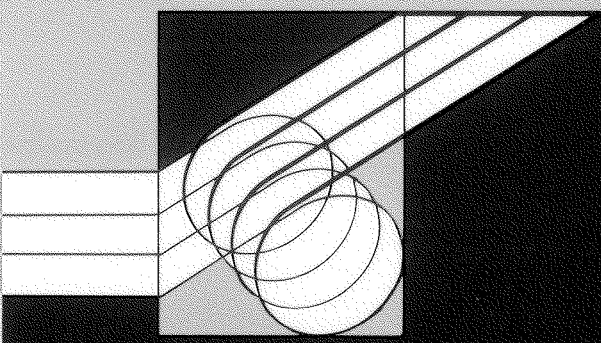
HEALTH EFFECTS INSTITUTE

H E I

RESEARCH REPORT No. 13



Effects of Nitrogen Dioxide on Alveolar Epithelial Barrier Properties

Edward D. Crandall, Ph.D., M.D.; Jeffrey M. Cheek, M.S.; Marian E. Shaw, M.D.; and Edward M. Postlethwait, Ph.D.

Division of Pulmonary and Critical Care Medicine
The New York Hospital
Cornell Medical Center
New York, NY 10021

Includes the Report of the Institute's Health Review Committee

The Health Effects Institute (HEI) is a non-profit corporation founded in 1980 to assure that objective, credible, high-quality scientific studies are conducted on the potential human health effects of motor vehicle emissions.

Funded equally by the U.S. Environmental Protection Agency (EPA) and 26 automotive manufacturers or marketers in the United States, HEI is independently governed. Its research projects are selected, conducted, and evaluated according to a careful public process, including a rigorous peer review process, to assure both credibility and high scientific standards.

HEI makes no recommendations on regulatory and social policy. Its goal, as stated by former EPA Administrator William D. Ruckelshaus, is "simply to gain acceptance by all parties of the data that may be necessary for future regulations."

Research Agreement No. 82-04
Designed and printed by Prime Communications, Inc.

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LIST OF ABBREVIATIONS

MEM	minimum essential medium
MEM-S	minimum essential medium plus newborn calf serum
NCS	newborn calf serum
R_t	tissue resistance
SCC	short circuit current
SEM	scanning electron microscopy
SPD	spontaneous potential difference

PREFACE

THE HEALTH EFFECTS INSTITUTE AND ITS RESEARCH PROCESS

The Health Effects Institute (HEI) is an independent nonprofit corporation which, according to its charter, is "organized and operated...specifically to conduct or support the conduct of, and to evaluate, research and testing relating to, the health effects of emissions from motor vehicles".

It is organized in the following ways to pursue this purpose:

INDEPENDENCE IN GOVERNANCE

HEI is governed by a four-member Board of Directors whose members are William O. Baker, Chairman Emeritus of Bell Laboratories and Chairman of the Board of Rockefeller University; Archibald Cox, Carl M. Loeb University Professor (Emeritus) at Harvard University; Donald Kennedy, President of Stanford University; and Charles Powers, President, Clean Sites, Incorporated. Professor Cox chairs the Board. These individuals, who select their own successors, were chosen initially, after consultation with industry and other individuals, by then Environmental Protection Agency Administrator, Douglas M. Costle.

TWO-SECTOR FINANCIAL SUPPORT

The Institute receives half of its funds from the United States government through the Environmental Protection Agency and half from the automotive industry. Twenty-six leading manufacturers of vehicles or engines that are certified for use on U.S. highways contribute to the Institute's budget, in shares proportionate to the number of vehicles or engines that they sell.

RESEARCH PLANNING AND PROJECT EVALUATION

HEI is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is developed by the Health Research Committee, a multi-disciplinary group of scientists knowledgeable about the complex problems involved in determining the health effects of mobile source emissions. The Committee seeks advice from HEI's sponsors and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee has defined an area of inquiry, the Institute announces to the scientific community that research proposals are being solicited on a specific topic. Applications are reviewed first for scientific quality by an appropriate expert panel. Then they are reviewed by the Health Research Committee both for quality and for relevance to the mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, which also reviews the procedures, independence, and quality of the selection process.

When a study is completed, a draft final report is reviewed by a separate HEI committee, the Health Review Committee. Members are expert scientists representing a broad range of experience in environmental health sciences. The Review Committee has no role in the review of applications or the selection of projects and investigators for funding. This Committee assesses the scientific quality of each study and evaluates its contribution to unresolved scientific questions.

Each funded proposal is assigned in advance of completion to a member of the Review Committee, who acts as "primary reviewer." When the draft report is received, the primary reviewer directs a peer review that involves: (1) referral of the report to appropriate technical experts and, when appropriate, (2) involvement of the Review Committee biostatistician to determine the appropriateness of the statistical methods used to evaluate the data. After the investigator has had a chance to comment on the technical evaluations, the primary reviewer drafts a review. This document is sent to the investigator for comment. It is subsequently examined by the full Review Committee and revised as necessary. The investigator's final report as well as the Review Committee's report are then made available to the sponsors and to the public after evaluation by the HEI Board of Directors.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing and nature of HEI report releases are tailored to ensure that the Review Committee's report does not interfere with the journal publication process. The report of the Review Committee will be as thorough as necessary to evaluate any individual report.

INTRODUCTION

In 1982, the Health Effects Institute requested applications to address cellular and biochemical markers that relate to nonneoplastic chronic lung diseases (RFA 82-4). One of the objectives of this RFA was the "development, calibration, and application of cellular and biochemical indicators of pulmonary disease." Edward D. Crandall, Ph.D., M.D., then Professor of Medicine at the University of California, Los Angeles, and now of New York Hospital, Cornell Medical School, proposed a five-year study to examine the effects of nitrogen dioxide and ozone on barrier characteristics of the pulmonary alveolar epithelium. The premise of this investigation was that information about these barrier properties would help us understand the basic functioning of the lung, as well as how changes in barrier properties might be related to pulmonary edema in humans. HEI accepted the proposal, and recommended several changes. The work would focus

exclusively on nitrogen dioxide, and would be limited to three years. The total cost of the work was $349,972. This project was initiated in September 1983, and the final report was first reviewed by the Health Review Committee in January 1987.

THE CLEAN AIR ACT

The Environmental Protection Agency (EPA) sets standards for motor vehicle emissions of oxides of nitrogen (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1977. Section 202(a)(1) directs the Administrator of the EPA to "prescribe (and from time to time revise)... standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgement cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a)(3) and 202(b)(1) impose specific requirements for reductions in motor vehicle emissions of oxides of nitrogen (and other pollutants), and provide EPA with limited discretion to modify those requirements.

The determination of the appropriate standards for emissions of oxides of nitrogen depends in part on an assessment of the risks to health it presents. Research on the effects of nitrogen dioxide on alveolar epithelium barrier properties, including the development of in vitro and in vivo models and improved research methods, can contribute knowledge useful in making the risk assessments needed for informed regulatory decision-making.

In addition, Section 109 of the Clean Air Act provides for the establishment of national ambient air quality standards to protect the public health. The current standards include one for nitrogen dioxide. Research on the effects of the type described above can contribute to the assessment of the appropriateness of these standards.

BACKGROUND

The health effects of high concentrations of air pollutants on the lung have been investigated extensively. However, the health effects of low or ambient concentrations of air pollutants are not clearly delineated. A major obstacle to such investigations is our inability to identify the early stages of injury that could serve as an indicator of a more serious or debilitating endpoint. The pulmonary epithelium, a single layer of cells that lines the pulmonary tract, plays an important role in protecting the underlying tissues and regulating the transport of fluid and solutes; therefore, damage to this permeability barrier may have significant health consequences. The study described in this report is an attempt to develop markers for detecting early stages of injury to the pulmonary alveolar epithelium.

The alveoli are thin-walled, sac-like structures that are exposed to the external environment on one side and are covered with capillaries on the other. The alveolar epithelium is composed of two types of cells. Type I pneumocytes cover 95 percent of the alveolar surface; these cells offer minimal resistance to diffusion of gases between the alveolar air space and capillary blood. The remaining 5 percent of the epithelial surface is covered with Type II pneumocytes, which replace Type I cells when the latter are damaged, and subsequently differentiate into Type I cells. In addition, Type II cells secrete a surfactant that lowers the surface tension of the alveolar fluid.

One of the earliest manifestations of lung injury from oxidant gases, such as nitrogen dioxide, is edema. Pulmonary edema is the abnormal accumulation of fluid in the airspaces of the alveoli; thus, it compromises the diffusion of oxygen into the blood. Edema may be life threatening in extreme cases. Although the detailed cellular mechanism edema induced by oxidant gases is not understood, it is believed that their oxidizing action leads to damage in the lipid bilayer of the cell membrane; the transport properties of the alveolar epithelium are altered as a result. Vascular fluid thus crosses the epithelial barrier and accumulates in the alveoli. As the alveoli fill up with fluid, oxygenation of the blood passing through the capillaries that surround the epithelium is adversely affected.

The normal exchange of fluids and solutes across the alveolar epithelium is stringently regulated by two kinds of transport mechanisms. Passive transport relies on concentration of bioelectrical gradients to move ions and small molecules. Active transport requires the expenditure of metabolic energy and frequently is facilitated by specific receptor proteins found in the cell membrane; this mechanism is used to transport ions and, perhaps, small and large molecules.

Dr. Crandall's objectives in the study described here were to assess alveolar epithelial permeability in cells cultured in vitro by looking at transport and the associated fluid transfer, and by measuring changes in bioelectric properties and ion fluxes. The investigation was based on two characteristics of epithelial cells. First, monolayers of Type II cells cultured on non-porous surfaces form fluid-filled hemicysts, or domes; these domes are thought to result from active solute transport from medium to substratum, with water following passively. In the current study, the number and volume of domes formed was used as a measure of integrity of barrier properties of the pneumocyte monolayer.

The second property of the alveolar monolayers examined was the bioelectric properties across the cellular layer adhering to a porous filter. Measurement of the bioelectric tissue resistance of such monolayers provides an indicator of epithelial barrier integrity; reduced tissue resistance implies a reduction in the barrier to passive transport of solutes. A reduction in another bioelectric property, the short-circuit current, is believed to indicate a net total reduction in active transport mechanisms.

INVESTIGATORS' REPORT

by Edward D. Crandall, Ph.D., M.D.; Jeffrey M. Cheek, M.S.; Marian E. Shaw, M.D.; and Edward M. Postlethwait, Ph.D.

Effects of Nitrogen Dioxide on Alveolar Epithelial Barrier Properties

ABSTRACT

This study analyzed the effects of nitrogen dioxide (NO_2) on alveolar epithelial permeability and transport properties. Primary cultured monolayers of rat Type II pneumocytes, cultured on both nonporous and porous surfaces, were used as models of isolated alveolar epithelium for in vitro exposure to nitrogen dioxide. The effects of nitrogen dioxide exposure for monolayers cultured on nonporous substrata were monitored by observing the changes in the net volume of fluid under the monolayer; for cells cultured on porous substrata, alterations in tissue bioelectric properties were noted.

As a first step, primary cultured monolayers of rat Type II pneumocytes plated on nonporous plastic Petri dishes were used to investigate the effects of nitrogen dioxide on alveolar epithelial barrier properties. Such monolayers form fluid filled domes that are thought to result from active solute transport from medium to substratum, with water following passively. We used dome formation as a transport marker. Five-day-old cultures were directly exposed to 30 ppm NO_2 in 5 percent CO_2 in air at 25°C, by cyclically tilting culture plates from side to side, so that both halves of the monolayer were exposed during each cycle. Exposures consisted of 10 cycles of four minutes each (two minutes per side), for a cell exposure time of 20 minutes. Control plates were simultaneously exposed to 5 percent CO_2 in air under identical conditions. One day after the exposure, nitrogen dioxide-exposed monolayers exhibited significant decreases in dome density and individual dome volume, compared to the controls. By 48 hours post-exposure, differences between nitrogen dioxide-exposed and control monolayers were less, but remained significant. These results showed that short-term sublethal exposures to nitrogen dioxide produce a decrease in dome formation in Type II alveolar epithelial cell monolayers. This finding is most likely due to a decrease in the active transepithelial sodium transport rate, or an increase in the permeability of cell membranes or tight junctions, or both. Addition of vitamin E-containing liposomes to the culture media 24 hours pre-exposure did not affect the nitrogen dioxide-induced decrease in dome formation, indicating that under these circumstances no protective effect was provided by the antioxidant.

Alteration of the isolation and culturing conditions of the cell monolayers generated cultures with differing susceptibility to nitrogen dioxide. Inhibition of dome formation 24 hours after exposure, compared to controls, was used to indicate the relation of dose to response of the monolayers to nitrogen dioxide. Cells that were isolated and cultured in Eagle's Minimum Essential Medium + 10% newborn calf serum (MEM-S) and were exposed for 20 minutes, demonstrated an apparent concentration to reduce dome formation by 50 percent (I_{50}) of 33 ppm, with a response threshold of approximately 10 ppm and a maximal response occurring at 40 ppm. Cells replated three hours post- plating and cultured in MEM-S supplemented with Ham's F-12 displayed an apparent I_{50} of 3 ppm, with a response threshold of less than 2 ppm and a maximal response occurring at 10 ppm. The reasons for this differing sensitivity are unclear at this time. However, the mechanism could involve differing nutrient components in the media, which may have participated in the production of reactive oxygen species during the nitrogen dioxide exposures.

Monolayers cultured on porous, crosslinked, collagen-coated Nuclepore filters were exposed to nitrogen dioxide by the same method as described above. For bioelectric measurements, the monolayers were mounted in Ussing chambers. Spontaneous potential differences and short circuit currents were measured, and tissue resistances were calculated. Bioelectric studies of control filters coated with crosslinked collagen revealed that monolayers exhibit spontaneous potential differences of 0.2 to 2.0 mV (apical side negative) and tissue resistances of 150-1000 Ω-cm^2, which are consistent with the development of monolayers with well-formed intercellular tight junctional complexes. Tissue resistance reflects the integrity of the tight junctional complexes that control paracellular permeability; short circuit current provides a measure of transcellular active ion fluxes. Monolayers exposed to 20 ppm nitrogen dioxide exhibited significant reductions in tissue resistance and nonsignificant reductions in short circuit current, compared to the controls. This suggests that nitrogen dioxide may primarily affect both epithelial active transport and passive ion transport via altered tight junctional pathways. It should be noted that a large decrease in tissue resistance could mask an effect on transcellular active ion transport because of a massive bi-directional ion leakage across the membrane. Further studies are necessary to delineate the relative dosimetry and specific mechanisms of oxidant (nitrogen dioxide)-induced effects on alveolar epithelial barrier characteristics.

INTRODUCTION

Nitrogen dioxide is a toxic atmospheric pollutant formed as a result of both automobile exhaust and industrial emissions. Its ability to alter, upon inhalation, normal pulmonary morphology and biochemistry has been well

detailed (Mustafa et al., 1978). Exposure to nitrogen dioxide results in marked morphological and physiological changes in airway epithelium (Kleinerman et al., 1977; Ranga et al., 1980; Jordon et al., 1983). Alveolar tissues also are affected because the low solubility of nitrogen dioxide allows for its penetration to distal areas. Inhalation of nitrogen dioxide causes reaction products to appear rapidly in the pulmonary circulation upon inhalation (Postlethwait, 1981). Alteration in protein clearance from parenchymal tissues has been reported (Sherwin et al., 1977), and high level exposures to nitrogen dioxide result in alveolar epithelial destruction, with resultant pulmonary edema (Spencer, 1968). Morphological studies reveal that cellular injury from chronic low-level exposures to nitrogen dioxide extends into the alveolar regions (Evans et al., 1973). Study of the characteristics of nitrogen dioxide toxicity to lung epithelia at various levels of exposure may be useful in elucidating mechanisms by which it induces cell injury.

The pulmonary alveolar epithelial barrier is a dominant factor in the maintenance of dry air spaces, with altered permeability likely involved in the generation of alveolar pulmonary edema. Because of the complex anatomy of the adult mammalian lung, investigations of the specific barrier properties of the intact alveolar epithelium have been difficult. As a result, our laboratory has used two models to study isolated alveolar epithelium: the hollow bullfrog lung, and primary cultured monolayers of mammalian Type II pneumocytes. When we used the latter preparation, the monolayers cultured on nonporous surfaces formed fluid-filled hemicysts, or domes. These domes are thought to result from active solute (sodium) transport from medium to substratum, with water following passively (Goodman, 1982; Mason et al., 1982; Goodman et al., 1983; Sugahara et al., 1984). This suggests that alveolar epithelium in vivo may actively remove solute from the alveolar air space, a process that could be important in the prevention and resolution of alveolar pulmonary edema in normal and pathologic states. Dome formation has been used in numerous epithelial cell culture systems to investigate active transepithelial transport properties (Misfeldt et al., 1976; Cereijido et al., 1978; Lever, 1979; Valentich et al., 1979).

Our research focused on the effects of nitrogen dioxide on active and passive transport properties of the alveolar epithelial barrier. These studies primarily involved isolated alveolar epithelial cells cultured on both porous and nonporous surfaces.

AIMS

HYPOTHESIS

Exposure to mobile source emissions adversely affects the barrier properties of mammalian alveolar epithelium, including both the active and passive transport characteristics.

SPECIFIC AIMS

1. To study the effects of nitrogen dioxide on monolayers of rat alveolar epithelial Type II pneumocytes plated on nonporous substrata, using dome formation as a transport marker.
2. To examine the effects of altered culture conditions on monolayer susceptibility to oxidant stress.
3. To investigate the effects of nitrogen dioxide on the bioelectric properties of monolayers plated on porous surfaces, and to determine the relative dosimetry and specific mechanisms by which nitrogen dioxide damages alveolar epithelial barrier properties.

METHODS

SOLUTIONS

Two balanced salt solutions were prepared according to the method of Dobbs et al. (1980). Solution I (pH 7.4 at 25°) contained 136 mM NaCl, 2.2 mM Na_2HPO_4, 5.3 mM KCl, 10 mM HEPES buffer, and 5.6 mM glucose. Solution II was identical to solution I, with the addition of 1.9 mM $CaCl_2$ and 1.3 mM $MgSO_4$. An emulsion of bovine serum albumin and fluorocarbon in solution II was prepared by sonication. An elastase solution (4 U/ml) was used to harvest the Type II cells (see description below). Elastase was obtained from Cooper Biomedical (Malvern, PA). Fluorocarbon was a gift from 3M (St. Paul, MN). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

CELL ISOLATION AND PLATING

A flow chart of the cell isolation procedure is given in Figure 1. Lungs were removed from 180 to 220 gm male specific-pathogen-free Sprague-Dawley rats (Hilltop Labs, Scottdale, PA), as previously described (Goodman et al., 1982). Solution I was used for perfusing the lungs to remove blood, and for repeatedly lavaging the airways to remove as many alveolar macrophages as possible. The excised lungs were filled with the fluorocarbon and albumin emulsion, and were incubated at 37°C for 20 minutes. After repeated lavaging with solution I to displace the emulsion, the lungs were filled with the elastase solution to total lung capacity (8 to 15 ml), and were incubated for 20 minutes at 37°C. Then the lungs were minced and sequentially filtered to obtain a crude cell mixture. Cell counts and viability were obtained using a hemocytometer and trypan blue dye exclusion.

A discontinuous metrizamide density gradient was prepared by layering metrizamide solutions ($p = 1.040$ over $p = 1.090$). The crude cell mix was layered onto the gradient, and was spun at 200g for 20 minutes at 4°C. Type II cells were found in a broad band throughout the gradient (Dobbs et al., 1980); alveolar macrophages, polymorphonuclear leukocytes, and clumped red blood cells were pelleted. Cells from the

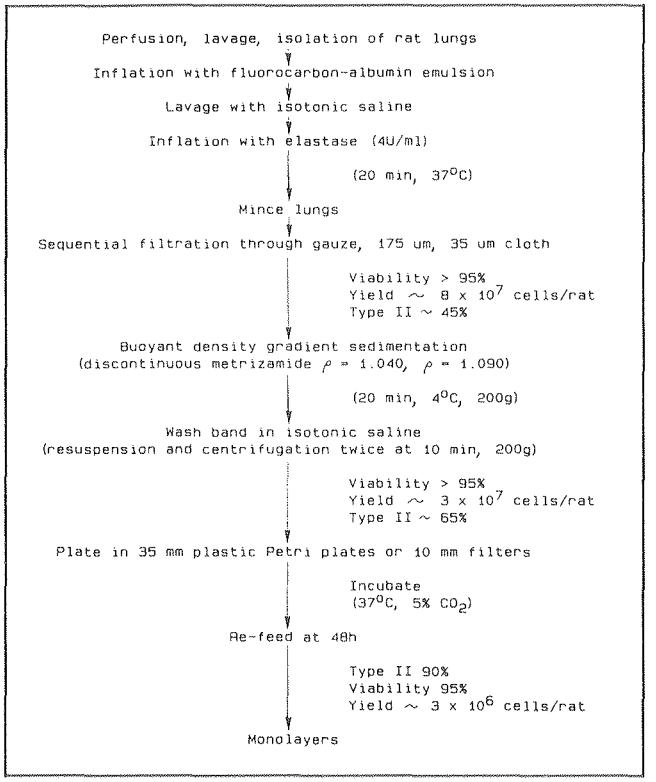

Figure 1. Flow chart of cell isolation procedure.

gradient band were removed and centrifuged twice, in cold solution II, for 10 minutes at 200g. The resulting Type II cell pellet (70 percent purity) was resuspended in Eagle's modified minimum essential medium (MEM), which contained 10 percent newborn calf serum (NCS) and 0.1 μM dexamethasone. Cells were plated on 35mm Falcon Petri dishes (Becton-Dickinson, Cockeysville, MD) or on 12 mm Nuclepore "filter cups" (see description below), at a density of 8 x 10^5 cells/cm^2. In some experiments, MEM was supplemented, at the ratio of 2 to 1, with Ham's F-12, and cells were replated after three hours. The nutrient medium was changed on the second day after plating; unattached blood cells were removed in the process. By the third day, the confluent monolayers contained more than 90 percent Type II cells with greater than 95 percent viability (Brown et al., 1984; Figure 1). The cultures were continuously incubated at 37°C in a 5 percent carbon dioxide in air incubator, except when they were removed for observations of dome formation (five minutes), or for exposure to experimental or control gases (two hours), or for bioelectric studies.

DOME DENSITY AND VOLUME DETERMINATIONS

The density of domes in the monolayers cultured in Petri dishes was determined by counting the number of domes on 10 random fields per plate, at a magnification of 200. The total number of domes on all fields was divided by the total area of the fields counted to calculate dome density. Dome diameter was estimated using a calibrated micrometer eyepiece, and dome height was determined using the calibrated microscope fine-focusing control. Individual dome volume (V) was calculated by assuming that each dome is a section of a sphere, using the equation

$$V = \pi h/6\ (h^2 + 3d^2/4),$$

where h = dome height and d = dome diameter. Total dome fluid volumes under the monolayers can be estimated from the dome densities multiplied by the individual dome volumes.

BIOELECTRIC MEASUREMENTS

Porous filter cups were prepared by affixing 0.22 μm Nuclepore filters to 12 mm Lexan rings with GE RTV Silicone Seal. Using a modification of the method of Cereijido et al. (1978), the cups were coated with a solution of rat tail collagen (3 mg/ml), which was attached to the filter by precipitation with ammonium hydroxide and crosslinked with glutaraldehyde. After thorough rinsing of the filter cups with MEM, the cells were plated on the filter cups, as previously described.

For bioelectric measurements (Figure 2), the filter cups were mounted in polyethylene adapters, so that the exposed tissue or monolayer surface area equalled 0.785 cm^2. Silicone high-vacuum grease (Dow-Corning, Midland, MI) was applied to the outside of the filter cups prior to mounting, to seal possible leaks. Once assembled in the polyethylene adapters, the filter cups were vertically mounted between two water-jacketed, conical Plexiglas hemichambers (Crandall et al., 1981; Figure 2). The hemichambers were filled with 8 to 12 ml of MEM without serum; a bubble-lift system, which used water-saturated 5 percent carbon dioxide-95 percent oxygen, maintained pH at 7.4 and fluid mixing. All measurements were performed at 37°C.

Spontaneous potential difference (SPD) across the monolayers was measured using two matched calomel half-cells (Fisher Scientific, Tustin, CA), whose output was amplified (DC preamplifier MPA-6, Transidyne General, Ann Arbor, MI) and recorded (Datamart 260 strip-chart, Linear Instrument, Irvine, CA). Each calomel electrode was connected to one hemichamber via a 4 percent agar-3M KCl bridge, whose tip was located about 2 mm from the tissue surface. The liquid junction potential of these bridges was less than 1 mV, and was adjusted or bucked to zero prior to mounting the tissue.

Direct current, including short circuit current (SCC), was generated by a 12-V dry battery, and was varied using a 10-turn 1-M Ω potentiometer. All currents were passed across the tissue through Ag/AgCl electrodes and connecting agar-KCl bridges. The current bridge tips were located about 5 cm from the tissue surface, at the apexes of the conical hemichambers. The

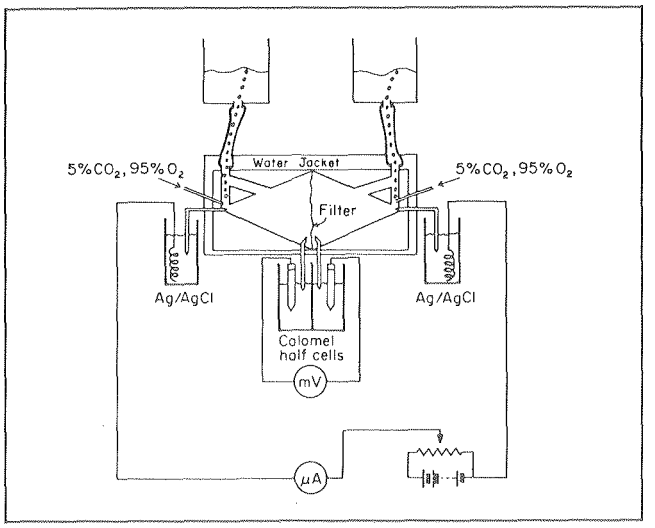

Figure 2. Schematic drawing of Ussing-type chamber. Monolayer on filter is mounted between two hemireservoirs of MEM. Transepithelial voltage, including spontaneous potential difference (SPD), is measured by calomel half cells and current (including short circuit current, or SCC) is passed through Ag/AgCl-agar bridge electrodes. Tissue resistance (R_t) is calculated from the relationship, $\Delta V/\Delta I$, where ΔV is the voltage deflection measured across the tissue in response to the current ΔI applied externally.

current was monitored by measuring the voltage drop across a 100-Ω resistor in the current circuit, and was recorded on the strip-chart recorder.

Tissue resistance (R_t) was calculated from the relation $R_t = dV/dI$, where dV is the voltage deflection in response to a small current dI (10 μA) across the tissue. All tissue resistance measurements were subsequently corrected for the solution resistance (80 Ω cm^2).

MONOLAYER EXPOSURE PROCEDURE

In experiments that used monolayers cultured on plastic dishes, dome density and volumes were measured on the fifth day after plating (Day 5), immediately before exposure to experimental or control gas mixtures. The time of exposure is referred to below as 0 hour. Dome density and volumes were measured again at 24 hours (Day 6) and 48 hours (Day 7) after exposure. Because the medium over the monolayers was changed every 48 hours, all plates were fed at 24 hours pre-exposure (Day 4) and 24 hours post-exposure (Day 6). For the bioelectric studies, spontaneous potential difference, tissue resistance, and short circuit current were measured at 24 hours post-exposure.

The exposure chambers consisted of two 150 mm inner diameter airtight Pyrex glass cylindrical vessels, each with inlet and outlet ports and a removable lid. Gas mixing inside the chambers was maintained by floating stir bars in the bottom of the chambers. The glass chambers were fixed onto magnetic stirrers, and the entire assembly was centered on a rocker platform that could be tilted by as much as 30° (Bellco Laboratories, Vineland, NJ). The exposure system materials were composed of glass, Teflon, and stainless steel, to minimize interactions with the nitrogen dioxide.

The inlet stream to both exposure chambers consisted primarily of 5 percent carbon dioxide in air, which was passed through a 0.22 μm filter for sterility, and was bubbled through sterile water in a gas washing bottle for humidification. In the experimental chamber, the desired concentration of nitrogen dioxide was obtained by diluting a concentrated source of nitrogen dioxide into the inlet stream. The appropriate amount of stock 900 ppm nitrogen dioxide in nitrogen (Matheson, Cucamonga, CA) was injected countercurrent into the inlet stream. The inlet stream with nitrogen dioxide then was passed through a mixing coil before it entered the exposure chamber. Both the inlet and outlet nitrogen dioxide concentrations were monitored by the Saltzman technique (Intersociety Committee for Air Sampling and Analysis, 1977). The control chamber was gassed only with humidified 5 percent carbon dioxide in air. The flow rates to both chambers, of 350 ml/min, were adjusted to allow 12 volume changes per hour.

The monolayers (either on plastic dishes or filter cups) were divided into two populations for exposure to either experimental or control gas mixtures. Prior to exposure, dome counts were taken on the plastic dishes. (At this time, multiple Ussing chamber measurements are not possible, so only post-exposure measurements were done on monolayers grown on filters.) Experimental and control monolayers were placed in their respective exposure chambers, and the inlet gases were allowed to equilibrate for approximately one hour. As soon as the outlet nitrogen dioxide concentration matched the inlet value in the experimental chamber, the monolayers were directly exposed to the gases by alternately tilting the chambers to an angle of 30 degrees. This temporarily removed the medium from the apical surface of one half of the monolayer, and allowed direct contact with the chamber gas. This method of exposure is similar to one previously reported by Rasmussen (1984).

The experimental cultures were exposed to various concentrations of nitrogen dioxide in 5 percent carbon dioxide in air at 25°C, for 10 cycles of 2 minutes per side, for a total cell exposure time of 20 minutes. Control cultures were simultaneously exposed to 5 percent carbon dioxide in air under identical conditions. This schedule allowed the direct interaction of gas with the monolayer, and minimized the damaging effects of cell drying. Additional control monolayers were kept in a 5 percent carbon dioxide incubator for the duration of the experiment, to determine if non-nitrogen dioxide related experimental conditions (e.g., temperature changes or mechanical handling) affected monolayer performance.

ANTIOXIDANT SUPPLEMENTATION STUDIES

To determine if providing antioxidants to the monolayer might be effective in preventing oxidant damage, we added vitamin E (primarily α-tocopherol, a lipid soluble antioxidant)

in some experiments to the nutrient medium prior to exposure to nitrogen dioxide. Vitamin E-containing liposomes were synthesized in the following manner. The appropriate amount of α-tocopherol (6 to 10 mg) was added to dipalmitoyl phosphatidyl choline (DPPC, 60 to 100 mg). This mixture was dissolved in chloroform, dried under nitrogen, and added to 15 ml of nutrient medium. The 10 percent α-tocopherol/DPPC liposomes then were prepared by sonication. The resulting liposome medium suspension was added to both control and experimental plates 24 hours prior to exposure (Day 4), to allow uptake of the liposomes by the cell monolayer to occur. Nitrogen dioxide exposures, and subsequent dome observations, then were conducted as described above.

DATA PRESENTATION

All values of experimental results (except where noted) are given as mean $\pm$ standard error of the mean. Sample points are defined as individual monolayers (i.e., n = one plate or monolayer), except where noted. Significant differences among groups were determined through the use of one way analysis of variance (ANOVA), with $p \leq 0.05$ as the significance level. In cases where the ANOVA F test indicated significant differences among the group means, specific differences between groups were determined by use of Scheffe's method.

RESULTS

PRELIMINARY STUDIES

As a first investigational approach, we exposed rat Type II alveolar epithelial cell monolayers, cultured on plastic plates, to nitrogen dioxide to determine whether or not effects on active transport could be an early marker of nitrogen dioxide toxicity. The development of a quantitatively reproducible system for exposing cultures to potentially toxic gases was a formidable task. One technical problem involved the determination of a suitable culture vessel for our exposure system. The Type II cell monolayer grows well in a tissue culture T-flask, but these are unacceptable for nitrogen dioxide exposure work because of size (the large number of cells required for confluent monolayers) and gas flow characteristics (difficult to expose monolayers to ambient atmosphere). As a result, despite successfully using monolayers cultured in T- flasks in numerous previous studies, we decided to plate the cells on 35mm Petri dishes.

Dome formation in the Petri dishes initially was variable and less dense than in the T-flasks, despite numerous maneuvers. We successfully and reproducibly cultured dome-forming monolayers on Petri dishes after we made significant alterations in our cell isolation and culture procedure, which included the use of higher purity cell suspensions for plating and increasing the volume of medium over the cell monolayer. Figure 3A shows a phase-contrast photomicrograph of a typical dome formed by a confluent monolayer of Type II alveolar epithelial cells. Figure 3B is a light photomicrograph of a cross-section from a typical dome from a Day 5 plate. Figure 3C is a transmission electron photomicrograph of the cell at the right corner of Figure 3B; Figure 3D depicts the tight junction of the cell shown in Figure 3C and an adjacent cell. These figures show that polarized alveolar epithelial cell monolayers are formed in primary culture, with the dome representing active sodium transport medium to substratum with water following passively.

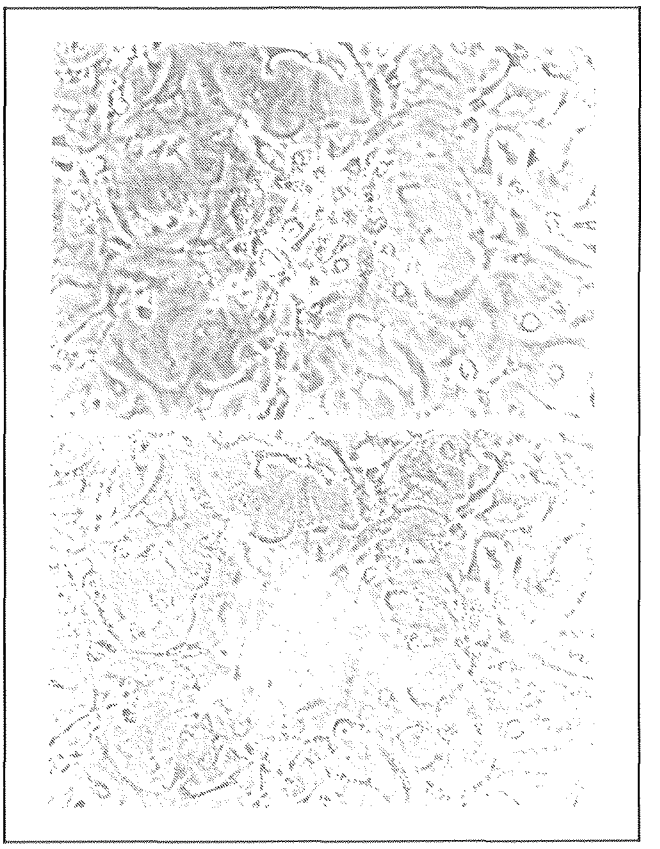

Figure 3A. Phase-contrast photomicrographs of a Type II alveolar epithelial cell monolayer. Top, focused on apex of dome; bottom, focused on base of dome.

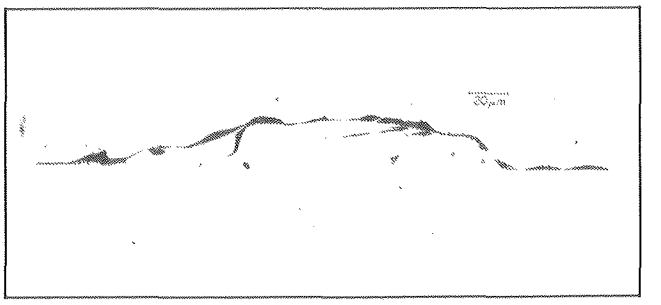

Figure 3B. Light micrograph of a cross-section of a typical dome from a Day 5 plate. Note that the dome consists of a monolayer of cells raised above the nonporous plastic surface. In culture, such domes are fluid-filled.

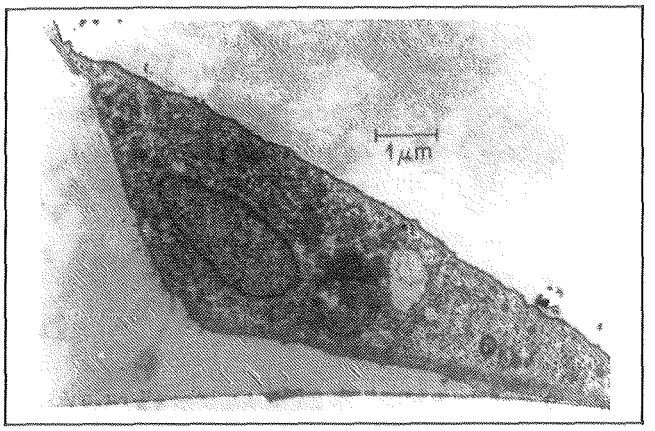

Figure 3C. Transmission electron micrograph of the cell at the right hand corner of the dome in Figure 3B. Note the presence of a lamellar inclusion body within the cell.

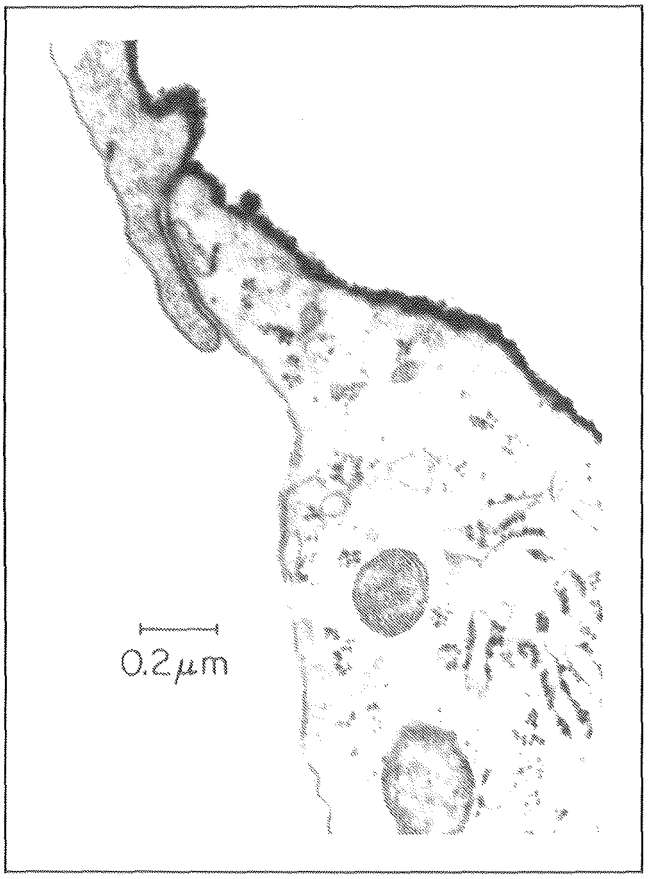

Figure 3D. Transmission electron micrograph of the tight junction between the cell shown in Figure 3C and an adjacent cell. Note the zonula occludens at the apical side of the junction.

Another problem that had to be solved was the development of an exposure system that would allow the interaction of nitrogen dioxide, a highly reactive and insoluble gas, with the cell monolayer. Initially, monolayers under the medium were exposed to 20 to 30 ppm nitrogen dioxide for up to four hours. This produced a change in color of the medium that contained the indicator (phenol red), even though no change in medium pH was measured. The exposure also produced a darkening color change in the medium even when the pH indicator was absent. No change in medium pH was detected during or after the exposures, which suggested that the color change was due to the formation of unknown nitrogen dioxide reaction products in the media. At least one of the reaction products was determined to be nitrite (NO_2-), which was produced in the medium at a rate of 15 μg/ml/hr. Despite the formation of nitrogen dioxide reaction products in the medium during these high level exposures, no differences in dome formation or monolayer viability were noted.

To check for the possible effect of a bolus of such nitrogen dioxide products in the medium, medium alone, which had been previously exposed for up to four hours as above, was fed to monolayers that had been maintained in the incubator. Again, no deleterious effects on the monolayers were observed. Based on the results of these experiments, we concluded that the thick overlying medium probably provides the cell monolayer with an insulating barrier against exposure to nitrogen dioxide, and that nitrogen dioxide reaction products formed in the medium are non-toxic. Consequently, our efforts turned to devising an exposure methodology that would circumvent this problem and would more accurately reflect the situation in the intact mammalian lung.

Because of the absence of toxicity associated with using the exposure protocol above, we developed an exposure system that allowed the direct interaction between the ambient gas and the cell monolayer (Figure 4). The culture plates were tilted from side to side during the exposure, by using a rocker platform. This allowed the removal of the medium from the apical surface of the monolayer, thus alternately exposing cells on each half of the plate directly to the ambient gas. Although

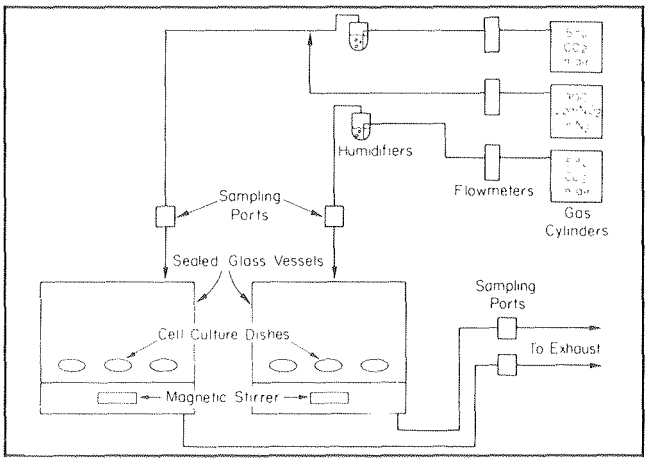

Figure 4. Schematic diagram of the NO_2 exposure system. Both exposure vessels and corresponding magnetic stirrers are supported on a rocker platform. This allows simultaneous tilting of experimental and control monolayers while facilitating gas mixing inside the chambers.

this approach overcame the separation of nitrogen dioxide from the cell monolayer created by the overlying thick layer of medium, it was still necessary to determine the appropriate parameters for monitoring toxic effects. The maximum amount of time that the monolayer could be exposed to gas (without ensuing cell drying and death) needed to be determined, as did the exposure concentration of nitrogen dioxide that would produce an acute effect. We used our exposure system initially at 30 ppm nitrogen dioxide for 20 minutes, to obtain quantitatively reproducible data on the effects of acute nitrogen dioxide exposure on dome formation in monolayers plated on nonporous plastic Petri dishes. The results of these studies are given below.

DOME STUDIES

In Figure 5, dome density in monolayers exposed to 5 percent carbon dioxide in air in the exposure chamber (exposure control plates, n = 27) is compared to that for monolayers maintained in the incubator for the duration of the experiment (n = 22). There were no significant differences in dome density or individual dome volume between the two groups at pre-exposure (0 hour), 24 hours or 48 hours after the exposure. Both groups demonstrated a slight but significant decrease in dome density 24 hours post- exposure (Day 6), compared to their respective 0 hour values. This decrease in dome formation probably reflects the requirement of the monolayers for both fresh nutrients and removal of metabolic wastes within 48 hours after the last medium change (which corresponds in Figure 5 to 24 hours after the exposure). All plates were fed again immediately after the 24 hour post-exposure observations (Day 6). By 48 hours after the exposure, dome densities in both groups had returned to their pre-exposure levels. These findings indicate that monolayer performance is not affected by non-nitrogen dioxide related parameters (e.g., temperature changes or handling), but can be influenced by the age of the medium.

The effect of nitrogen dioxide exposure on dome density is shown in Figure 6. There was no significant difference between the experimental and control monolayers at 0 hour. Twenty-four hours after exposure, the number of domes on nitrogen dioxide-exposed plates (n = 27) was 65 ($\pm$7) percent (mean $\pm$ sem) of those on control plates (n = 27). All plates were fed immediately after the 24 hour counts. By 48 hours post-exposure, dome density in nitrogen dioxide-exposed monolayers had increased to 79 ($\pm$ 2) percent of control values. The differences at both 24 hours and 48 hours between the two groups were significant.

Figure 7 shows the effect of nitrogen dioxide exposure on individual dome volume. Pre-exposure values for both groups were almost identical, at 6.0 ($\pm$ 0.6) x 10^5 μm^3. (Sample size, n, represents an individual dome; for the controls, n = 120, and for the experimental group, n = 124.) Twenty-four hours after exposure, the control values remained essentially unchanged, at 6.8 ($\pm$ 0.7) x 10^5 μm^3 (n = 105), but dome

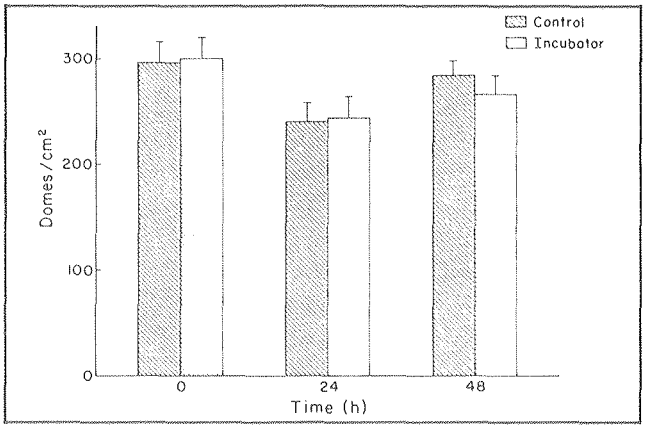

Figure 5. Dome density in incubator vs. control monolayers. Vertical bars represent standard error of the mean. No significant differences were noted between populations at 0, 24, or 48h (Appendix A).

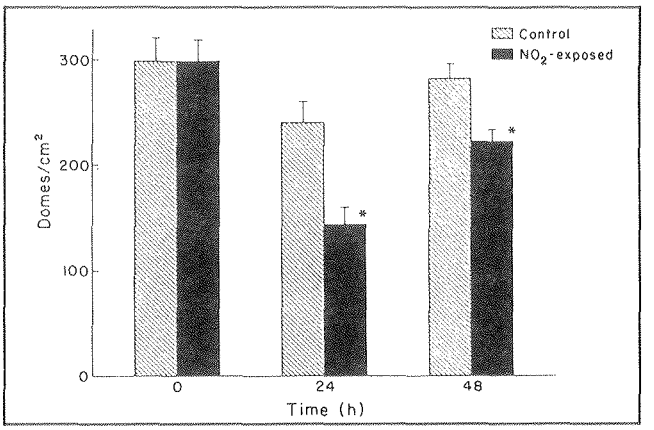

Figure 6. Effects of NO_2 exposure on dome density. Experimental monolayers were exposed to 30 ppm NO_2 for 20 minutes. Vertical bars represent standard error of the mean. Significant differences between control and NO_2-exposed populations are noted by (*).

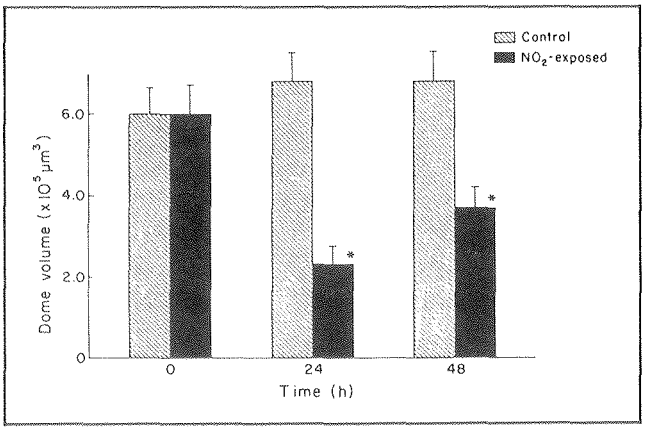

Figure 7. Effects of NO_2 exposure on individual dome volume. Experimental monolayers were exposed to 30 ppm NO_2 for 20 minutes. Vertical bars represent standard error of the mean. Significant differences between control and NO_2-exposed populations are noted by (*).

volumes in nitrogen dioxide-exposed monolayers had decreased significantly (in comparison to controls), to 2.3 ($\pm$0.5) x 10^5 μm^3 (n = 25). Finally, at 48 hours post-exposure, control values were equal to their 24 hour volumes (n = 97), but mean dome volumes of the nitrogen dioxide-exposed monolayers increased slightly over their 24 hour values, to 3.7 ($\pm$0.5) x 10^5 μm^3 (n = 31), although they remained significantly below the 48 hour control values.

In Figure 8, the effect of nitrogen dioxide exposure on total fluid volume under the monolayers is shown. The two groups yielded very similar pre-exposure mean values of 17.8 ($\pm$2.9) x 10^7 μm^3 /cm^2. By 24 hours post-exposure, control values decreased slightly, to 16.3 ($\pm$2.8) x 10^7 μm^3 /cm^2, but total fluid volume under nitrogen dioxide-exposed monolayers had fallen markedly, to 3.3 ($\pm$1.2) x 10^7 μm^3 /cm^2. Forty-eight hours after exposure, control mean total volume was 19.3 ($\pm$2.1) x 10^7 μm^3 /cm^2, and the nitrogen dioxide-exposed monolayer values increased somewhat (relative to their 24-hour volumes), to 8.2 ($\pm$1.1) x 10^7 μm^3 /cm^2. At both 24 hours and 48 hours post-exposure, mean total fluid volumes under nitrogen dioxide-exposed monolayers were significantly below those of control monolayers. These data suggest that nitrogen dioxide exposure inhibits active transport or increases solute permeability across the monolayers, or both (see below).

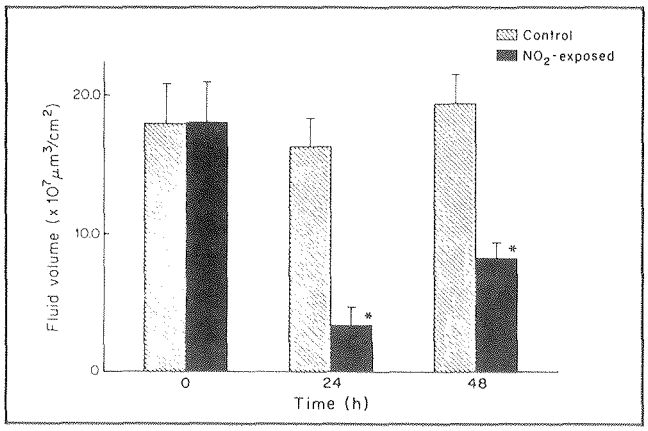

Figure 8. Effects of NO_2 exposure on total fluid volumes under the monolayers. Experimental monolayers were exposed to 30 ppm NO_2 for 20 minutes. Vertical bars represent standard errors. Significant differences between control and NO_2-exposed populations are noted by (*).

ANTIOXIDANT SUPPLEMENTATION STUDIES

Having found an effect of acute nitrogen dioxide exposure using the parameters described above (30 ppm nitrogen dioxide, 20 minutes), we wondered if providing antioxidants to the monolayer might be effective in preventing damage. To date, we were only able to investigate the effects of adding one protective factor (vitamin E) to the nutrient medium in an attempt to modify damage to the alveolar epithelial cells. Following the protocol described above, we supplemented the nutrient medium with a concentration (10 μg/mg protein) of vitamin E equivalent to up to 50 times the amount found in whole lung homogenate. In these experiments, dome formation in α-tocopherol-supplemented monolayers was inhibited to the same degree as in nonsupplemented monolayers. These results suggest that, under these experimental conditions, exogenous vitamin E offers no protection to the cell monolayer against nitrogen dioxide toxicity. It is difficult to determine if the lack of an observed effect resulted from an insufficient uptake of vitamin E by the cell monolayer, or from nitrogen dioxide-induced oxidation of cellular components that were not protected by supplemental α-tocopherol. It is possible that other antioxidants (e.g., ascorbate) would be more effective in ameliorating the effect of nitrogen dioxide on dome formation.

EFFECTS OF ALTERING CULTURE CONDITIONS ON MONOLAYER SENSITIVITY TO NITROGEN DIOXIDE

A preliminary project in our laboratory, which was being conducted concurrently with this research, investigated the effects of an alternate cell culture condition on Type II cell attachment to nonporous substrata. It was determined that the combination of supplementing Eagle's modified minimum essential medium with Ham's F-12 at the ratio of 2 to 1 (plus newborn calf serum to a final concentration of 10 percent), followed by replating the cell suspension three hours after the initial plating, yielded monolayers that actively formed domes by Day 2 post-plating. (Replating involves an additional purification step through differential adherence, in which adherent macrophages and fibroblasts are removed.) Conversely, cultures plated in minimum essential medium plus 10 percent newborn calf serum alone (MEM-S) did not form domes until Day 3 or 4. Subsequent experiments pursued the effect of altered culture conditions on monolayer sensitivity to nitrogen dioxide exposure.

To investigate the relative dosimetry of the Type II cell monolayers to a given nitrogen dioxide insult, inhibition of

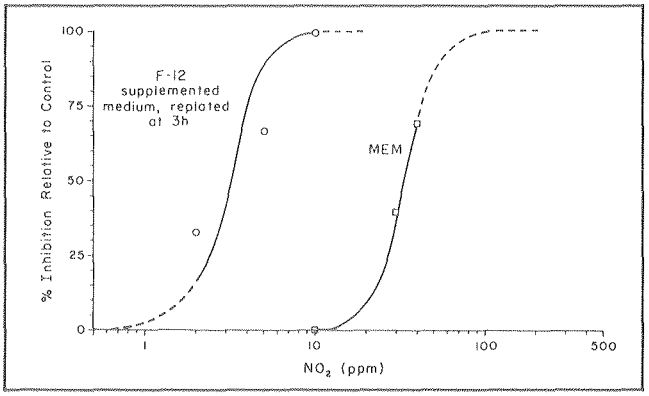

Figure 9. Dose-response of monolayers grown on plastic surfaces and exposed to NO_2 for 20 minutes. The percent reduction in dome density relative to controls was used to indicate response.

dome formation (relative to exposure controls) was used to indicate the dose-response of the monolayers to nitrogen dioxide. Cells were isolated and cultured in MEM- S, and were exposed to various concentrations of nitrogen dioxide (2 to 50 ppm) for 20 minutes. For these monolayers, the dose-response curve demonstrated an apparent I_{50} of 33 ppm 24 hours after exposure (Figure 9). Under these culture exposure conditions, the response threshold was approximately 10 ppm, with the maximal response beginning around 40 ppm. When the cells were replated at three hours post-plating and cultured in MEM supplemented at the ratio of 2 to 1 with Ham's F-12 (plus 10 percent serum), increased sensitivity of the monolayers was unexpectedly noted upon exposure. Here, the apparent I_{50} was 3 ppm, with a response threshold of less than 2 ppm and maximum response beginning at 10 ppm (Figure 9). The reasons for this differing sensitivity are not clear. However, one possibility is that Ham's F-12 contains iron and hypoxanthine, both of which may have participated in the production of reactive oxygen species, and thereby may have generated an increased oxidant stress (relative to the MEM-S cultures) during the nitrogen dioxide exposures (see below).

DEVELOPMENT OF CELL CULTURES ON POROUS FILTERS

The final phase of this project centered around the development of Type II cell cultures grown on porous filters. Although dome formation in cultures grown on plastic plates is a useful indicator of active transport, it does not readily allow one to measure the bioelectric properties or quantitate active transport, as do cultures grown on porous filters. However, Type II alveolar epithelial cells do not readily attach to bare filters, so the proper filter coating or substrate needed to be developed, along with an adequate technique for mounting the filters in an Ussing chamber.

Previous studies attempted to grow cultures on Millipore filters placed in the bottom of a 35 mm Petri dish. Once the cultures were established, the filters were removed and clamped directly into an Ussing chamber that used "o" rings and silicone grease to create tight seals around the tissue being studied. The results from these initial studies were very inconsistent (Goodman et al., 1983). Little spontaneous potential was generated, and low resistances were noted. It was decided that the clamping procedure was likely causing damage at the monolayer edges, thereby opening channels for free ion flow. Furthermore, problems with substrate characteristics led to uncertainty in the adequacy of confluent monolayer formation.

Recent modifications have facilitated the development of more successful filter preparations. The problem of edge damage was eliminated by adapting the Ussing chamber to accept small Lexan rings. Filters were glued to the bottom of the rings, effectively creating filter cups. The isolated cells were seeded into the cup and the whole cup was placed in the medium for incubation. For Ussing chamber studies, the sides of the cups (the Lexan rings) were clamped, which eliminated any edge damage to the monolayers.

It was determined that, for Type II cell monolayers, the most readily obtainable and satisfactory substrate is rat tail collagen. Collagen is acid extracted from rat tail tendons, dialyzed, and diluted to the proper concentration (approximately 3 mg/ml). We found that the two best ways of applying the collagen to the filters were either simple submersion of the filter into the native collagen solution followed by immediate use, or chemically crosslinking the collagen onto the filters. (Crosslinking involves precipitating the solubilized collagen onto the filter through an exposure to ammonia fumes, followed by glutaraldehyde application, which crosslinks the collagen.)

Experiments were performed to evaluate and improve the preparation of filters. Filters (of both Millipore and Nuclepore variety) were prepared using various collagen concentrations with native or crosslinked coatings, and were evaluated for uniformity of coating using scanning electron microscopy (SEM). The results were not definitive, although they were useful. Filters that necessarily must be kept wet (i.e., with native collagen) uniformly developed large artifacts during sample preparation for scanning electron microscopy. Air-drying native filters resulted in the collagen lifting off the filters, and dehydration in ethanol (through normal electron microscopy sample preparation) washed the filters free of collagen. The crosslinked filters fared better; the air-dried filters appeared to have thick uniform collagen layers on their surfaces. (Ethanol dehydration also resulted in the collagen lifting off the filter surface.) The Nuclepore filters appeared to coat more uniformly than the Millipore type, so it was decided to proceed, using Nuclepore filters only. A typical crosslinked collagen coated Nuclepore filter is shown in Figure 10.

Because it was unclear from the scanning electron microscopy studies which substrate was most desirable, it was decided to attempt plating cells on both native and crosslinked collagen filter cups. Initially, the cells were plated on both native and crosslinked collagen coated filter cups in a manner similar to plating on plastic dishes (see Methods). The cultures were maintained in a 37°C incubator with a 48 hour refeeding interval. Bioelectric studies of filters prepared with collagen crosslinking revealed that monolayers develop spontaneous potential differences of 0.2 to 2.0 mV (apical side negative), and tissue resistances of 150 to 1000 Ω-cm^2. A transmission electron micrograph of a section of one such filter is shown in Figure 11. On the other hand, monolayers on native collagen filter cups failed to consistently generate either significant spontaneous potential differences or tissue resistances. It was decided that using crosslinked collagen filter cups provides the most desirable means of conducting mechanistic studies of nitrogen dioxide toxicity on alveolar epithelium via bioelectric measurements.

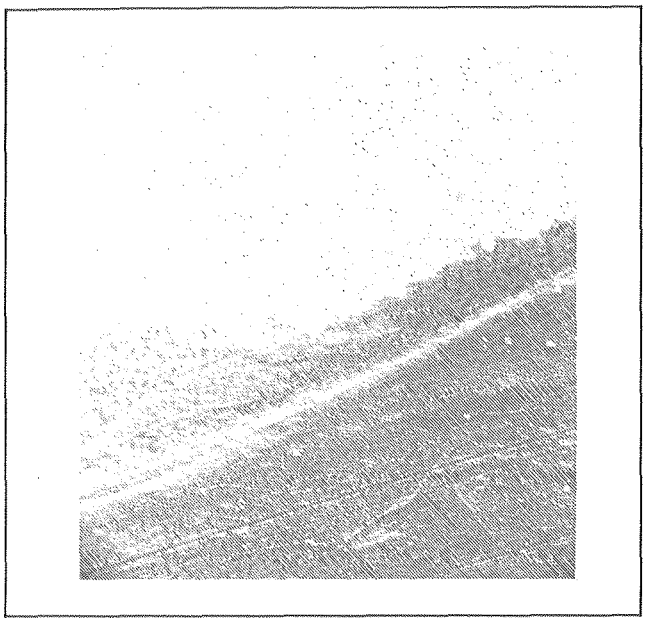

Figure 10. Scanning electron micrograph of a crosslinked collagen-coated Nuclepore filter. 600x. Note lower half of filter, which is covered by a uniform layer of collagen, contrasted with the upper half, which was left uncoated for comparison.

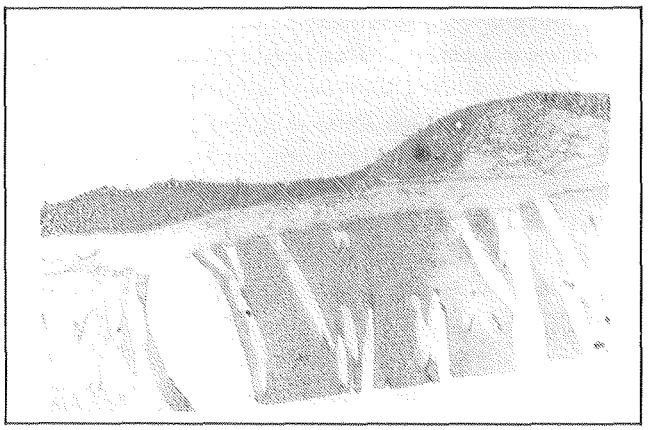

Figure 11. Type II cells on collagen-coated filter. Transmission electron micrograph of primary monolayer culture of rat alveolar epithelial cells on Nuclepore filter. Note typical microvilli, lamellar bodies, and tight junctions.

BIOELECTRIC STUDIES

Monolayers cultured on filters with crosslinked collagen were exposed to nitrogen dioxide in a manner similar to those on plastic plates. Results from these acute exposures (20 ppm nitrogen dioxide, 20 minutes) are shown in Table 1. A comparison of spontaneous potential difference and tissue resistance values for incubator controls and monolayers exposed to 5 percent CO_2 in air (exposure controls) demonstrates somewhat lower values for both parameters in the case of the exposure controls. This probably reflects some effect of the exposure procedure, which may slightly affect the integrity of the monolayer, previously unseen in experiments using cells cultured on nonporous plates. However, monolayers exposed to nitrogen dioxide consistently demonstrated significant drops in tissue resistance and reductions (nonsignificant to date) in short circuit current relative to both incubator and exposure controls. These findings suggest that nitrogen dioxide may primarily increase paracellular permeability, in addition to decreasing transcellular active transport.

Table 1. Effects of NO_2 on Bioelectric Properties of Filter Cup Monolayers[a,b]

	Incubator Control	Exposure Control	Experimental[c]
SPD (mV)	0.41 ± 0.12	0.26 ± 0.07	0.10 ± 0.01
SCC ($\mu A/cm^2$)	1.48 ± 0.16	1.46 ± 0.12	1.22 ± 0.16
R_t (Ω-cm^2)	271 ± 62	186 ± 46	83 ± 9*
n	17	17	15

a Values are given as mean ± standard error of the mean
b Monolayers were cultured in MEM + 10% NCS + 0.1 μM dexamethasone
c Monolayers were exposed to 20 ppm NO_2 in 5% CO_2/air by cyclic tilting for 20 minutes
* Significant decrease ($p < 0.05$), relative to control

DISCUSSION

MECHANISM OF NITROGEN DIOXIDE-INDUCED TRANSPORT INHIBITION

The results of the studies using monolayers grown on nonporous plastic plates demonstrate that short-term exposures to 30 ppm nitrogen dioxide produce a decrease in dome formation in pulmonary Type II alveolar epithelial cell monolayers 24 hours after exposure, with partial recovery seen at 48 hours. The reduction in dome formation in nitrogen dioxide-exposed monolayers is evidenced by a decrease in dome density and individual dome volume, which results in a marked fall in the total amount of fluid under the monolayers. This result is most likely a manifestation of either a reduction of active sodium transport by the monolayers, or an increase in the leakage of transported fluid back through the monolayer into the medium. The nitrogen dioxide-mediated effect may be due to (1) an inhibition of membrane Na^+, K^+-ATPase, which causes a decrease in the active transepithelial sodium transport rate, or (2) an increase in the permeability of cell membranes or intercellular tight junctions to solutes or water, or both. One or both of these effects could serve to lower the net volume of fluid under the monolayer.

Data from experiments involving monolayers cultured on porous collagen-coated filters correlate with the results obtained with dome formation studies. Interpretations of bioelectric measurement data suggest that nitrogen dioxide

may affect both epithelial active transport or passive ion transport, or both, through altered tight junctional pathways. Tissue resistance provides an indication of the tightness of the monolayer; i.e., it reflects the integrity of the tight junctional complexes between cells that control paracellular permeability. Short circuit current is a measure of net transcellular active ion fluxes. High short circuit current values may thus indicate a high rate of active transport across the epithelial membrane. Monolayers exposed to nitrogen dioxide consistently demonstrated significantly decreased tissue resistance and small reductions in short circuit current relative to the controls. These findings suggest that nitrogen dioxide may damage both paracellular tight junctions and, perhaps, transcellular transport membrane pumps (e.g., Na^+, K^+- ATPase).

It should be noted that a large increase in paracellular permeability (decrease in tissue resistance) could mask an effect on transcellular active ion transport because of massive bidirectional ion leakage across the membrane. As a result, further studies on bioelectric properties and other parameters (such as radiolabeled inulin permeability experiments in conjunction with morphologic studies of damaged monolayers) are necessary to delineate the specific mechanisms of nitrogen dioxide injury effects on alveolar epithelial barrier characteristics (e.g., integrity of tight junctions).

At the membrane molecular level, organic free radical generation is probably involved in initiating this nitrogen dioxide-related effect. Free radicals are able to abstract hydrogen from biomolecules and initiate a chain of damaging reactions in tissue, including the disruption of membrane lipids and proteins (Mustafa et al., 1979). The peroxidation of membrane lipids (Roehm et al., 1971), and the ensuing disruption of orderly membrane structure, may lead to an increase in the permeability of alveolar epithelium at the membrane or tight junctional level. Additionally, lipid peroxidation could reduce the rate of active transport through direct protein (Na^+,K^+-ATPase) inactivation due to free radical attack, or by derangement of the surrounding lipid environment (Mead, 1976). Direct oxidation of functional groups (e.g., sulfhydryls) by nitrogen dioxide (Mustafa et al., 1980) might be expected to result in decreased active transport rates, although increases in paracellular permeability might occur as well. Either of these free radical-initiated mechanisms for nitrogen dioxide toxicity would lead to decreased transport or increased permeability across the cell monolayer, or both. It will be advantageous in future studies to include biochemical approaches (e.g., detection of lipid peroxidation products), as well as bioelectric and morphologic findings, to further clarify the specific mechanisms of nitrogen dioxide injury to alveolar epithelium.

The dose-response studies that compared differing culture techniques demonstrate that altering the isolation and culturing conditions of the cell monolayers generates cultures with differing susceptibility to nitrogen dioxide. In these experiments, the inhibition of dome formation, compared to controls, was used as an index of monolayer sensitivity to nitrogen dioxide exposure. Cells isolated in Eagle's Modified minimum essential medium supplemented with Ham's F-12 (plus 10 percent serum) and replated three hours after plating displayed greater than an order of magnitude increase in sensitivity to nitrogen dioxide, when compared to cells isolated and cultured in minimum essential medium (plus 10 percent serum) alone. To date, the mechanism of this differential sensitivity remains uncertain. The fact that Ham's F-12 contains iron and hypoxanthine, components that may facilitate the production of reactive oxygen species upon exposure to nitrogen dioxide, provides a plausible hypothesis to explain the increased susceptibility to oxidant stress. This finding of differential sensitivity to an oxidant insult increases the complexity of interpreting the mechanistic explanations that attempt to describe nitrogen dioxide-related toxic interactions, and requires that further studies on relative dosimetry be performed.

RELEVANCE OF FINDINGS TO OTHER INVESTIGATIONS

Results from previous studies indicate that exposure to nitrogen dioxide can lead to extensive pulmonary injury, because of nitrogen dioxide's oxidation properties and free radical potential. Sagai et al. (1984) showed that chronic exposures to low levels of nitrogen dioxide induce the formation of lung lipid peroxides in vivo, as evidenced by the presence of maldonaldehyde (a three carbon decomposition product of lipid peroxides) in lung homogenate following nitrogen dioxide exposure. A number of other membrane components, in addition to lipids, are oxidized by nitrogen dioxide. For example, DeLucia et al. (1972) demonstrated that both protein and nonprotein reduced sulfhydryl groups in lung homogenate decreased after oxidant exposure. Specifically, both glutathione and protein sulfhydryl groups may be oxidized to the corresponding disulfide by nitrogen dioxide (Menzel, 1976). It follows that nitrogen dioxide-induced free radical attack leading to either alterations in membrane structure or inactivation of membrane proteins could serve to increase epithelial permeability or alter active transport rates, or both.

Several investigators have shown that acute high level exposure to nitrogen dioxide compromises the barrier properties of airway epithelium. Ranga et al. (1980) demonstrated that exposing hamsters to 15 ppm nitrogen dioxide for 14 days resulted in increased transepithelial permeability to horseradish peroxidase in trachea and bronchi. Using freeze-fracture replicas of bronchiolar epithelium, Case et al. (1982) showed that the tight junctional network was disrupted after exposure to 28 ppm nitrogen dioxide for 48 hours, even after a 48 hour recovery period. Subsequently, Gordon et al. (1983) found that bronchiolar tight junctions in hamster lungs were highly permeable to horseradish peroxidase following exposure to 28 ppm nitrogen dioxide for 48 hours. These

results indicate that bronchiolar epithelium is highly sensitive to nitrogen dioxide-induced injury. Comparable morphologic studies on alterations in alveolar epithelial permeability caused by nitrogen dioxide exposure are not available.

The complex anatomy of adult mammalian lung complicates the study of nitrogen dioxide toxicity in intact alveolar epithelium. As a result, such studies require the use of models of isolated alveolar epithelium. The primary cultured monolayer of Type II pneumocytes is a particularly attractive model for investigating alveolar epithelial permeability properties. The role of the Type II cell in the synthesis of pulmonary surfactant (Kikkawa et al., 1975), as well as its differentiation capability for replacing oxidant-injured Type I cells (Evans et al., 1975), emphasizes the importance of this cell in alveolar structure and function. Additionally, the relative ease of Type II cell isolation and culture from adult mammalian lung facilitates short-term toxicity studies (Diglio et al., 1977).

Previous investigations have provided indirect information that whole lung nitrogen dioxide exposures can affect Type II cell function, such as surfactant production. For example, Blank et al. (1978) observed an increase in phospholipid accumulation in rat lung homogenate after nitrogen dioxide exposure. However, investigations of specific effects of direct nitrogen dioxide toxicity to Type II pneumocytes alone are limited to one previous study, in which Wright et al. (1982) demonstrated an increase in phospholipid biosynthetic enzyme activities in isolated rat Type II cells after in vivo exposure to nitrogen dioxide. This study represents an initial approach to defining the effects of nitrogen dioxide on Type II cells and the barrier function of mammalian alveolar epithelium.

Previous investigators used in vitro exposure systems to study the effects of nitrogen dioxide on various cell types. Using a strain L cell line, Pace et al. (1961) exposed cultures under a layer of medium (containing serum) to concentrations as high as 4100 ppm nitrogen dioxide, before noting any deleterious effects. When the medium overlying the cells was replaced with a thin film of balanced salt solution, exposure to 100 ppm nitrogen dioxide for 30 minutes resulted in cell death. Although the use of a different exposure methodology precludes any direct comparison with our results, it is interesting to note the high concentration of nitrogen dioxide required to produce an effect when cells are protected by even a thin layer of a balanced salt solution.

More recently, Samuelsen et al. (1978) developed a cell exposure system in which cells are grown on membrane filters that are constantly perfused from the basolateral surface during exposure, to prevent cell drying. By monitoring the cytotoxic effects on V-79 cells, it was determined that exposure to concentrations as low as 0.12 ppm nitrogen dioxide for six hours resulted in cell death. Although this report noted the effects of low concentrations of nitrogen dioxide closer to ambient levels, no provisions were made for mechanistic or functional studies. The advantages of using the system employed in this study include the versatility of preparing actively transporting Type II cell monolayers on nonporous as well as porous surfaces, and the ability to investigate early physiologic effects. Further studies on these simplified systems, with future extension to correlative studies in intact mammalian lungs, will be necessary to fully understand the mechanism by which nitrogen dioxide damages alveolar epithelium, relative dosimetry among simpler and more complex models, and the contribution of ambient nitrogen dioxide exposure to human health.

In summary, we demonstrated that acute sublethal exposures to nitrogen dioxide result in a decrease in the net volume of fluid under monolayers of pulmonary Type II alveolar epithelial cells cultured on nonporous substrata. Additionally, when cells are grown on porous filters for use in bioelectric studies and are subsequently exposed to nitrogen dioxide, both short circuit current and tissue resistance values decrease relative to controls, which indicates that nitrogen dioxide may primarily damage active ion transport processes, as well as paracellular tight junctions. These observed reductions may be physiologically significant, because the exposure of alveolar epithelium to nitrogen dioxide could result in impaired clearance of alveolar fluid from air spaces in vivo. A reduction in active solute transport by alveolar epithelium, or an elevation in ion permeability of the epithelial barrier, or both, would probably produce such an effect. The mechanism of nitrogen dioxide injury may involve free radical-initiated oxidation and peroxidation of membrane components including Na^+, K^+-ATPase, and unsaturated lipids, respectively. Dose to response relations suggest that nitrogen dioxide levels close to ambient concentrations may lead to deleterious effects on alveolar epithelium. When correlated with the results obtained from studies involving other models, as well as in vivo exposures, the use of an in vitro exposure system may enable us to develop specific information for predicting oxidant effects in complex systems.

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APPENDIX A

Dome density, incubator vs. exposure control groups. Data are presented as mean ± standard error of the mean for each experiment. Sample size (n) = number of monolayers. No differences were noted between monolayers kept in the incubator for seven days and those exposed to 5% CO_2 in air (exposure controls). This finding indicates that there were no effects on dome formation due to the exposure procedure itself.

Experiment	Group	n	0 hr domes/cm^2	24 hr domes/cm^2	48 hr domes/cm^2	
1	Incubator	2	242 ± 58	125 ± 75	***	
	Exposure control	4	217 ± 48	121 ± 36	***	
2	Incubator	6	358 ± 25	278 ± 41	311 ± 16	
	Exposure control	6	428 ± 22	311 ± 46	300 ± 12	
3	Incubator	4	321 ± 62	358 ± 24	354 ± 28	
	Exposure control	4	321 ± 22	342 ± 24	383 ± 32	
4	Incubator	2	350 ± 0	200 ± 50	258 ± 42	
	Exposure control	4	350 ± 22	225 ± 32	283 ± 12	
5	Incubator	4	238 ± 24	212 ± 8	200 ± 18	
	Exposure control	5	180 ± 14	230 ± 16	240 ± 8	
6	Incubator	4	254 ± 53	192 ± 20	183 ± 29	
	Exposure control	4	242 ± 22	179 ± 23	217 ± 24	
TOTAL	Incubator	22	301 ± 19	244 ± 20	266 ± 18	(n = 20)
	Exposure control	27	296 ± 21	240 ± 19	284 ± 14	(n = 23)

*** 48hr observations not taken.

APPENDIX B

Dome density, exposure control vs. experimental (NO_2-exposed) groups. Data are presented as mean ± standard error of the mean for each experiment. Sample size (n) = number of monolayers. Dome formation in monolayers exposed to 30 ppm NO_2 was significantly decreased ($p < 0.05$) compared to control values at 24 and 48 hours post-exposure.

Experiment	Group	n	0 hr domes/cm^2	24 hr domes/cm^2	48 hr domes/cm^2	
1	Exposure control	4	217 ± 48	121 ± 36	***	
	Experimental	4	242 ± 44	96 ± 32	***	
2	Exposure control	6	428 ± 22	311 ± 46	300 ± 12	
	Experimental	6	417 ± 40	103 ± 28	219 ± 12	
3	Exposure control	4	321 ± 22	342 ± 24	383 ± 32	
	Experimental	4	317 ± 18	287 ± 42	300 ± 19	
4	Exposure control	4	350 ± 22	225 ± 32	283 ± 12	
	Experimental	4	358 ± 20	129 ± 17	225 ± 25	
5	Exposure control	5	180 ± 14	230 ± 16	240 ± 8	
	Experimental	5	177 ± 11	163 ± 14	203 ± 19	
6	Exposure control	4	242 ± 22	179 ± 23	217 ± 24	
	Experimental	4	246 ± 21	117 ± 14	167 ± 25	
TOTAL	Exposure control	27	296 ± 21	240 ± 19	284 ± 14	(n = 23)
	Experimental	27	297 ± 20	146 ± 16	222 ± 11	(n = 23)

*** 48hr observations not taken.

APPENDIX C

Individual dome volume, exposure control vs. NO_2-exposed groups. Data are presented as mean ± standard error of the mean for each experiment. Sample size (n) = number of domes. Individual dome volumes in monolayers exposed to 30 ppm NO_2 were significantly lower than ($p < 0.05$) those in control monolayers at 24 and 48 hours post-exposure.

Experiment	Group	0 hr n	0 hr mean ± sem	24 hr n	24 hr mean ± sem	48 hr n	48 hr mean ± sem
1	Exposure control	77	7.66 ± 0.91	70	8.60 ± 1.00	58	7.15 ± 1.02
	Experimental	80	7.56 ± 1.08	8	2.80 ± 0.49	1	3.40
2	Exposure control	28	1.69 ± 0.22	21	2.34 ± 0.44	23	5.87 ± 1.49
	Experimental	25	2.45 ± 0.40	15	1.60 ± 0.30	19	3.53 ± 0.62
3	Exposure control	15	5.91 ± 0.90	14	5.00 ± 1.10	17	7.10 ± 1.31
	Experimental	19	4.37 ± 0.76	2	6.31 ± 4.38	11	4.20 ± 1.06
TOTAL	Exposure control	120	5.96 ± 0.63	105	6.73 ± 0.73	97	6.86 ± 0.74
	Experimental	124	6.04 ± 0.73	25	2.34 ± 0.45	31	3.72 ± 0.50

APPENDIX D1

Dose-response of monolayers cultured in MEM + 10% NCS. Dome densities are presented as experimental mean ± standard error of the mean. Inhibition of dome formation was used to indicate the dose response of monolayers 24 hours after exposure to 10-40 ppm NO_2 for 20 min.

Experiment	ppm NO_2	Group	n	0 hr domes/cm^2	24 hr domes/cm^2	% Inhibition at 24 hr
1	10	Exposure control	2	325 ± 8	92 ± 59	0
		NO_2-exposed	3	244 ± 39	111 ± 43	
2	40	Exposure control	2	166 ± 16	83 ± 0	70
		NO_2-exposed	2	225 ± 58	25 ± 8	
3-8	30	Exposure control	27	296 ± 21	240 ± 19	39
		NO_2-exposed	27	297 ± 20	146 ± 16	

APPENDIX D2

Dose-response of monolayers cultured in MEM + F-12 with Ham's F-12 and replated after 3 hours. Dome densities are presented as experimental mean ± standard error of the mean. Inhibition of dome formation was used to indicate the dose response of monolayers 24 hours after exposure to 2-10 ppm NO_2 for 20 min.

Experiment	ppm NO_2	Group	n	0 hr domes/cm^2	24 hr domes/cm^2	% Inhibition at 24 hr
1	10	Exposure control	3	261 ± 24	250 ± 17	100
		NO_2-exposed	4	258 ± 46	0	
2	5	Exposure control	5	377 ± 24	190 ± 21	66
		NO_2-exposed	5	373 ± 25	63 ± 20	
3	2	Exposure control	2	280 ± 27	250 ± 67	33
		NO_2-exposed	2	283 ± 31	167 ± 16	

APPENDIX E

Bioelectric properties of filter cup monolayers, incubator vs. exposure control vs. experimental groups. Data are presented as mean ± standard error of the mean for each experiment. Sample size (n) = number of monolayers. Tissue resistance (R_t) was significantly decreased ($p < 0.05$) in monolayers exposed to 20 ppm NO_2 compared to control values at 24 hours post-exposure. Experimental monolayers also demonstrated reduced short circuit current (SCC) relative to controls.

			24 hr post-exposure measurements		
Experiment	**Group**	**n**	**SPD (mV)**	**R_t (Ω-cm^2)**	**SCC(μA/cm^2)**
1	Incubator	3	1.25 ± 0.39	716 ± 178	1.70 ± 0.09
	Exposure control	2	0.84 ± 0.42	496 ± 198	1.60 ± 0.20
	Experimental	***	***	***	***
2	Incubator	4	0.23 ± 0.07	152 ± 58	1.73 ± 0.40
	Exposure control	5	0.11 ± 0.02	79 ± 9	1.46 ± 0.18
	Experimental	5	0.11 ± 0.02	76 ± 26	1.11 ± 0.13
3	Incubator	5	0.23 ± 0.03	222 ± 74	1.07 ± 0.10
	Exposure control	5	0.26 ± 0.08	257 ± 95	1.15 ± 0.13
	Experimental	5	0.08 ± 0.02	56 ± 12	1.66 ± 0.27
4	Incubator	5	0.22 ± 0.05	148 ± 38	1.58 ± 0.24
	Exposure control	5	0.18 ± 0.04	102 ± 16	1.73 ± 0.22
	Experimental	5	0.10 ± 0.01	89 ± 13	1.17 ± 0.18
TOTAL	Incubator	17	0.41 ± 0.12	271 ± 62	1.48 ± 0.13
	Exposure control	17	0.26 ± 0.07	186 ± 46	1.46 ± 0.10
	Experimental	15	0.10 ± 0.01	83 ± 9	1.22 ± 0.16

*** No NO_2 exposures were performed in this experiment.

INVESTIGATORS' OBJECTIVES

Previous studies from numerous laboratories have suggested that certain gases and vapors contained in mobile source emissions, or their reaction products, alter the barrier properties of mammalian alveolar epithelium and cause pulmonary edema. However, the specific effects of these pollutants on the barrier properties of alveolar epithelium are difficult to evaluate because of technical difficulties that result from the complexity of the intact mammalian lung. Dr. Crandall's laboratory has had experience with two models for studying isolated alveolar epithelium: primary cultured monolayers of mammalian Type II pneumocytes, and the hollow bullfrog lung.

This investigation addressed specific aims designed to test the hypothesis that mobile source emissions adversely affect the barrier properties of alveolar epithelium, including both active and passive transport characteristics. Specifically, the stated aims of the investigators were:

1) to study the effects of nitrogen dioxide (NO_2) on monolayers of rat alveolar epithelial Type II pneumocytes plated on non-porous substrata, using dome formation as a transport marker;
2) to examine the effects of altered culture conditions on the monolayer's susceptibility to oxidant stress; and
3) to investigate the effects of nitrogen dioxide on the bioelectric properties of monolayers plated on porous surfaces to determine the relative dosimetry and specific mechanisms by which nitrogen dioxide damages alveolar epithelium barrier properties.

This list of specific aims, included in the final report, is considerably reduced from those initially proposed by the investigators. The original objectives included experiments with Type II cells isolated from nitrogen dioxide-exposed rats and studies with bullfrog lungs in organ cultures. This was an ambitious research proposal for the time and effort initially proposed. However, the investigators encountered unforeseen methodological difficulties with the dome formation experiments and, with HEI's approval, concentrated their efforts on the three specific aims listed above.

SUMMARY OF INVESTIGATORS' CONCLUSIONS

To investigate the effect of nitrogen dioxide on dome formation, Type II alveolar cell monolayers were cultured on non-porous surfaces, and the number and volume of the domes formed was determined. The cells were exposed to nitrogen dioxide for twenty minutes, followed by 24 and 48 hour incubations in normal growth conditions. At the end of the incubation period, the number and volume of domes were determined again. From these experiments the authors concluded that:

1) Control cultures that were exposed to air (which contained only 5 percent carbon dioxide) did not differ from unexposed control cell cultures in the number and volume of domes formed and maintained.
2) Monolayers exposed to 30 parts per million (ppm) of nitrogen dioxide had 35 percent fewer domes than controls at 24 hours after exposure, and 21 percent fewer domes than controls at 48 hours after exposure. The dome volume in the nitrogen dioxide-exposed monolayers was less than 50 percent of control at 24 hours and had recovered only slightly by 48 hours.
3) From a dose-response study that used exposure concentrations of 10, 30, and 40 ppm nitrogen dioxide in the standard Eagle's minimum essential medium with 10 percent newborn calf serum (MEM-S), the concentration of nitrogen dioxide estimated to cause 50 percent reduction in domes was 33 ppm, with a response threshold of approximately 10 ppm, and a maximal response at around 40 ppm.
4) When the cells were replated at three hours post plating and then cultured in the MEM-S medium supplemented 2:1 with Ham's F-12 medium, the concentration calculated to cause 50 percent reduction in dome formation was only 3 ppm, as determined by tests at 2, 5, and 10 ppm. This replating and supplementation procedure appears to have increased the sensitivity of the cells to the nitrogen dioxide-induced reduction of dome formation by nearly an order of magnitude. The authors speculate that this may be due to the presence of iron and hypoxanthine in the Ham's F-12 medium, and that these constituents may have participated in the production of reactive oxygen species and thereby generated increased oxidant stress during the nitrogen dioxide exposures. However, according to the investigators, experiments to substantiate this hypothesis were beyond the scope of the current project and were not attempted.
5) The addition of alpha-tocopherol (vitamin E) to the monolayer cultures did not alter the effect of nitrogen dioxide on dome formation. This negative finding was not interpreted mechanistically by the authors because they did not have direct evidence that the vitamin E was taken up into the cell membranes nor that there was any change in the reactive components that might have been induced by nitrogen dioxide.

Experiments to investigate the effect of nitrogen dioxide on the bioelectric properties of cells were more limited. For these experiments, monolayers cultured on porous filters were

suspended between electrodes. Monolayers exposed to 20 ppm nitrogen dioxide had consistently reduced tissue resistance, which was statistically significant, and also had some reduction in short circuit current, which was not statistically significant. Because decrements in tissue resistance are indicative of increased passive diffusion of solute, the authors conclude that, in addition to decreasing transcellular active transport, nitrogen dioxide may increase paracellular permeability. The authors also conclude that the effect of nitrogen dioxide exposure on bioelectric properties are consistent with the findings of reduced dome formation. They propose that nitrogen dioxide may damage both paracellular tight junctions, and possibly, transcellular transport membrane pumps, such as Na^+, K^+-ATPase.

TECHNICAL EVALUATION

ASSESSMENT OF METHODS AND STUDY DESIGN

During the course of this study, a considerable amount of time was spent on development of methods that were reliable and reproducible. One of the major problems was the suitability of chambers for culturing cells and for exposure to gaseous nitrogen dioxide. Standard protocols for cell culture require a constant immersion of the cells under culture medium. However, the medium represented a barrier to contact of the noxious gas with the cells. The problem was overcome by rocking the cell layers in and out of the medium. In this way, the cells were alternately bathed and directly exposed to nitrogen dioxide-contaminated air. Thus, the investigators were able to study the effects of concentrations of nitrogen dioxide that were much lower than were required to cause any change when exposure was through the culture medium. A further modification, the miniaturization of the culture dishes, provided a practical means for studying several monolayers under conditions that appear to be both relatively reproducible and quite resistant to manipulative variations. The authors are to be complimented for their perseverence, and for describing their methods in detail in their final report.

The reproducibility of the methods reported here can be judged by an inspection of the data summarized in the Appendices. Dome formation by monolayers exposed to air differs little, if at all, from dome formation in monolayers kept in the incubator. However, there is a slight, but reproducible, decrement in dome formation at 24 hours. The authors suggest that this probably reflects the requirements of monolayers for both fresh nutrients and removal of metabolic wastes. After a change of medium at 24 hours, dome densities return to the 0 hour level by 48 hours.

The experiments to determine dose response could have been better designed. With the exception of 30 ppm nitrogen dioxide in the standard medium tests, there were relatively few monolayers tested. The interpretation of the results of these experiments is further complicated by very large decreases observed in some cases in the dome density at 24 hours. Also, the observations were not extended to 48 hours. Figure 9, based on the data in Appendices D1 and D2, therefore, summarizes preliminary data; the precise values for the concentrations required to reduce dome formation by 50 percent should not be given undue emphasis. It does appear, however, that the replating of the cells and the supplementation of the medium with Ham's F-12 greatly increased the sensitivity of the monolayers to nitrogen dioxide, as determined by dome formation.

The methodological approach for measuring the bioelectric properties of the filter cup monolayers appears to be logically conceived, and the apparatus seems to be carefully developed and used. The data summarized in Table 1 indicated that the tissue resistance parameter alone was significantly reduced by exposure for 20 minutes to 20 ppm of nitrogen dioxide. The authors concluded from this finding that increased passive diffusion of solutes and water, possibly due to the effect of nitrogen dioxide on the paracellular tight junctions, may be an important mechanism of reduced dome formation. The small reduction of short circuit current, which the authors suggest is due to reduction in cellular transport, i.e., active transport, is not statistically significant. Whether or not the small reduction is of functional significance remains to be determined by future experimentation. It appears that one of the major contributions of the studies on the bioelectric properties is the development of a method for measuring these properties in small monolayer cultures.

Some statistical design and data analysis problems emerged in the course of reviewing this project. The investigators measured dome densities immediately before exposure to the test atmospheres, and at 24 and 48 hours after exposure. There were concerns that the analysis conducted by the investigators did not adequately take these time variables into account. Observations made at three different times on the same experimental unit cannot be considered independent. Ideally, a different form of analysis, sometimes described as "split-plot," might have been performed, although a number of other two-dimensional tests also might have been appropriate. The investigators acknowledged the validity of this criticism, but remained firm in their belief, for plausible biochemical reasons, that the effect of nitrogen dioxide exposure would far outweigh any effect of time, i.e., the aging of the test medium.

In analyzing the data, the choice of the basic unit for statistical treatment in the experiments also was questioned. The investigators have analyzed the data using individual dome density and volume data. Measurement of average mean dome volume/monolayer might have been more appropriate, given the design of the study. The investigators, however, did the analysis again using the suggested unit of treatment, and found that the basic conclusions remained unchanged.

INTERPRETATION OF RESULTS

The investigators conclude that exposure of monolayers of rat Type II pneumocytes to nitrogen dioxide results in a reduction of the barrier properties of this model of alveolar epithelium, and that, with manipulation and supplementation of culture medium, these monolayers can be made sensitive to the effects of nitrogen dioxide that approach minimally effective concentrations observed in other systems. This is a potentially important preliminary observation, and this investigation appears to have made significant progress in the development of a model test system that might allow more detailed mechanistic studies or lead to methods to test for damage to alveolar epithelium. However, there was not enough time in this project to thoroughly test the system and validate it against other model systems, as well as against intact animal exposures.

The investigators hypothesize that the reduction in dome formation upon exposure to nitrogen dioxide may reflect the formation of a reactive derivative of nitrogen dioxide or an activated endogenous constituent, which ultimately results in a reduction in active transport via the sodium-potassium ATPase system. The authors speculate that substances may be present in Ham's F-12 medium which enhance the formation of reactive radicals with nitrogen dioxides. However, in an attempt to examine the role of reactive oxygen intermediates on dome formation, it was found that addition of the antioxidant vitamin E had no influence on the effect of nitrogen dioxide. It is not certain, however, that the vitamin E added to the medium was available to the cells.

Most of the experiments conducted in this investigation used nitrogen dioxide concentrations of 20 or 30 ppm. These exceed realistic ambient air pollution concentrations by at least an order of magnitude. In this context, the observation of an effect on dome formation at 2 ppm in the modified replating Ham's F-12 medium experiments is particularly interesting because this exposure level is quite close to ambient air pollution conditions. No attempt was made in this study to relate the concentrations of nitrogen dioxide that were used in the in vitro exposure experiments to calculated alveolar concentrations in intact animals or humans exposed to nitrogen dioxide. Ultimately, this is a key practical calculation.

The investigators' finding of reduced tissue resistance in the bioelectric properties experiments seems to be substantiated by the data. However, since a statistically significant reduction in short circuit current was not observed, it is difficult to interpret the reduction in tissue resistance. As the authors suggest, further studies on bioelectric properties and other measurements, such as radio-labeled inulin transport, are necessary to further explore this phenomenon.

ATTAINMENT OF STUDY OBJECTIVES

The investigators have developed a suitable method for demonstrating that nitrogen dioxide exposure at moderate concentrations reduces dome formation in epithelial monolayers, and that dome formation is valuable as a type of transport marker. They also have demonstrated that alterations of culture conditions can markedly affect the monolayer's susceptibility to the effects of the oxidant. Investigations on the effects of nitrogen dioxide on bioelectric properties of monolayers plated on porous surfaces resulted in the development of an improved procedure for establishing the monolayer culture on a filter surface and in the assembly of a reasonable apparatus for testing bioelectric properties. The application of the methodology demonstrated some alteration in bioelectric properties with one concentration level of nitrogen dioxide. However, as discussed above under "Investigators' Objectives," the results of the experiments reported here only partially met the original goals of the investigators. This was largely because of several methodological difficulties faced by the investigators. Although these difficulties appear to have been successfully resolved during the course of this project, the investigators were not able to attempt some of their other objectives. In particular, the studies fall short of determining the specific mechanisms by which nitrogen dioxide damages alveolar epithelial barrier properties.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The significance and value of the measurements of dome formation is controversial among experts in the field. Some think that the use of dome formation as a marker of transport of solutes and water is not entirely satisfactory because dome formation is believed to depend in part upon weak attachment of the epithelial cells to the substratum. However, the authors believe that dome formation does reflect the integrity of the normal barrier function of an epithelial membrane, and that the reductions in both the number and volume of the domes are indicative of either a reduction in the active transport of solute (with water following) from the medium to the substrata, or a loss of the barrier to passive transport of solutes and water (possibly through changes in tight junctions).

It appears that dome formation, and the reduction of dome formation, are related to the overall barrier properties of the epithelial membrane. The authors emphasize that this suggests a reduction in the sodium-potassium ATPase pump mechanism, i.e., the active transport mechanism. They do acknowledge, however, that it could also be a breakdown of the barrier to passive diffusion. The relative importance of either of the proposed mechanisms was not delineated in this investigation.

If the model system described here is deemed sufficiently relevant to the evaluation of the effects of nitrogen dioxide or other oxidant gases on lung properties and function in the intact organism, a number of experiments can be undertaken to characterize and explore the mechanism of action of nitrogen dioxide. There are specific examples of research that might be conducted to validate and to increase the utility of the model system described here.

1. Further investigation could be carried out to determine the reason why the replating and Ham's F-12 supplementation increased the sensitivity of monolayers to dome formation. There could be a systematic investigation to explore: a) whether other manipulations can substantially alter the susceptibility to this effect of nitrogen dioxide, and b) whether these manipulations will provide insight into the mechanism by which nitrogen dioxide causes the reduction in dome formation.
2. A sufficiently standardized sensitive culture system could be used to conduct corollary biochemical experiments that would include the measurement of: active transport enzyme systems; the status of sulfhydryl groups in the cells; and the measurement of transport of labeled inulin or other model molecules.
3. Additional morphologic or electron microscopic studies could be undertaken to better define the status of the paracellular tight junctions, and to determine if any ultrastructural changes at this or other sites in the membrane can be correlated with alterations in bioelectric properties or fluid accumulation parameters in the intact monolayers.
4. Experiments on cultured cells from animals exposed to nitrogen dioxide could be performed to determine whether adverse effects in vivo are detectable in vitro and, if so, whether these changes are persistent. The use of the isolated frog alveolar sac also might be a useful test model to correlate with the rat pneumocyte monolayer model tested here.
5. Experiments or literature reviews could be used to determine if the concentrations of nitrogen dioxide that are effective on the monolayer model system may be achieved in the alveoli of intact animals exposed to reasonable concentrations of nitrogen dioxide.

CONCLUSIONS

The findings reported in this investigation are preliminary, and are not sufficiently understood or explained to be translatable directly into risk assessment efforts. Most of the work reported in this study was performed at levels of nitrogen dioxide that are too high in the context of human exposure. However, the unexpected, and as yet unexplained, observation of a reduction in dome formation under certain special culture conditions at 2 ppm nitrogen dioxide is potentially important. Although 2 ppm nitrogen dioxide is high for usual community concentrations of nitrogen dioxide, it is close to the realm of human exposure in industrial settings or other localized environments. Therefore, further information developed through continuing studies like those initially proposed by the investigators, and reiterated and extended in the suggestions above, might place this model test system in an important position for use in risk assessments.

Although the study and the results obtained thus far have no direct application in risk assessment, that does not mean that they do not have important potential in that regard. Properly designed experiments that will provide mechanistic and correlative dose-response data could place the model system reported here in a useful position for applied purposes. It seems possible that the model system described here might become useful in making dose-effect extrapolations and species correlations if we both understood the mechanism of nitrogen dioxide-induced alteration in cellular transport, and we could relate dome formation to this process. It also is likely that if the model system is to be useful in risk assessment applications in the future, it will have to be supplemented by appropriate and sufficient correlative data on more complex systems.

ABOUT THE AUTHORS

Edward D. Crandall received a Ph.D. in Chemical Engineering at Northwestern University in 1964, and an M.D. at the University of Pennsylvania in 1972. In 1983, he was appointed the Director of the Will Rogers Institute Pulmonary Research Laboratory at the University of California at Los Angeles. Dr. Crandall has recently moved to Cornell University, where he is Professor of Medicine and Chief of the Division of Pulmonary and Critical Care Medicine. Dr Crandall's research interests include lung epithelial transport properties and responses to lung injury.

Jeffrey M. Cheek is a doctoral candidate majoring in Environmental and Occupational Health Science/Pathology at the School of Public Health at the University of California at Los Angeles. He also is currently a Research Associate at the Department of Medicine, Cornell University. His research interest is the effects of nitrogen dioxide exposure on alveolar epithelial cells.

Edward M. Postlethwait received a Ph.D. in Environmental Science/Toxicology from the University of California at Los Angeles in 1983. Prior to this, Dr. Postlethwait served as a biologist at the Pulmonary Pharmacology Branch of the National Institute for Environmental Health Sciences in North Carolina and at the Health Effects Research Laboratory at the U.S. Environmental Protection Agency, North Carolina. He recently has moved to the University of Texas Medical Branch where he is Associate Professor of Research Medicine in the Division of Pulmonary Medicine. His current research interests include the effects of oxidant pollutants on the lungs.

Marian E. Shaw received her M.D. from the University of Texas, Southwestern Medical School, Dallas, Texas in 1979. She is presently Clinical Instructor in Medicine at the University of Washington, Seattle.

FOR FURTHER INFORMATION CONTACT:

The HEALTH EFFECTS INSTITUTE
215 First Street
Cambridge, MA 02142
(617) 491-2926

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RESEARCH REPORT No. 13: October, 1987