.
   Alveolar wall thickening of the mouse lung following ozone and/or nitrogen dioxide

4. Sherwin, R.P. and Richters, V.
Alveolar wall thickening of the mouse lung following ozone and/or nitrogen dioxide exposures at ambient levels. Part II. Nature of the alveolar wall thickening.

5. Richters, V. and Sherwin, R.P.
The effect of ambient levels of ozone (0.2ppm) and nitrogen dioxide (0.3ppm) on the mouse lung. Image analysis quantitation of Type 2 Cells and alveolar walls.