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Mechanisms of Nitrogen Dioxide Toxicity in Humans

Mark J. Utell, Mark W. Frampton, Norbert J. Roberts Jr., Jacob N. Finkelstein, Christopher Cox, and Paul E. Morrow
Departments of Medicine, Biophysics, Pediatrics, and Biostatistics, University of Rochester School of Medicine and Dentistry, Rochester, NY

Includes the Commentary of the Institute's Health Review Committee

Research Report Number 43

HEALTH EFFECTS INSTITUTE

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HEALTH REVIEW COMMITTEE'S COMMENTARY Health Effects Institute

Mechanisms of Nitrogen Dioxide Toxicity in Humans

Mark J. Utell[1], Mark W. Frampton, Norbert J. Roberts Jr., Jacob N. Finkelstein, Christopher Cox, and Paul E. Morrow

ABSTRACT

These studies were undertaken to evaluate short-term respiratory effects and identify markers of nitrogen dioxide toxicity during exposures designed to approximate realistic conditions. With the development of bronchoalveolar lavage as a clinical investigative technique, the evaluation focused on the assessment of effects induced at the alveolar level. The exposure protocols were designed to assess the duration of nitrogen dioxide–induced effects and determine exposure-response relationships.

Groups of normal, nonsmoking volunteers of both sexes between the ages of 18 and 40 years, without airway hyperreactivity, constituted the study population. The exposure protocols required a total of three to five days for each subject, depending on the timing of bronchoalveolar lavage. Subjects were exposed to nitrogen dioxide or air for three hours in a double-blind, randomized fashion in a 45-m^3 environmental chamber, with intermittent exercise sufficient to quadruple minute ventilation. Pulmonary function was measured during and after exposure, and airway reactivity to carbachol was assessed before and after exposure. Lavaged cells were examined for their capacity to inactivate influenza virus and secrete IL-1 in vitro. Cell-free lavage fluid was analyzed for total protein, albumin, α_2-macroglobulin, arylsulfatase, and α_1-protease inhibitor.

The studies were undertaken in three phases, each of approximately one year's duration. In Phase 1, 15 subjects were exposed to a background concentration of 0.05 parts per million[2] (ppm) nitrogen dioxide and to three 15-minute peaks of 2.0 ppm, and underwent bronchoalveolar lavage 3.5 hours after nitrogen dioxide exposure. During Phase 2, 8 subjects were exposed to continuous 0.60 ppm nitrogen dioxide and underwent bronchoalveolar lavage 18 hours later. Finally, in Phase 3, 15 subjects were exposed to continuous 1.5 ppm nitrogen dioxide and underwent bronchoalveolar lavage 3.5 hours after exposure.

No significant symptomatic or pulmonary function changes could be detected in response to any of the nitrogen dioxide exposures. However, a small but significant increase in airway reactivity was observed in normal subjects after exposure to 1.5 ppm nitrogen dioxide. Following the highest dose of carbachol (10 mg/mL), the forced expiratory volume in one second decreased 7.5 $\pm$ 1.1 percent after nitrogen dioxide exposure compared to 4.8 $\pm$ 1.1 percent after exposure to air ($p < 0.05$). No symptoms were induced in any of the groups by the carbachol exposures. Analyses of cells recovered by bronchoalveolar lavage during all three phases revealed no differences in total cell recovery, cell viability, or differential cell counts. Even the 1.5-ppm nitrogen dioxide exposures produced no significant alteration in α_2-macroglobulin, protein, or the albumin concentrations in the lavage fluid.

Because influenza virus is a common respiratory pathogen that routinely infects both the upper and lower respiratory tract and the alveolar macrophage may have a role in limiting viral infection, we examined influenza virus inactivation by nitrogen dioxide–exposed alveolar macrophages. In the Phase 1 and Phase 2 protocols, no differences were observed in virus inactivation between air and nitrogen dioxide exposures. This contrasted with previous studies in our laboratory, in which alveolar macrophages obtained by bronchoalveolar lavage 3.5 hours after a three-hour exposure to 0.6 ppm nitrogen dioxide tended to inactivate influenza virus in vitro less effectively than did alveolar macrophages collected after air exposure. In the Phase 3 studies at 1.5 ppm nitrogen dioxide, remaining infectious virus titers were consistently greater after nitrogen dioxide exposure than after air, but the difference did not reach statistical significance.

In summary, low-level continuous exposure to nitrogen dioxide caused a slight increase in airway reactivity, in agreement with other recent studies. Collectively, this study and prior studies provide some evidence that alveolar macrophages obtained by bronchoalveolar lavage 3.5 hours after exposure to nitrogen dioxide might inactivate influenza virus in vitro less effectively than do cells collected after air exposure. The methodology utilized in these studies should prove valuable in further characterizing pollutant effects on respiratory defense mechanisms and may potentially be extended to susceptible populations.

[1] Correspondence may be addressed to Dr. Mark J. Utell, Department of Medicine, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Box 692, Rochester, NY 14642.

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

INTRODUCTION

The relationship between fossil fuel combustion and the production of nitrogen dioxide as primary and secondary pollutants is well established (Morrow 1984). All high-temperature combustion processes involving air have the potential for producing the oxides of nitrogen, primarily nitric oxide (NO). By photochemical conversion involving ozone (O_3), hydrocarbons, and other agents, NO is converted in the atmosphere to nitrogen dioxide (NO_2) and, to varying extents, to other oxides. Because NO_2 is dominant among pollutants in the production and persistence of health-related effects, a high priority for research on NO_2 has emerged. This research potentially may have a major impact on emissions standards for internal combustion engines and stationary power sources.

Although NO_2 is an important component of both outdoor and indoor air, the status of NO_2 as a contaminant affecting the public health is uncertain. On the basis of epidemiological studies of air pollution, a plausible association between NO_2 levels and adverse health effects has been established. Most recent studies of NO_2 epidemiology emphasized indoor sources in order to estimate exposure and focused on respiratory symptoms and illnesses and on levels of pulmonary function. In a four-year longitudinal study, Melia and coworkers (1977, 1979) reported higher rates of respiratory illness in children from households with gas stoves and high ambient NO_2 levels than in those from households with lower levels of NO_2. Speizer and colleagues (1980) initially reported significant reductions in pulmonary function in children from homes with gas stoves, but adjustment for confounding variables, expansion of the populations, and extension of follow-up intervals eliminated statistical differences. Other studies have been equally inconclusive (Samet et al. 1987). When evidence on the effects of NO_2 exposure is interpreted within the context of the limitations of the individual studies, the finding of small or insignificant effects of exposure should be anticipated (Samet and Utell 1990). In combination, bias resulting from misclassification and bias from inadequate sample size reduce the likelihood of demonstrating adverse effects of NO_2 by using epidemiologic methods. However, as appropriately emphasized by Samet and associates (1987), the potential public health impact of even a small NO_2 effect is substantial given that childhood respiratory illnesses are so common.

Animal studies have provided the strongest evidence for a relationship between exposure to NO_2 and increased susceptibility to respiratory infections. Mice exposed to NO_2 at levels as low as 0.50 ppm and then challenged with aerosolized bacteria showed increased mortality when compared with air-exposed animals (Ehrlich 1966; Gardner et al. 1982). Evidence indicates that the mechanism of this increased mortality is impaired killing of the inhaled microorganisms in the lung, most likely by the alveolar macrophage. Goldstein and coworkers (1973) administered aerosols of radiolabeled *Staphylococcus aureus* to mice, exposed them to NO_2, and demonstrated impaired pulmonary bactericidal activity without impairment in physical clearance of organisms. Jakab (1987) similarly found that exposure of mice to NO_2 at levels up to 20 ppm for four hours did not affect physical translocation of inhaled bacteria, yet NO_2 impaired bactericidal activity at levels as low as 4 ppm. Since the alveolar macrophage is the most important alveolar defense against inhaled microorganisms, these studies suggest NO_2 may primarily affect alveolar macrophage function.

It is likely that most human respiratory infections are caused by viruses (Monto and Ullman 1974). The few animal studies addressing this question provide evidence that NO_2 impairs antiviral defenses. Squirrel monkeys exposed to NO_2 at 5 and 10 ppm after challenge with influenza virus experienced a greater mortality than did animals exposed to the virus only (Henry et al. 1970). Fenters and colleagues (1973) exposed squirrel monkeys to 1 ppm NO_2 for 16 months, then repeatedly challenged them with influenza virus, and found an increased antibody response in exposed versus control monkeys. Rose and coworkers (1988) explored the effects of repeated daily exposure of 5 ppm NO_2 on susceptibility of mice to cytomegalovirus infection. Nitrogen dioxide–exposed mice became infected using a smaller viral inoculum than did air-exposed mice, and NO_2 depressed phagocytosis of colloidal gold by macrophages in vivo. Virus was found to be associated with macrophages in all animals exposed to NO_2, whereas macrophage-associated virus was not detected in any air-exposed animal given the same viral inoculum.

Direct effects on alveolar macrophage function by inhalation of near-ambient levels of NO_2 have been demonstrated in animals. These include decreased intracellular killing of bacteria (Gardner 1984), decreased random mobility and phagocytic capacity (Schlesinger 1987b), decreased interferon production in response to parainfluenza infection (Valand et al. 1970), and increased adsorption and internalization of parainfluenza-3 virus (Williams et al. 1972). These findings could be explained by oxidant-induced alterations in the cell membrane of alveolar macrophages, which lead to increased viral attachment and subsequent internalization, coincident with impairment of the cellular response to the viral challenge. Collectively, these animal studies provide strong evidence that NO_2 impairs the ability of alveolar macrophages to defend the host against bacterial and viral challenges.

Few human exposure studies are available that examine the interaction between pollutant exposure and respiratory infection. Utell and colleagues (1980) found that subjects with naturally acquired influenza infections demonstrated increased airway responsiveness to a particulate nitrate aerosol. In contrast, two more recent studies failed to demonstrate an effect of oxidant exposure in a chamber on experimentally induced upper respiratory virus infection (Kulle and Clements 1987; Henderson et al. 1988). These studies used viruses incapable of infecting the lower respiratory tract and do not exclude the possibility that oxidants impair lower airway defense mechanisms. Indeed, studies in our laboratory (Smeglin et al. 1986; Frampton et al. 1989c) found that alveolar macrophages obtained by bronchoalveolar lavage 3.5 hours after a three-hour exposure to 0.60 ppm NO_2 tended to inactivate influenza virus in vitro less effectively than did cells collected after air exposure. Four of nine subjects accounted for the observed impairment in virus inactivation; cells from these four subjects demonstrated an increase in interleukin-1 (IL-1) production after NO_2 as opposed to air, whereas the five remaining subjects had decreased IL-1 production after NO_2.

Nitrogen dioxide, unlike O_3, is slightly soluble in water and is the acid anhydride of both nitrous and nitric acid; consequently, it is incorrect to consider NO_2 simply as an oxidant gas. Convincing experimental demonstrations that NO_2 and O_3 produce similar biological effects through different mechanisms have been reported by Goldstein and associates (1977) and by Rietjens and coworkers (1986) using alveolar macrophage culture systems. In addition, Pryor and colleagues (1976, 1982) have described different biochemical reactions for NO_2 and O_3.

Dosimetric models (Miller et al. 1982) indicate that exercise increases the uptake of NO_2 primarily in the distal lung; therefore, traditional pulmonary function tests, most sensitive to changes in larger airway function, may be a relatively insensitive means of detecting the earliest responses. The adaptation of clinical investigative tools such as bronchoalveolar lavage permits new approaches to examining mechanisms of low-level, pollutant-induced effects.

AIMS

The principal objective of this project was to examine pulmonary responses of humans to NO_2 at levels that might be reasonably encountered and are, therefore, relevant to actual environmental exposures. Our studies were designed to evaluate the development of injury-related processes at low levels of NO_2 exposure using bronchoalveolar lavage to sample critical targets of oxidant effects in the lower respiratory tract. Three specific goals for our studies were proposed:

1. To evaluate the short-term respiratory and clinical effects of NO_2 by determining alterations in pulmonary function tests, increases in airway reactivity, or development of symptoms after exposure.
2. To evaluate NO_2 toxicity by quantification of inflammatory cells, measurement of protein and enzyme levels, and assessment of functional activity and immunological concentration of α_1-protease inhibitor (α_1-PI) in bronchoalveolar lavage fluid.
3. To evaluate the relationship between NO_2 exposure and enhanced susceptibility to viral infections by examining alterations in two defense mechanisms: the ability of lavaged lung leukocytes to inactivate influenza virus and the ability of lavaged macrophages to produce IL-1.

The foregoing objectives were expected to contribute at least partial answers to the following questions: Is the clinical tool of bronchoalveolar lavage useful in assessing NO_2 toxicity? Do pulmonary inflammatory changes develop after acute NO_2 exposure? How important is the timing of lavage in assessing the inflammatory response? Are the inflammatory changes linked with the airway hyperreactivity provoked by carbachol? Are peak NO_2 exposures on a low-level background more likely to produce airway changes than are unchanging, continuous exposures? Is the in vitro inactivation of virus by cells exposed in vivo a useful model for examining human host defenses?

Providing partial answers to these questions would extend the role of controlled human studies from pulmonary mechanics into the arena of cell biology. Because we used a double-blind design with a crossover with pure air, there was the possibility of directly comparing results from control and exposure bronchoalveolar lavages. Clearly there was no expectation that this group of studies by their design and findings would directly affect regulatory decisions on NO_2. However, we envisioned that these studies could constitute an important contribution to a developing data base of human and animal experimental findings and epidemiological studies.

METHODS

This section consists of a general overview of the study design, followed by a detailed description of the study protocol and specific research methods. The project consisted of three separate but related studies, referred to as Phase 1, Phase 2, and Phase 3.

STUDY DESIGN

The study used a double-blind, crossover design and was performed over a period of three years. Studies consisted of three-hour exposures to air or NO_2 in a controlled human environmental chamber. On the exposure days, subjects exercised intermittently; underwent pulmonary function tests before, during, and immediately after exposure; were given a carbachol challenge test after exposure; and were questioned regarding symptoms. Bronchoalveolar lavage samples were collected either 3.5 or 18 hours after exposure to assess prompt and delayed local pulmonary responses.

In Phase 1, subjects were exposed to three 15-minute, 2.0-ppm "peaks" of NO_2 on a background of 0.05 ppm NO_2. Bronchoalveolar lavage was performed 3.5 hours after the exposure. In Phase 2, the three-hour exposure was to continuous 0.60 ppm NO_2 and lavage was performed 18 hours later. In the final year, Phase 3, NO_2 concentrations were increased to 1.5 ppm and lavage was performed 3.5 hours later. Features of these exposure protocols, including estimates of NO_2 intake, are summarized in Table 1.

Exposure Conditions

The concentrations of NO_2 required to produce airway responses in healthy and susceptible humans remain controversial, and the optimal timing for bronchoalveolar lavage following exposure is virtually unexplored. The levels of NO_2 exposure used in this project were selected on the basis of available studies in animals and humans at higher and lower exposure concentrations. For example, infectivity studies in animals at levels of 0.5 ppm NO_2 suggested that peak concentrations of NO_2 produced greater mortality rates than did continuous exposure (Gardner et al. 1979). Another study using exposure of rabbits to as little as 0.3 ppm NO_2 for a total of four hours resulted in impaired random mobility and phagocytic capacity of alveolar macrophages obtained by prompt lavage (Schlesinger 1987b). Human studies have focused on pulmonary function responses. Several studies have demonstrated changes in lung function of asthmatic individuals exposed to NO_2 concentrations ranging from 0.1 to 0.5 ppm (Orehek et al. 1976; Kleinman et al. 1983; Bauer et al. 1986; Mohsenin 1987; Morrow and Utell 1989), but several other studies using between 0.3 and 4.0 ppm NO_2 for comparable periods reported no effects (Hackney et al. 1978; Sackner et al. 1981; Linn et al. 1985). At the time of this protocol design, only one preliminary report in humans was available incorporating in vivo exposure and lavage to examine NO_2 effects on human alveolar macrophages. In that report, Smeglin and colleagues (1986) suggested that exposure to 0.60 ppm NO_2 may impair the antiviral activity of alveolar macrophages obtained by lavage 3.5 hours after exposure. From a review of the pertinent, but generally controversial data, we deduced that intermittent peak exposures were more likely to produce effects than continuous exposures. Therefore, the peaks protocol in Phase 1 was designed to provide a comparable concentration × time (C × T) product with the continuous 0.60 ppm NO_2 study (Frampton et al. 1989c). Observations by Koren and colleagues (1989) that low-level O_3-induced injury was detectable by bronchoalveolar lavage 18 hours after exposure persuaded us to delay the lavage until 18 hours after the exposure in Phase 2. Prior to the final phase of this project, Mohsenin and Gee (1987) reported a significant decrease in functional activity of α_1-PI recovered by lavage three hours after exposure to 3.0 to 4.0 ppm NO_2 in humans. Therefore, the Phase 3 protocol incorporated an intermedi-

Table 1. Summary of Exposure-Lavage Protocols

Protocol	NO_2 Concentration	Total Mean Intake Estimate[a,b]	BAL[c], Hours after Exposure
Peaks protocol (Phase 1)	0.05 ppm (0.09 µg/L) with 2.0 ppm (3.76 µg/L) peaks	5,051 µg	3.5
Continuous 0.60 ppm NO_2 (Phase 2)	0.60 ppm (1.13 µg/L)	3,288 µg	18
Continuous 1.5 ppm NO_2 (Phase 3)	1.50 ppm (2.82 µg/L)	8,206 µg	3.5

[a] Total intake estimate: Approximation of total mean intake of NO_2 for each protocol was based on product of concentration (µg/L) × time of exposure (min) × mean minute ventilation ($\dot{V}_E$, L/min). Mean $\dot{V}_E$ was 7 L/min during rest and 40 L/min during exercise and did not differ between protocols. Total intake was higher in the peaks protocol than in the continuous 0.60 ppm NO_2 protocol because 30 min of exercise time occurred during the 2.0 ppm NO_2 peaks. For example, calculation of intake for the peaks protocol was as follows: 0.09 µg/L × 115 min × 7 L/min = 72 (0.05 ppm, rest); 0.09 µg/L × 20 min × 40 L/min = 72 (0.05 ppm, exercise); 3.76 µg/L × 15 min × 7 L/min = 395 (2.0 ppm, rest); 3.76 µg/L × 30 min × 40 L/min = 4,512 (2.0 ppm, exercise); total = 5,051 µg.

[b] Estimates of actual NO_2 deposition for individual and group exposures were calculated from subjects' data and are given in Appendix D.

[c] BAL = bronchoalveolar lavage.

ate level of 1.5 ppm NO_2 in an effort to explore the exposure-response relationship between 0.6 and 4.0 ppm NO_2.

Double-Blind Crossover Approach

The studies were all of a crossover design, whereby each subject was exposed either to the NO_2 test gas or to purified air on each of two occasions separated by at least two weeks. The order of exposure to NO_2 or air was randomized so that half of the group received either NO_2 or air initially.

All studies were conducted under double-blind conditions in which the subject and clinical investigative team (M. J. Utell, M. W. Frampton, J. N. Finkelstein, N. J. Roberts Jr., and D. M. Speers) responsible for pulmonary tests, bronchoalveolar lavage, and analyses of lavage samples were unaware of whether the exposure was to NO_2 or purified air. Only the chamber operations and analytical team (P. E. Morrow, F. R. Gibb, and H. Beiter) were aware of the exposure conditions, and this codified information was not released until the entire experimental portion of each phase was completed.

Environmental Chamber

All exposures were conducted in an environmental chamber (Utell et al. 1984; Morrow and Utell 1986) located in the University of Rochester Medical Center's Clinical Research Center (CRC). The chamber facility consisted of the 45-m^3 exposure chamber with an attached instrument room, an adjacent environmental control area, an adjoining bathroom, and an air lock entry (Figure 1). The exposure chamber was a single-pass system operating at approximately 10 m^3/min air flow. The air was first passed through a series of pollutant absorbers and high-efficiency filters and then conditioned as to temperature and humidity before it was admitted to the chamber through five ceiling diffusers. Before the air intake system divided and went to the diffusers, there was an injection point at which NO_2 was dynamically added and diluted. For these studies, NO_2 was injected at 0.1 to 6.4 L/min (0.05 to 2.0 ppm NO_2). The chamber air was removed through five floor-level exhaust outlets by an exhaust fan, thereby keeping the chamber at a negative pressure of 0.01 to 0.02 inches of water with respect to the CRC area.

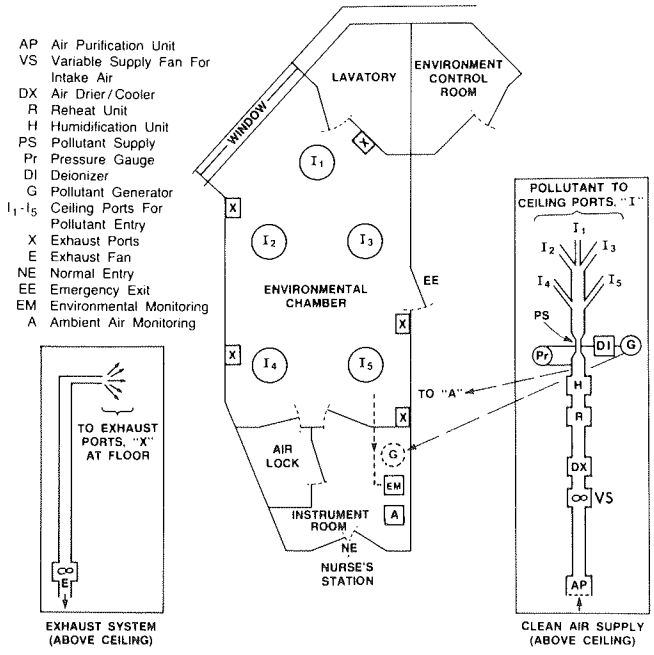

Figure 1. Schematic design of the 45-m^3 environmental chamber facility in the Clinical Research Center. The middle diagram indicates the major areas of the exposure facility: the environmental chamber, instrument room, environment control room, and lavatory. The independent ventilation system for the chamber is shown in two insets: the inset to the right depicts the air intake, purification, and conditioning system; the inset to the left depicts the exhaust portion of the system.

The mixing characteristics of the chamber under a constant 10 m^3/min air flow were excellent, with 90 percent of the steady-state NO_2 concentration achieved in approximately four minutes. Concentration differences in NO_2 throughout the chamber were within ± 5 percent of the mean concentration.

Although the capability existed to set the relative humidity and temperature of the environmental chamber over a wide range, all NO_2 studies were conducted in a comfort zone of 40 ± 5 percent humidity and 70° ± 2°F (21° ± 1°C) temperature.

Subject Selection

Healthy, nonsmoking volunteers were recruited from the Rochester metropolitan area primarily by solicitations in University of Rochester publications. The volunteers, 18 to 40 years of age, were accepted if they fulfilled the following criteria: asymptomatic and no history of recurring respiratory disease; spirometry within the normal values based on the standards published by Morris and coworkers (1971); residual volume within 30 percent and total lung capacity within 15 percent of predicted values (Goldman and Becklake 1959); airway conductance in the normal range published by Briscoe and DuBois (1958); lack of response to carbachol challenge at concentrations below 1.0 percent; and absence of an acute respiratory infection within the preceding six weeks. Detailed characteristics of the study group are given in the Results section.

In addition, at initial interviews, a standardized questionnaire (Lebowitz et al. 1975) was completed by each subject (Appendix G)[3] and a complete respiratory and cardiac his-

[3] Appendix G is available on request from the Health Effects Institute.

tory was taken. A general physical examination was also performed. Information pertaining to lifestyle factors of possible relevance to NO_2-induced responses (for example, smoking habits of spouse or family, use of vitamin C and E, home cooking with gas, and kerosene space heating) was also collected (Appendix B), but volunteers were not excluded on the basis of their answers.

Volunteers were informed of the study purposes, the experimental protocols and procedures, and the potential risk from participation in the study. The study protocols were approved by the Committee on Investigation Involving Human Subjects at the University of Rochester. Each participant was informed of his/her right to withdraw from the study at any time; all participants completed the entire protocol. Each subject signed an informed consent statement prior to participation (Appendix E)[4].

Experimental Protocol

In all cases, the overall protocol required a minimum of three days to complete; for studies with bronchoalveolar lavage 18 hours after exposure, five days of volunteer time was required (Appendix C). The first day was devoted to the preexposure base-line measurements. On the second day, the first of the three-hour exposures was conducted and lavage was performed 3.5 hours after exposure. A third day was required for lavage 18 hours after the first exposure to determine if there were latent inflammatory responses. On the fourth day, we conducted the second three-hour exposure to NO_2 or purified air, depending on which of these gases was used in the initial exposure, and lavage was performed 3.5 hours later. Finally, the fifth day was devoted to completing lavage 18 hours after the second exposure. The two exposure days were separated by a minimum of two weeks.

Evaluation Criteria

Responses in these studies were based on two distinct criteria: first, pulmonary function and airway reactivity testing in which the individual subject's responses were assessed before (base line) and after NO_2 exposure and were compared to the responses obtained during control (air) exposures. Airway reactivity testing was performed with carbachol. The pulmonary function assessments included airway resistance and spirometric data. Specific methodologies and equipment used are described under Exposure and Evaluation Methods.

The second, more extensive set of criteria for determining NO_2-induced responses was based on analysis of bronchoalveolar lavage specimens. Four general analyses were carried out to assess oxidant toxicity: (1) measurements of inflammatory response including total cell counts, cell viability, and differential cell counts; (2) changes in protein and enzymes in lavage fluid, specifically, total proteins, albumin, α_2-macroglobulin, and arylsulfatase; (3) assessment of antiviral activity of lavaged leukocytes, namely, the ability of lavaged alveolar macrophages to inactivate influenza virus and produce IL-1; and (4) measurements of functional activity of α_1-PI in lavage fluid. The α_1-PI studies were performed by Dr. David Johnson at East Tennessee State University and have been published (Johnson et al. 1990). The lavages were performed on both exposure days and assessments compared the exposure and control days. Specific methodologies and equipment used are described below.

EXPOSURE AND EVALUATION METHODS

Standard Three-Hour Protocol

Before the start of each NO_2 or pure air exposure, the relative humidity and temperature in the chamber were established by setting the desired values in the environmental control room.

All chamber instrumentation was calibrated after the chamber air intake was adjusted to approximately 10,000 L/min by the variable-speed fan. The incoming purified air was then checked to verify that background levels of air pollutants were minimal, that is, at or below detection limits with respect to particles, sulfur oxides, nitrogen oxides, and O_3: specifically, less than 4 μg particles/m^3, approximately 0.01 ppm NO_2, and less than 0.005 ppm for O_3 and sulfur dioxide.

By conducting the standardized NO_2 span gas through a manifold, the chamber NO_2 analyzer was checked and the span adjusted as needed. The procedure was repeated for "zero" air. (Details of calibration are provided in the Quality Assurance section.) Concurrently, all physiological instrumentation was turned on, and calibration procedures were begun according to standard operating procedures for each instrument.

Upon entering the chamber, subjects were provided with the abdominal and thoracic bands used with the inductive plethysmograph (Model 300 SC, Respitrace, Ardsley, NY). The terminals of each band were connected to oscillators and each subject was "initialized," that is, the bands were calibrated to conform to the individual. Elastic bandages and an elastic net shirt were then placed over the bands to stabilize their positions. With the subject seated, the oximeter probe was attached to the ear of the subject, and the

[4] Appendix E is available on request from the Health Effects Institute.

Respitrace calibration was continued by the use of a calibrated spirometer.

With these calibrations completed, a blood sample was drawn if the protocol (Phase 3) required it. A nurse from the CRC checked the subject, and brief ventilatory recordings were made to check Respitrace settings. Preexposure baseline pulmonary function tests were then begun. Airway resistance, thoracic gas volume, and flow-volume curves were determined in the Emerson (Cambridge, MA) air-conditioned, integrated-flow, pressure-corrected, whole-body plethysmograph (Leith and Mead 1973). Multiple measurements of resistance or its reciprocal, conductance, and thoracic gas volume were rapidly performed during panting (DuBois et al. 1956; Leith and Mead 1973). Using the body plethysmograph in connection with a flow-meter at the mouth (pneumotachograph No. 7322, Fleisch, Lausanne, Switzerland) and an X-Y storage oscilloscope (Textronix Inc., Portland, OR) display of flow versus volume, maximum flow-volume curves were constructed. A maximal expiratory flow-volume curve was obtained by recording as the subject inspired to total lung capacity and forcefully expired to residual volume. From the expiratory flow-volume curve, we measured expiratory reserve volume in order to obtain residual volume from functional residual capacity.

After these measurements were made in the body plethysmograph, spirometry was performed using a Medical Graphics Corporation (St. Paul, MN) Microloop system. This system incorporates a pneumotachograph interfaced with a microcomputer to measure flow rates and integrates signals to measure volumes. After tidal breathing, subjects inspired to total lung capacity and expired forcefully to residual volume to provide forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) measurements, which were expressed in liters corrected to body temperature and pressure, saturated with water vapor (BTPS) conditions. Lung volumes, including vital capacity, residual volume, and total lung capacity, were readily determined from the foregoing maneuvers. All measurements were made in triplicate and the mean value was calculated.

After the pulmonary function testing was completed, the NO_2 or air exposure was begun. The subjects began a period of exercise for 10 minutes, approximately every 30 minutes, at a workload designed to quadruple minute ventilation (the specific workload was determined on the screening evaluation). During the "peak" exposures, exercise periods were timed to coincide with the higher NO_2 concentrations, thus maximizing total exposure. Pulmonary function tests were measured immediately after each of the first three exercise periods and at the end of the exposure. Symptom reports were completed at termination of the exposure. The subject exited the chamber, and the NO_2 supply was turned off if it was in use. After approximately 10 minutes, background levels of pollutants in the chamber were rechecked and all recordings were collected and properly identified. The exposure protocols are described in detail in Appendix C.

Bronchial Challenges

Inhaled carbachol aerosol is a parasympathomimetic bronchoconstrictor agent utilized for airway reactivity testing. To assess inherent base-line reactivity, carbachol challenges were performed for each subject during the initial screening session. Aqueous propylene glycol alone and then two concentrations of carbachol in propylene glycol (2.5 mg/mL and 5.0 mg/mL) were given at 10-minute intervals. In preliminary testing, a fall in FEV_1 of greater than 15 percent or in specific airway conductance (SG_{aw}) of greater than 40 percent after either of the two carbachol concentrations resulted in exclusion of the subject from the study on the basis of base-line airway hyperreactivity. In our laboratory, normal subjects begin to demonstrate changes in lung function at concentrations of carbachol greater than 10 mg/mL. In addition, carbachol testing was performed to assess acute changes in airway responsiveness after pollutant exposure. Similar to its sensitivity in stable asthma, increased airway reactivity may be a more sensitive indicator of pollutant-induced effects than are standard pulmonary function tests. Therefore, on all exposure days, the subject was reevaluated at the conclusion of the three-hour exposure (with the exception of the "delayed" lavage study) to detect the presence of any subclinical change in airway hyperreactivity induced by the air or NO_2 exposure. An additional, higher dose of carbachol (10 mg/mL) was used to construct a dose-response curve. The number of exposure concentrations used in these cases was determined by the responsiveness of the subject; that is, if after any dose of carbachol, the FEV_1 fell by 15 percent or more, or the SG_{aw} fell by 40 percent or more, the testing was discontinued.

The particle size distribution and aerosol output of a Dautrebande D31 nebulizer (R.E. Reynolds Co., Rochester, NY) were predetermined for the 80 percent (v/v) aqueous propylene glycol solutions of carbachol before their use with subjects. For the challenge, each subject took five deep breaths from functional residual capacity to total lung capacity with a five-second breath hold. Thereby, the entire output of the generator (1.5 L/min) was inhaled during each inspiratory maneuver; the supplemental air required by each individual was drawn through a side tube that continuously purged the aerosol delivery mouthpiece. In this manner, a 0.25 percent (w/v) carbachol solution, for example, was found to have a carbachol output of approximately

25 μg/min. At the average mass median aerodynamic diameter, the droplet aerosol (determined by the aqueous propylene glycol) of 0.8 μm (σ_g = 2.5), the subject typically deposited 65 percent of the aerosol while breathing orally. Assuming 100 percent aerosol deposition, the average dose of carbachol delivered to the airways from the 0.25 percent carbachol solution was approximately 16 μg. Since aerosol output and droplet size were independent of the carbachol concentration, the carbachol output varied with the carbachol concentration in the generator; therefore, a challenge performed with a 1 percent carbachol solution produced an average deposited dose of 64 μg of carbachol. Measurements of airway resistance and spirometry were performed before testing and three minutes after each aerosol administration.

Bronchoalveolar Lavage

Using the standard procedure for bronchoalveolar lavage in our laboratory (Ettensohn and Roberts 1983), the lavage was performed 3.5 or 18 hours after the exposure. Premedication was limited to atropine, 0.75 mg administered intravenously; oxygen was given by nasal cannula during the procedure. After topical anesthesia of the upper airways with 2 percent lidocaine, a flexible fiberoptic bronchoscope (FB-19H, o.d., 6.3 mm Pentax, Orangeburg, NY) was inserted orally and gently wedged in a subsegmental airway of the inferior segment of the lingula. Lidocaine (1 percent) was applied through the bronchoscope to suppress cough. Three 50-mL aliquots of sterile normal saline were sequentially instilled and immediately withdrawn under gentle suction: the position of the bronchoscope was not altered until the lavage procedure was complete. Lavage fluid return with this technique averaged 65 percent of volume instilled. The same lingular subsegment was entered during each of the two lavages for each subject. For subjects studied in Phase 3, the 1.5 ppm NO_2 protocol, a second lavage was performed in a subsegment of the right middle lobe immediately after lavaging the lingula in order to increase the yield of cells for further studies. Lavage was completed in all subjects, and there were no complications.

After completion of the lavage, the fluid was immediately transported to the laboratory for processing. It was pooled, filtered through four layers of sterile gauze to remove debris and mucus, and then centrifuged at 500 × *g* for 20 minutes to remove cells. The supernatant fluid was then stored at −70°C for subsequent analysis. The specific methodology for the assays performed on lavaged cells and fluid is detailed below.

Cell Recovery. Lavage has been used effectively to evaluate and quantify the types of effector cells in lung fluid. In these studies, the total cell counts were obtained from a dilution of the resuspended cell pellet. The lavaged cells were washed three times in phosphate-buffered saline (PBS), resuspended in medium M199 (supplemented with penicillin, 100 units/mL) and streptomycin (50 μg/mL), and counted in a hemocytometer (Model 1483, American Optical, Piscataway, NJ). Cytospin smears (Model 7, Shandon Scientific Co., Astmoor, Runcorn, Cheshire) for differential cell counts were prepared using DIFF Quick stain (American Scientific Products, McGraw, IL); 1,000 cells were counted.

Cell viability was determined by exclusion of trypan blue. For subjects in the Phase 3 protocol, who had two segments lavaged, cells from both segments were pooled for counting, but biochemical analyses were performed only on fluid returned from the lingula for consistency with Phase 1 and Phase 2 protocols. After counting, lavaged cells were diluted to 1×10^6 viable cells/mL.

Exposure of Lavaged Cells to Influenza Virus. Influenza A/AA/Marton/43 H1N1 virus was grown in allantoic cavities of 10-day-old embryonated hens' eggs, stored at −70°C, and titered at $10^{8.0}$ to $10^{8.2}$ 50 percent tissue culture infectious dose ($TCID_{50}$)/mL when assayed in Madin-Darby canine kidney (MDCK) cells (Roberts and Steigbigel 1978; Roberts et al. 1979). Aliquots (1 to 1.2×10^6) of lavaged cells were incubated with virus at a multiplicity of infection (MOI) of 1 or 10 in serum-free medium for 1 hour at 37°C in a 5 percent carbon dioxide atmosphere (Roberts et al. 1979). These samples of virus-exposed cells, as well as control samples consisting of virus and medium without cells, were then supplemented with 5 percent fetal bovine serum (FBS), divided into six aliquots, incubated from zero to five days at 37°C, and then collected and frozen at −70°C until assayed for virus.

Assay for Infectious Influenza Virus. Confluent monolayers of MDCK cells were grown in 24-well cluster dishes (Tobita et al. 1975; Frank et al. 1979) in minimal essential medium (MEM) containing 10 percent FBS, penicillin (100 units/mL), streptomycin (50 μg/mL), and amphotericin B (2.5 μg/mL). Aliquots of lavage cell culture fluids to be assayed for virus were serially diluted 10-fold in Earle's balanced salt solution (EBSS), inoculated in quadruplicate (0.2 mL/well), and incubated at 37°C for 1 hour, with agitation every 15 minutes to ensure an even distribution of inoculum and to maintain moisture on cell surfaces. The cell monolayers were then washed with PBS and were overlaid with agarose-containing MEM, antibiotics, and trypsin (1 mg/mL, Worthington TPCK, Freehold, NJ), without FBS. After three days of further incubation, the cells were fixed with 10 percent formalin, the agarose layer was removed, and the wells were washed with water. A 0.3 percent methylene blue stain was added for 15 minutes, the cultures were washed and air-dried, and the viral plaques were counted. Results were recorded as

the mean values of quadruplicate determinations for each assay.

Assays for Interleukin-1 Activity and Concentration. Cells obtained by bronchoalveolar lavage were exposed or sham-exposed for 1 hour at 37°C in serum-free medium to influenza virus (MOI = 1), as described above. The cells were then washed three times with PBS and incubated at 37°C for 18 to 24 hours in M199 supplemented with 5 percent FBS. The culture supernatant fluids were collected, depleted of cells by centrifugation, and stored at −70°C until assayed. Interleukin-1 activity of these culture fluids was measured using the standard mouse thymocyte comitogen assay (Scala and Oppenheim 1983) in which thymocytes obtained from 6- to 8-week-old C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME) were cultured for 72 hours at 1×10^6 cells/well in 96-well, flat-bottomed plates (3596, Costar, Cambridge, MA) in the presence of 5 μg/mL phytohemagglutinin (PHA) and serial dilutions of the test samples. Each sample was run in triplicate. The thymocytes were pulsed terminally with tritiated thymidine, collected with a semiautomatic cell harvester (Brandell, Gaithersburg, MO), and counted using a liquid scintillation counter. All assays included (as a standard) a purified IL-1 preparation (Ultrapure IL-1, Genzyme, Boston, MA) and relevant controls, that is, culture medium alone and medium with PHA but without cell-derived supernatants. Previous experiments have shown that influenza virus alone does not induce thymocyte proliferation in this assay.

In addition to the biological assay of IL-1 activity, alveolar macrophage culture supernatants from subjects in Phase 3 were assayed for levels of interleukin-1β (IL-1β) using an enzyme-linked immunosorbent assay (ELISA)(Cistron, Inc., Winooski, VT). One hundred microliters of each culture supernatant, or dilutions of IL-β for construction of a standard curve, were added in duplicate to 96-well microtiter plates precoated with monoclonal antibody to IL-1β and incubated for two hours at 37°C. Wells were washed three times and 100 μL of polyclonal rabbit anti-IL-1 was added to each well. After another two-hour incubation and multiple washes, 100 μL of anti-rabbit IgG conjugated to horseradish peroxidase was added to each well. An enzyme substrate consisting of *o*-phenylenediamine and hydrogen peroxide was then added, and the color change was determined using a microtitration plate reader at a wavelength of 490 nm.

Assay for Viral Protein Synthesis in Alveolar Macrophages. To determine whether or not exposure to NO_2 alters the susceptibility of alveolar macrophages for infection with influenza virus in vitro, techniques were developed during Phase 3 to measure viral genome expression in lavage cells. Alveolar macrophages were exposed or sham-exposed to influenza virus, washed, incubated for 0 to 24 hours in medium M199 with 5 percent FBS, and then pelleted and resuspended in serum-free medium with 80 μCi (2.45×10^7 Bq) of ^{35}S-methionine for two hours (Mock et al. 1987). The cells were again washed and lysed with a buffer containing sodium dodecyl sulfate (SDS) for subsequent analysis by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Laemmli 1970; Mock et al. 1987). Viral protein synthesis was quantified using scanning laser densitometry (Zienek Model SCR-2 B/iD, Biomed, Fullerton, CA).

Assay of Protein and Enzymes in Lavage Fluid. Total protein was measured by the method of Lowry and colleagues (1951), using crystalline bovine serum albumin as the standard. Albumin and α_2-macroglobulin levels in lavage fluid were measured using a modified ELISA, employing appropriate commercially obtained polyclonal antibodies (Organon Teknika-Cappel, Malvern, PA). The samples to be tested were adsorbed onto 96-well microtiter plates. After extensive washing and blocking with heat-inactivated sheep serum, the samples were incubated with the appropriate primary antibody. After additional washes, the plates were then incubated with a 1:1,000 dilution of secondary antibody conjugated with biotin. The amount of either albumin or α_2-macroglobulin bound to antibody was detected by adding avidin-conjugated alkaline phosphatase and its substrate, *p*-nitrophenyl phosphate. Change in optical density was detected with a Bio-Tek ELISA plate reader set at 405 nm. Results were compared against standard curves of albumin or α_2-macroglobulin to convert to micrograms.

Arylsulfatase activity in lavage fluid was determined according to the method of Wasserman and Austen (1976). Lavage fluid was concentrated 10-fold using a Centricon 10 concentrator (W.R. Grace and Company, Danvers, MA) with a molecular weight cutoff of 10 kDa. Specific concentration for each sample was determined by the ratio of total protein in the concentrated versus the unconcentrated sample.

QUALITY ASSURANCE

The quality assurance plan instituted for this study was directed primarily at establishing the NO_2 exposure levels by using the National Institute of Standards and Technology (formerly the National Bureau of Standards) standards for traceable NO_2, the U.S. Environmental Protection Agency (EPA) Reference Methods for NO_2 analysis, and adjunctive standardization procedures using EPA-equivalent methods. We concurrently monitored the performance of the air purification system, which required EPA Reference Methods for oxides of nitrogen (NO_x), sulfur oxides, and O_3. We also established standardization and calibration methods for particulate matter in the unoccupied chamber. The basic

requirements, directed at achieving reliable and fully documented NO_2 exposure conditions, were, therefore, performed according to EPA-recommended procedures, but we extended our calibration and standardization procedures, for which there are no specific EPA guidelines, to include environmental instruments and all physiological testing instrumentation.

A quality assurance organizational plan was made, with H. Lisman in charge of the instrumentation and H. E. Beiter in charge of the operational areas. Instrumentation documentation comprised spare-parts inventories, service records, and preventive maintenance schedules; operations included the design and use of data books, analog and digital printouts, data storage, verification of calibration, and standardization procedures. All documentation was compiled and maintained in a quality assurance manual.

The specific quality assurance requirements for NO_2 standardization and calibration are as follows. Prestandardized NO_2 was obtained from Ideal Gas Products, which later became Alphagas (Edison, NJ), and from Scott Specialty Gases (Plumsteadville, PA) as NO_2 in air. Because ambient NO_x levels were also measured, NO in nitrogen was obtained from the same suppliers. Both gases were traceable by the standards set by the National Institute of Standards and Technology.

For the chamber operation, the NO_2 was purchased from Air Products (Allentown, PA) at a requested concentration of 3,000 ppm, whereas for the standardization procedures, NO_2 concentrations of between 3 and 5 ppm were specified. Direct standardization of the undiluted 3,000 ppm NO_2 was not practicable; consequently, the exposure chamber concentration depended on the calibration and verification of the EPA Reference Standard, that is, the Monitor Labs NO/NO_x Analyzer model 8840 (San Diego, CA).

When a standardizing NO_2 gas mixture was acquired, it normally came with a concentration value having five significant figures, for example, 4.9452 ppm. This standardizing gas was first measured by the chemiluminescent method (by the EPA Reference Standard Monitor 8840) and by several Beckman Model 952 NO/NO_x analyzers (Fullerton, CA), the latter being calibrated to agree with the Monitor 8840. The Beckman instruments provided a convenient basis for conducting further standardization and calibration procedures outside the chamber area, where the Monitor 8840 was unavailable. These further tests of the standardization NO_2 gas included comparative measurements by a Monitor Labs Calibrator 8550 with a standardized NO_2 permeation tube (VICI Metronics, Santa Clara, CA) and by measuring the standardization gas using a modified EPA-equivalent method. In our laboratory, the latter method was predicated on the chemiluminescent measurement of the NO_2 concentrations before and after the chemical absorbers, which operate at a sampling rate of 100 mL/min or slightly less, established by bubble-flow metering. The change in NO_2 concentration adjusted to the sampled volume provided a basis for comparing the absorbed NO_2 to a calibration curve prepared with sodium nitrite, which in turn was verified, after oxidation of nitrite to nitrate with hydrogen peroxide, by using an ion chromatograph (Model 16, Dionex, Sunnyvale, CA).

Consistency between the NO_2 methods was considered the basis for verification of the National Institute of Standards and Technology traceable NO_2 gas and for fine adjustments of the NO/NO_x analyzers if needed. Whenever a major inconsistency (more than 10 percent difference) between the value obtained and the manufacturer's standardization occurred, we reordered the standardizing NO_2 gas and resolved the problem.

After the standardizing gas was validated and the NO/NO_x analyzers were determined to be in calibration, the chamber NO_2 concentration was established on the basis of a 10^4 air dilution of 3,000 ppm NO_2 (0.1 to 6.4 L NO_2/min diluted by 10 m^3 air/min). Slight variations are normally found because the NO_2 source gas is only nominally 3,000 ppm and because there are small losses of NO_2 within the system. These variations in chamber NO_2 levels are adjusted then to the desired NO_2 concentrations by slight alterations in the rotational rate of a variable-speed intake fan, or by slight changes in metering the 3,000 ppm NO_2 into the diluting gas, or by both procedures, the former being less coarse than the latter.

The standardized NO_2 gas, which serves as the NO/NO_2 analyzer span gas, is stable over several months of storage; however, we reordered both the standardized NO_2 gas and NO_2 permeation tubes approximately every six months. Having an overlap period during which a previously validated standardization of NO_2 gas was still usable while acquiring a new standardization gas further simplified the validation and calibration procedures.

Data Handling and Statistical Methods

Data collection and storage were performed in accordance with guidelines established by quality assurance and "Good Laboratory Practices" (Salem 1987). All data books were custom-made for each study, numbered, and bound. All entries were made in ink, dated, signed by the responsible person or persons, and periodically verified by the quality assurance officer (H. E. Beiter). All digital and analog recordings were similarly identified and filed. Data entry and initial data processing were accomplished by a technician trained in biostatistics (D. M. Speers) using a Digital Pro 350 Computer (Boston, MA) and RS1 data management, statis-

tics, and graphics software (BBN, Cambridge, MA) under the direction of Dr. C. Cox, Associate Professor of Biostatistics. All records were identified only by code numbers and treated as confidential information.

The basic design of the studies summarized in this report is a two-period crossover design. Carryover effects were minimized by requiring at least two weeks between exposures for each subject. The appropriate statistical analysis for comparison of the two exposures in this design is a crossover *t* test (Armitage and Berry 1987), which is a paired *t* test that takes into account the fact that one group of subjects received air first, while the other received NO_2 first. This test differs from an ordinary paired test in that it uses a pooled error estimate from the two groups receiving the two treatments in each of the two different orders. In this sense the test allows for period effects, which are removed from the error estimate. For some analyses the numbers of subjects in one of these two groups were too small (for example, 2) to compute a reliable variance. In such cases the ordinary paired *t* test was used for significance testing. By using each subject as his or her own control, the *t* test exploits the design of the study to the greatest possible extent. The study design avoided the confounding influence of intersubject variability. For certain endpoints, 95 percent confidence intervals were calculated for the difference between air and NO_2 exposures, in order to give an estimate for the possible size of the NO_2 effect taking account of the uncertainty in the data. The width of the confidence interval gives a quantitative measure of the extent to which the study has delimited the size of the effect. If the two exposures are not significantly different, but the confidence interval includes clinically significant differences, then the study cannot rule out a clinically meaningful effect, despite the negative result of the significance test.

Another feature of these studies that complicated the statistical analysis was that measurements on each subject were repeated at intervals for a total of three or four times. Our basic analysis was to perform the *t* test for each repeated measurement. An alternative analysis that has been used in such situations is the repeated measures analysis of variance (ANOVA). This analysis makes use of the combined data from all repeated measurement periods. However, because of the crossover design employed in this study, and the extra assumptions required by the ANOVA, the *t* test was considered the more informative of the two methods of analysis.

Data on pulmonary function tests were expressed as a percentage of change from base-line values for analysis. The reason for this is that effects are best measured as changes from individual base lines, which are highly variable, and changes from base line are proportional, reflecting inherent differences in lung capacity, based to a considerable extent on size. Statistical analyses were applied to the standardized data. Major comparisons were made for the following response parameters measured for all groups during air and NO_2 exposures: physiologic responses (FVC, FEV_1, and SG_{aw}), influenza virus inactivation, IL-1 production, cell retrieval, total protein, α_2-macroglobulin, and arylsulfatase. Data on cell counts and virus titers were square root and log transformed, respectively, for analysis in order to stabilize the variance and make the data more normally distributed. For the same reasons, the arcsin square root transformation was used for data in the form of proportions or ratios. Symptom scores were summed for tabular presentation and were not analyzed statistically. For evaluating the results of statistical tests, $p > 0.1$ was described as nonsignificant, $p \leq 0.1$ to 0.05 was considered possibly significant, $p < 0.05$ to 0.01 was considered probably significant, $p < 0.01$ to 0.005 was significant, and $p < 0.005$ was highly significant. The outcome of statistical testing of important findings is presented in the Results section.

RESEARCH TEAM

The three-year clinical study involved many individuals with complementary roles. The personnel and their designated responsibilities are given in Appendix A.

RESULTS

The principal findings for each of the three study phases are summarized first. After these summaries, detailed analyses and selected data from each phase are presented in tabular and graphic form to document the principal findings and to facilitate comparison among points of special interest in the exposure protocols. Representative findings are presented initially for pulmonary function measurements, bronchial challenge, and symptomatology. Subsequently, analyses from bronchoalveolar lavage, including cell recovery, proteins and enzymes measured in lavage, influenza virus inactivation, and IL-1 production are described in this section.

In Appendix F[5], selected raw data documenting the results are collated in tables.

PHASE 1: PEAKS PROTOCOL

During the first phase of this study, subjects were exposed to a low background level of 0.05 ppm NO_2 and three 15-

[5] Appendix F is available on request from the Health Effects Institute.

minute peaks of 2.0 ppm NO_2 (Figure 2). The NO_2 peaks coincided with exercise on the bicycle ergometer. The findings for this 15-subject group were uniformly negative under exposure conditions that led to an NO_2 uptake (dose) of 5,634 ± 1,274 μg (mean ± SD) (Appendix D) delivered at a mean rate of 3.8 μg/min during rest and 22 μg/min during exercise. No significant changes from base-line values or between air and NO_2 exposure were found in any pulmonary function assessment during or after NO_2 exposures. Bronchial challenges uniformly failed to reveal any change in airway responsiveness after air or NO_2 inhalation.

Bronchoalveolar lavage was performed 3.5 hours after exposure. Analysis of cells recovered after lavage revealed insignificant differences in total cell recovery, cell viability, or differential cell counts, between air and NO_2. Analyses of lavage fluid showed no differences in protein concentration or enzyme levels. No differences were seen between air and NO_2 exposure for influenza virus inactivation or IL-1 secretion by alveolar macrophages.

PHASE 2: CONTINUOUS EXPOSURE TO 0.60 PPM NITROGEN DIOXIDE, DELAYED LAVAGE

Experiments performed during Phase 1 and in previous studies (Frampton et al. 1989c) utilized bronchoalveolar lavage 3.5 hours after the experimental exposure. However, oxidant exposure can induce the release of mediators from cells that require many hours to produce an influx of inflammatory cells such as lymphocytes, neutrophils, or monocytes-macrophages. These studies, with bronchoalveolar lavage performed 18 hours after exposure, were designed to look for more delayed responses to the oxidant exposure. During this second phase, subjects were exposed to 0.60 ppm NO_2 for three hours and exercise was performed for 10 minutes, every 30 minutes, on the bicycle ergometer. The findings for this eight-subject group were generally negative under exposure conditions that led to an NO_2 uptake (dose) of 3,440 ± 504 μg (mean ± SD) (Appendix D) delivered at a mean rate of 7.9 μg/min during rest and 45.2 μg/min during exercise. No significant changes were observed in pulmonary function from base-line values or by comparing air-exposure responses to NO_2-exposure responses.

Bronchoalveolar lavage was performed eighteen hours after exposure. Analysis of cells recovered again revealed no significant differences in total cell recovery, cell viability, or differential cell counts between air and NO_2. No changes were observed in lavage fluid levels of protein or enzymes. No differences were seen between air and NO_2 exposure for virus inactivation or IL-1 secretion by alveolar macrophages. These findings contrasted with earlier observations in our laboratory that alveolar macrophages obtained by lavage 3.5 hours after exposure to 0.60 ppm NO_2 tended to inactivate influenza virus in vitro less effectively than cells collected after air exposure (Frampton et al. 1989c).

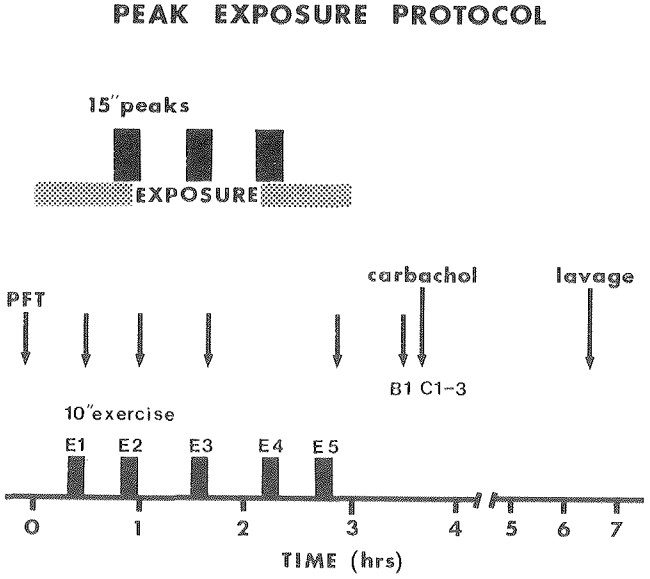

Figure 2. Peaks protocol design. The 15-minute 2.0 ppm NO_2 peaks occurred at 45 minutes, 85 minutes, and 125 minutes of exposure. Exercise was performed during each of the peaks and at two other times during exposure. PFT = pulmonary function tests; B1 is the measurement prior to bronchial challenge; C1, C2 and C3 are measurements after three carbachol challenges.

PHASE 3: CONTINUOUS EXPOSURE TO 1.5 PPM NITROGEN DIOXIDE, IMMEDIATE LAVAGE

The final protocol was designed to study responses to a higher NO_2 level in order to reexamine earlier observations, to determine whether or not exposure-response relationships are evident, and to explore possible NO_2 effects on interactions between alveolar macrophages and influenza virus. During Phase 3, subjects were exposed to 1.5 ppm NO_2 for three hours. The findings for this group of 15 subjects provided the most significant effects detected. Dosimetric estimations for the group indicated an NO_2 uptake of 8,123 ± 967 μg (mean ± SD) delivered at a mean rate of 19.7 μg/min during rest and 192.8 μg/min during exercise. Bronchial challenges were performed 30 minutes after exposure. After inhalation of 10 mg/mL carbachol, small but significantly greater reductions in both FVC and FEV_1 occurred after NO_2 exposure compared with air exposure. By crossover paired t tests, the group showed a significantly greater decrement in FVC (see Figure 5, $p = 0.005$) and FEV_1 (see Figure 6, $p = 0.034$) after NO_2 exposure plus carbachol than after air exposure plus carbachol. No other functional parameters, symptom scores, or data from bron-

chial challenges were significantly different when NO_2- and air-exposure values were compared.

Bronchoalveolar lavage was performed 3.5 hours after exposure. Once again, analysis of cells recovered revealed no differences in total cell recovery, cell viability, or differential cell counts, between air and NO_2 exposure. No changes were seen in lavage levels of protein concentrations and IL-1 production. Studies of virus inactivation differed from Phase 1 and Phase 2 studies by using earlier sampling times of incubation (0, 4, 18, 36, and 60 hours vs. 0, 18, 36, 60, 84, and 108 hours, Phase 3 vs. Phases 1 and 2, respectively). The means of remaining infectious virus titers were greater after NO_2 exposure than after air, but the difference did not reach statistical significance. In additional studies, virus-infected alveolar macrophages consistently showed synthesis of new proteins not observed in sham-exposed cells.

Table 2. Characteristics of Individual Subjects

Subject	Age (yr)	Sex	Height (cm)	FVC (L)	FVC (% predicted)	FEV_1 (L)	FEV_1 (% predicted)	FEV_1/FVC (× 100)	FEV_1 After Carbachol[a]
Phase 1									
1	26	F	156	3.01	85	2.86	99	95	1.0
2	27	M	187	5.09	86	4.29	89	84	−6.5
3	23	M	180	5.33	95	4.74	104	89	−3.2
4	22	M	179	5.82	105	4.69	104	81	1.7
5	21	F	171	3.61	88	3.26	96	90	−3.7
6	24	F	175	4.55	109	3.75	109	82	−0.8
7	28	M	174	4.72	93	3.89	95	83	−0.8
8	27	M	185	5.43	93	5.10	108	94	−5.0
9	37	M	174	4.73	94	3.92	100	83	−2.0
10	24	M	180	5.03	90	4.50	100	90	−2.0
11	23	M	175	4.56	87	4.20	98	92	−1.7
12	31	F	171	3.82	97	3.55	108	93	−4.0
13	27	M	189	5.58	92	4.80	98	86	−5.2
14	19	M	179	5.81	94	4.37	95	82	−3.0
15	18	M	175	5.49	101	5.15	117	94	−0.4
Phase 2									
1	31	M	180	5.70	105	4.40	99	77	−0.9
2	28	M	185	5.43	93	5.10	108	94	−5.0
3	33	F	161	3.74	108	3.27	114	87	0.0
4	33	F	165	3.62	100	2.97	100	82	−5.0
5	39	M	175	4.09	85	3.58	93	88	−0.6
6	33	F	169	4.75	126	3.68	119	78	−0.8
7	26	M	180	5.03	90	4.50	100	90	−2.0
8	38	M	174	4.25	89	3.46	90	82	1.0
Phase 3									
1	29	M	182	5.50	99	4.16	92	76	0.7
2	27	M	180	5.03	90	4.50	100	90	−2.0
3	24	M	168	5.09	106	4.22	108	83	−4.0
4	25	M	188	5.88	97	5.09	104	87	−0.6
5	25	M	173	5.17	102	3.93	95	76	0.5
6	21	M	180	5.67	100	4.59	100	81	−3.0
7	21	M	179	5.31	94	4.37	95	82	−3.0
8	22	M	175	5.06	96	4.40	102	87	−2.0
9	20	F	166	4.32	110	3.53	108	82	−10.0
10	26	M	185	6.69	115	5.82	123	87	−0.7
11	25	M	169	4.34	90	3.80	97	88	−5.0
12	21	M	185	6.06	101	5.04	103	83	0.6
13	25	F	169	4.06	103	3.62	111	89	−0.6
14	21	M	181	5.20	91	4.46	96	86	0.7
15	20	F	165	3.09	79	2.81	87	91	−2.0

[a] FEV_1 after carbachol represents the percentage of change in FEV_1 from base line after inhalation of five breaths of carbachol at 5.0 mg/mL.

However, overall viral protein synthesis by virus-exposed macrophages was not altered by the NO_2 exposure.

REPRESENTATIVE FINDINGS

A summary of the individual subjects' characteristics for each of the three protocols is given in Table 2. All of the normal subjects were nonsmokers and none required any chronic medications.

Pulmonary Function and Airway Reactivity

Although the results of pulmonary function testing and carbachol challenge at the end of "peak" exposures (Phase 1) were consistently negative, with all air-exposure and NO_2-exposure comparisons insignificantly different, selected presentations of the FVC and FEV_1 values are given in Figures 3 and 4, respectively, and in Table 3. The data in the figures are expressed as mean percentage of change (± 1 SEM) from base-line preexposure values during and after three-hour exposures to 0.05 ppm NO_2 with 2.0 ppm NO_2 peaks or to air. The abscissa denotes specific measurement events in the protocol. For the peaks protocol, measurements E1, E2, E3, and E5 denote measurements made after exercise during the three-hour exposure. Peak exposures immediately preceded E2 and E3; no measurements were made after the fourth exercise period, which preceded the final 2.0 ppm NO_2 peak exposure. Measurement B1 was obtained immediately prior to bronchial challenge, which was 30 minutes after completion of the exposure, whereas

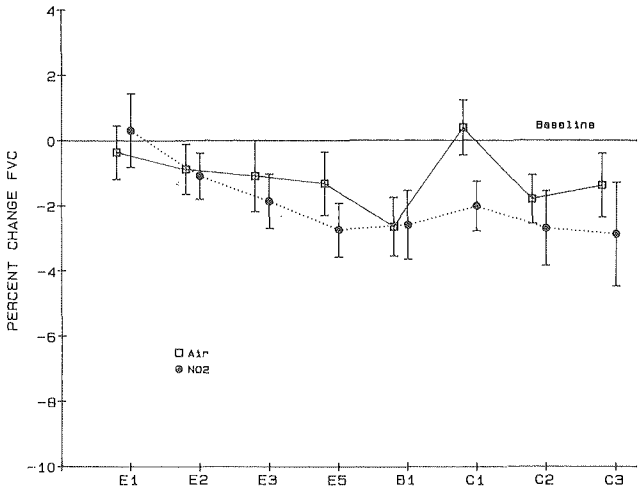

Figure 3. Peaks protocol, FVC. The percentages of change in FVC values measured during the exposures to NO_2 and air, compared to their respective base-line values, are depicted as means ± 1 SEM. E1, E2, E3, and E5 are measurements made after exercise periods during exposure. B1 measurements were obtained before bronchial challenge 30 minutes after the end of exposure. C1, C2, and C3 are measurements made after successive carbachol challenges.

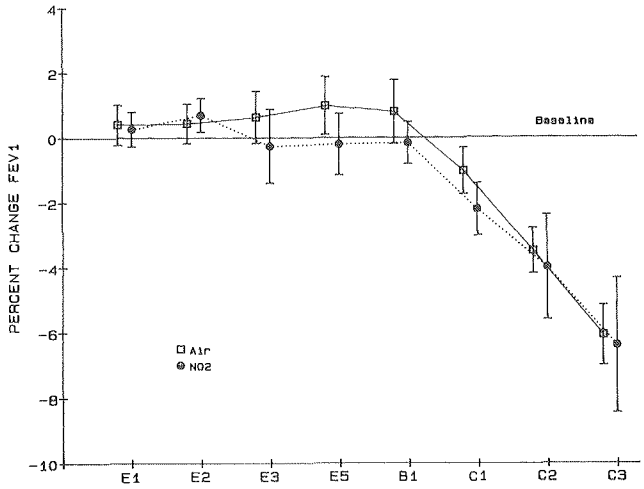

Figure 4. Peaks protocol, FEV_1. The percentages of change in FEV_1 values measured during the exposures to NO_2 and air, compared to their respective base-line values, are depicted as means ± 1 SEM. Although carbachol challenges (C1, C2, and C3) led to small reductions in FEV_1, the NO_2- and air-exposed subjects responded similarly.

measurements C1, C2, and C3 were obtained after successive bronchial challenges with carbachol aerosols.

For the three-hour exposure to 0.60 ppm NO_2 (Phase 2), no significant alterations in pulmonary function were observed during or after exposure (Table 3). Carbachol challenge was not performed after exposure in this phase, because we had previously demonstrated that 0.60 ppm NO_2 did not alter airway reactivity in a similarly selected group of normal volunteers (Frampton et al. 1989c).

As in the previous phases of these studies, no alterations in pulmonary function were observed in Phase 3 during or after exposure to 1.5 ppm NO_2 (Table 3). Figures 5 and 6 show the percentage of change in FVC and FEV_1, respectively, from preexposure base line in response to increasing concentrations of carbachol. The abbreviations on the abscissa again denote specific measurement events, as described for Figures 3 and 4. After inhalation of 10 mg/mL carbachol, small but significantly greater reductions in both FVC and FEV_1 occurred after exposure to 1.5 ppm NO_2 than after exposure to air. The individual changes in FEV_1 in response to 10 mg/mL are shown in Figure 7; 11 of 15 subjects showed a greater decrease in FEV_1 after NO_2 exposure than after air exposure. Most subjects had a decrease in FEV_1 of less than 10 percent, although for one subject FEV_1 decreased by almost 20 percent after exposure to NO_2. No subject experienced dyspnea in response to the carbachol challenge.

Symptom Responses

Symptoms experienced during pollutant or air exposure

Table 3. Pulmonary Function and Airway Reactivity After Exposure to Nitrogen Dioxide or Air[a]

	Peaks Protocol			Continuous 0.60 ppm NO_2			Continuous 1.5 ppm NO_2		
	FVC (L)	FEV_1 (L)	SG_{aw} (cm H_2O/L/sec)	FVC (L)	FEV_1 (L)	SG_{aw} (cm H_2O/L/sec)	FVC (L)	FEV_1 (L)	SG_{aw} (cm H_2O/L/sec)
Base line									
Air	4.78 ± 0.21	4.05 ± 0.16	0.356 ± 0.024	4.54 ± 0.29	3.80 ± 0.19	0.378 ± 0.025	5.04 ± 0.23	4.22 ± 0.18	0.297 ± 0.016
NO_2	4.78 ± 0.21	4.11 ± 0.15	0.367 ± 0.022	4.54 ± 0.30	3.78 ± 0.19	0.375 ± 0.030	5.05 ± 0.25	4.20 ± 0.19	0.293 ± 0.015
After last exercise period[b]									
Air	4.72 ± 0.21	4.11 ± 0.17	0.337 ± 0.018	4.59 ± 0.29	3.80 ± 0.19	0.367 ± 0.031	4.99 ± 0.22	4.29 ± 0.18	0.285 ± 0.017
NO_2	4.65 ± 0.21	4.09 ± 0.17	0.350 ± 0.024	4.55 ± 0.28	3.82 ± 0.18	0.358 ± 0.032	4.96 ± 0.24	4.29 ± 0.19	0.275 ± 0.015
After carbachol (10 mg/mL) challenge[c]									
Air	4.59 ± 0.21	3.85 ± 0.16	0.294 ± 0.029	ND[d]	ND	ND	4.91 ± 0.22	4.10 ± 0.18	0.226 ± 0.019
NO_2	4.52 ± 0.21	3.84 ± 0.18	0.271 ± 0.030	ND	ND	ND	4.73 ± 0.23	3.93 ± 0.18	0.204 ± 0.025
p =	0.32	0.008	0.08	ND	ND	ND	0.005	0.03	0.13

[a] Results are expressed as means ± SEM.

[b] Compares the differences (air − NO_2) of the percentage of change between base line and exercise, for each protocol and each pulmonary function variable; $p > 0.1$ for all comparisons.

[c] Compares the differences (air − NO_2) of the percentage of change between base line and carbachol challenge, for each protocol and each pulmonary function variable; p values in table.

[d] ND = not done.

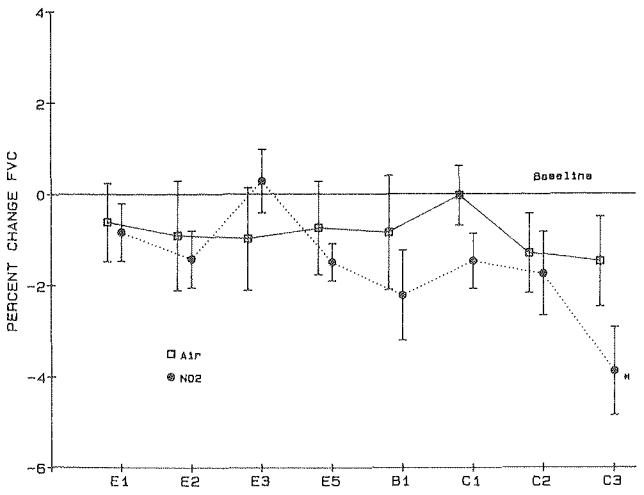

Figure 5. The 1.5 ppm NO_2 protocol, FVC. The percentages of change in FVC values measured during the exposures to NO_2 and air, compared to their respective base-line values, are depicted as means ± 1 SEM. *Measurement C3, the highest concentration of carbachol, after NO_2 exposure is significantly different (p = 0.005) from that measured after air exposure.

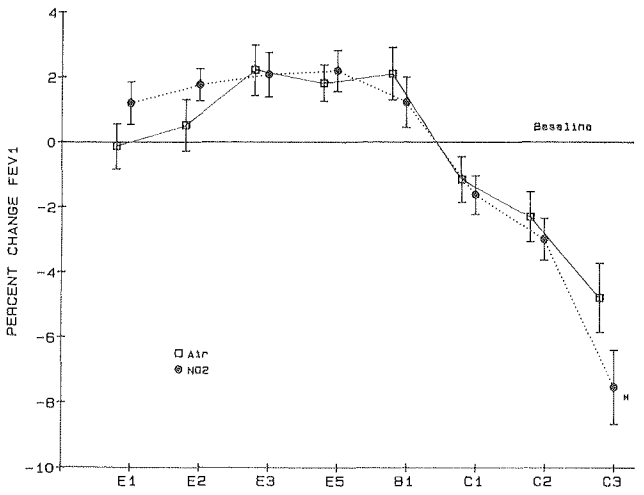

Figure 6. The 1.5 ppm NO_2 protocol, FEV_1. The percentages of change in FEV_1 values during the exposures to NO_2 and air, compared to their respective base-line values, are depicted as means ± 1 SEM. *Measurement C3, the highest concentration of carbachol, after NO_2 exposure is significantly different (p = 0.034) from that measured after air exposure.

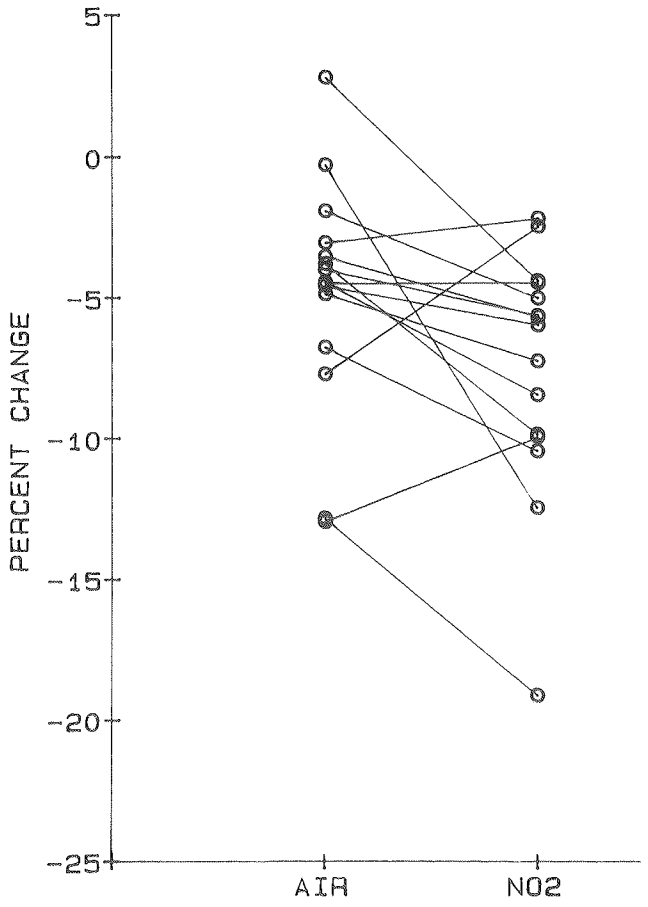

Figure 7. The 1.5 ppm NO_2 protocol, individual changes in FEV_1 for 15 subjects after 10 mg/mL carbachol for air and NO_2 exposures. Of 15 subjects, 11 showed a greater decrease in FEV_1 after NO_2 than after air exposure.

were recorded by questionnaire (Appendix B) immediately after each exposure for the three phases of this study. Subjects were polled with regard to a variety of symptoms, including respiratory and upper airway irritation, headache, and fatigue, and were asked to grade the severity of the symptoms. There were no differences in symptoms reported after air and NO_2 exposures for any of the protocols (Table 4). In addition, subjects were asked whether or not they could detect an odor or taste in the exposure atmosphere. Only in the "peaks" protocol did half of the group correctly identify the NO_2 exposure day; that is, 7 of 15 subjects detected an odor on the NO_2 day, and 2 of 15 detected an odor on the air day (Table 4). The individuals who detected the odor did not demonstrate the greatest changes in airway reactivity.

Cell Recovery

Analyses of cells recovered by bronchoalveolar lavage revealed no significant differences in total cell recovery, cell viability, or differential cell counts between air and NO_2 for the 2.0 ppm NO_2 peaks, the continuous 0.60 ppm NO_2, or the 1.5 ppm NO_2 (Table 5). This remained true when cell retrieval was expressed as cells per milliliter of recovered lavage fluid. Alveolar macrophages were the predominant cells obtained (94 percent), similar to findings after bronchoalveolar lavage of healthy humans without environmental exposures (Ettensohn and Roberts 1983). During Phase 3, we lavaged two lung segments and pooled the cells prior to counting to permit additional analyses of alveolar macrophage function. Thus, total cell recovery was higher for Phase 3 than for previous phases. Lavage supernatant fluids were not pooled; fluid volume and biochemical analyses were from the lingula only, which was always lavaged first.

Lavage Fluid Proteins and Enzymes

The volume of bronchoalveolar lavage fluid and quantification of total protein and albumin in the fluid obtained for each of the exposure protocols are shown in Table 6. No significant effect of NO_2 exposure on total protein or albumin

Table 4. Summary Scoring of Symptoms Reported by Subjects After Exposure to Nitrogen Dioxide or Air

	Reported Symptom Score/ Total Symptom Score Possible[a]		Subjects Reporting Unusual Taste or Smell/Total No. of Exposed Subjects[b]	
	Air	NO_2	Air	NO_2
Phase 1 (n = 15)	6/900	9/900	2/15	7/15
Phase 2 (n = 8)	12/480	6/480	1/8	1/8
Phase 3 (n = 15)	7/900	12/900	0/15	2/15

[a] Sum of symptom scores for all participants/total maximum score possible; 12 symptoms were scored: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe; 5 = incapacitating. Symptom questionnaire is included in Appendix B. For Phase 1, total symptom score: 15 subjects × 12 symptoms × 5 (maximum score) = 900.

[b] Number of subjects reporting an unusual taste or odor during exposure/total number of subjects exposed.

Table 5. Cells Obtained by Bronchoalveolar Lavage After Exposure to Nitrogen Dioxide or Air[a]

	Total Cells ($\times 10^6$)		Viability (%)		Macrophages (%)		Lymphocytes (%)		Neutrophils (%)	
	Air	NO_2	Air	NO_2	Air	NO_2	Air	NO_2	Air	NO_2
Peaks protocol[b]	14.4 ± 1.6	14.1 ± 1.6	88.4 ± 1.9	87.4 ± 1.3	95.3 ± 0.8	95.2 ± 0.5	3.7 ± 0.7	3.7 ± 0.5	0.5 ± 0.1	0.6 ± 0.1
Continuous 0.60 ppm NO_2[c]	16.1 ± 1.7	15.6 ± 1.3	92.3 ± 1.6	91.5 ± 2.2	95.0 ± 0.6	95.3 ± 1.2	3.3 ± 0.5	3.5 ± 1.1	0.8 ± 0.2	0.6 ± 0.2
Continuous 1.5 ppm NO_2[b]	30.3[d] ± 4.3	28.2 ± 3.3	96.9 ± 0.6	96.2 ± 0.5	93.3 ± 1.2	93.6 ± 1.2	4.8 ± 1.0	5.0 ± 1.2	1.1 ± 0.4	0.6 ± 0.1

[a] Values are expressed as means ± SEM.

[b] Lavage 3.5 hours after exposure. Air vs. NO_2, $p < 0.1$.

[c] Lavage 18 hours after exposure. Air vs. NO_2, $p < 0.1$.

[d] Cells from two lung segments were pooled in this protocol, accounting for higher total cell counts.

Table 6. Lavage Fluid Proteins After Exposure to Nitrogen Dioxide or Air[a]

	Volume Returned (mL)		Total Protein (μg/mL)		Albumin (μg/mL)	
	Air	NO_2	Air	NO_2	Air	NO_2
Peaks protocol[b]	103 ± 3	101 ± 4	135 ± 13	133 ± 11	84 ± 6	83 ± 5
Continuous 0.60 ppm NO_2 (late lavage)[b]	106 ± 4	101 ± 5	135 ± 10	129 ± 9	82 ± 8	94 ± 12
Continuous 1.5 ppm NO_2[b]	113 ± 4	116 ± 2	133 ± 12	125 ± 9	112 ± 13	103 ± 9

[a] Values are expressed as means ± SEM.

[b] Air vs. NO_2, $p < 0.1$.

was observed in any protocol. Examination of results after exposure to air shows that the three protocols were comparable, although subjects from the 1.5 ppm NO_2 protocol tended to show a slightly greater volume of fluid return and higher albumin values.

To assess possible changes in other proteins in lavage fluid, we utilized an immunological staining technique to identify the serum proteins that had been separated by SDS-PAGE out of bronchoalveolar lavage fluid. In a previous study (Frampton et al. 1989a), visual comparison of stained gels after air and 0.60 ppm NO_2 exposure suggested a pollutant-associated increase in a serum protein with molecular weight approximately 700 kDa, migrating in a position identical to serum α_2-macroglobulin. Therefore, an ELISA was developed to quantify α_2-macroglobulin in these samples and in lavage fluid from the subsequent exposure protocols.

α_2-Macroglobulin was measurable in all samples in nanogram quantities (Table 7). Frampton and associates (1989a) observed that continuous exposure to NO_2 at 0.60 ppm with lavage 3.5 hours later resulted in a 47 percent mean increase in α_2-macroglobulin when compared with exposure to air ($p = 0.01$). Six of the eight subjects showed increases in association with exposure to NO_2. This contrasted with α_2-macroglobulin levels in lavage fluid obtained after exposure to intermittent peaks of NO_2, which showed no change from control results. In addition, when broncho-

Table 7. Lavage Fluid Levels of α_2-Macroglobulin After Exposure to Nitrogen Dioxide or Air[a]

	Air	NO_2
Peaks protocol[b]	30 ± 1	30 ± 3
Continuous 0.60 ppm NO_2 (late lavage)[b]	35 ± 6	31 ± 3
Continuous 1.5 ppm NO_2[b]	43 ± 2	44 ± 3
Continuous 0.60 ppm NO_2 (early lavage)[c]	20 ± 1	29 ± 2

[a] Values are expressed as means ± SEM in nanograms per milliliter.

[b] Air vs. NO_2, $p < 0.1$.

[c] Data from Frampton and associates (1989a). Air vs. NO_2, $p = 0.01$.

alveolar lavage was performed 18 hours after a continuous exposure to 0.60 ppm NO_2, five of eight subjects showed decreases in α_2-macroglobulin after the exposure, but there was no significant change for the group as a whole. Similarly, α_2-macroglobulin levels in lavage fluid were unaltered by continuous exposure to 1.5 ppm NO_2.

Levels of α_2-macroglobulin after the air exposures for each protocol showed an increase over time during the course of these studies, from a mean of 20 ± 1 ng/mL for the previous 0.60 ppm NO_2 protocol (Frampton et al. 1989a) to a mean of 43 ± 2 ng/mL for the Phase 3 protocol (Table 7). This was, presumably, due to changes in sensitivity of the primary antibody, because a repeat assay of lavage fluids from the original 0.60 ppm NO_2 protocol performed with the same reagent and lot of primary antibody used to assay fluid from the Phase 3 protocol yielded levels comparable to the Phase 3 protocol: 48 ± 5 ng/mL after air exposure in the initial 0.60 ppm NO_2 protocol versus 43 ± 2 ng/mL after air exposure in the Phase 3 protocol. Consistency of measurements of α_2-macroglobulin for each study group was assured by concurrent assays of samples obtained after air and NO_2 exposure.

Arylsulfatase activity was detectable in lavage fluid for 12 of the 15 subjects in Phase 1. Failure to detect activity in three subjects was, presumably, related to a delay in the assay, because it was subsequently found that arylsulfatase activity decreased with time even when samples were stored at −70°C. The average arylsulfatase activity showed no difference in lavage fluid between exposures (25.1 ± 6.9 units [SEM] vs. 28.0 ± 6.1 units, air vs. NO_2, $p > 0.1$). Arylsulfatase activity was not detectable in lavage fluid from the Phase 2 studies, possibly because activity was lost during storage at −70°C. Although arylsulfatase activity was found in all samples of lavage fluid from the Phase 3 protocol, it was unchanged between the air and NO_2 exposures.

Influenza Virus Inactivation

The effects of in vivo exposure to NO_2 on the ability of alveolar macrophages to inactivate influenza virus in vitro were assessed in all 15 subjects in Phase 1 (Figure 8, Table 8). Virus inactivation studies were performed at MOIs of both 1 and 10. We found an MOI of 10 was required to permit persistence of countable virus beyond two or three days of incubation. Figure 8 gives the mean virus titer measured over time after in vitro infection of alveolar macrophages obtained by lavage 3.5 hours after exposure to air or peak NO_2; all results shown represent an MOI of 10. In all experiments, virus titers decreased with time, indicating little or no release of new infectious virus. The initial titers and rate of decline in titers in assays with alveolar macrophages, when compared with virus cultured in the absence of cells

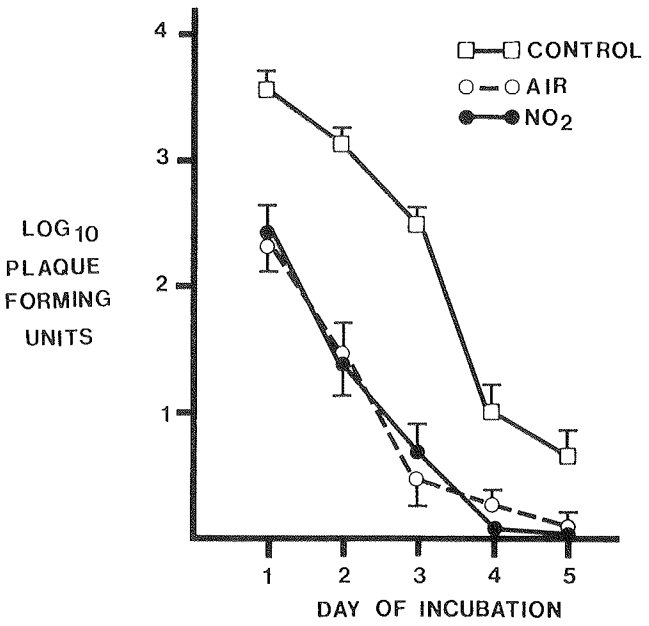

Figure 8. Peaks protocol, influenza virus inactivation. Cells obtained by bronchoalveolar lavage 3.5 hours after exposure to air or NO_2 were incubated with influenza virus, divided into five aliquots, incubated from one to five days at 37°C, and subsequently assayed for infectious virus. Control samples consisted of virus in medium without cells. Results are expressed as means ± SEM. Means and standard errors for these data are presented in Table 8.

(control), suggested uptake and inactivation of the virus by alveolar macrophages. The concurrent assays with virus alone suggested that further declines in titers indicated heat inactivation as well. Nonetheless, there were no consistent differences between titers of virus in NO_2 and air exposures after culture with alveolar macrophages on any day of incubation. In Phase 2, inactivation of influenza virus by alveolar macrophages lavaged 18 hours after exposure to 0.60 ppm NO_2 was again examined for all subjects. Virus titers declined on each day of incubation, indicating no net release of infectious virus (Table 8); a virus-to-cell ratio of 1:10 was used. Again, no differences were seen between air and NO_2 exposures in the titer of remaining infectious virus.

For Phase 3, inactivation of influenza virus by alveolar macrophages was assessed in vitro, as in previous experiments. Remaining infectious virus was quantified at five time points during incubation, but sampling times differed from previous phases such that early effects could be more accurately assessed (0, 4, 18, 36, and 60 hours in Phase 3 vs. 0, 18, 36, 60, 84, and 108 hours in Phases 1 and 2). The means of remaining infectious virus titers were greater after NO_2 exposure than after air, but the differences did not reach statistical significance (p = 0.26 at 36 hours) (Table 8, Figure 9). When compared with air, NO_2 exposure was associated with increased virus titers in seven subjects, de-

Table 8. Inactivation of Influenza Virus[a]

Hours of Incubation	Peaks Protocol[b]		Continuous 0.60 ppm NO_2[b]		Continuous 1.5 ppm NO_2[b]	
	Air	NO_2	Air	NO_2	Air	NO_2
0	2.64 ± 0.36	3.30 ± 0.15	3.29 ± 0.18	3.15 ± 0.26	3.68 ± 0.15	3.66 ± 0.08
4	ND[c]	ND	ND	ND	3.01 ± 0.32	3.28 ± 0.09
18	2.32 ± 0.21	2.42 ± 0.25	1.28 ± 0.40	1.64 ± 0.45	2.73 ± 0.29	3.00 ± 0.10
36	1.48 ± 0.27	1.39 ± 0.27	1.00 ± 0.39	0.99 ± 0.37	2.28 ± 0.28	2.67 ± 0.10
60	0.47 ± 0.20	0.68 ± 0.24	0.29 ± 0.29	0.75 ± 0.36	1.98 ± 0.30	2.06 ± 0.28
84	0.26 ± 0.12	0.09 ± 0.07	0.27 ± 0.27	0.24 ± 0.22	ND	ND
108	0.10 ± 0.10	0.00 ± 0.00	0.27 ± 0.27	0.16 ± 0.16	ND	ND

[a] Results are expressed as means ± SEM in $\log_{10}$ plaque-forming units.

[b] Air vs. NO_2 at each incubation period, $p < 0.1$.

[c] ND = not done.

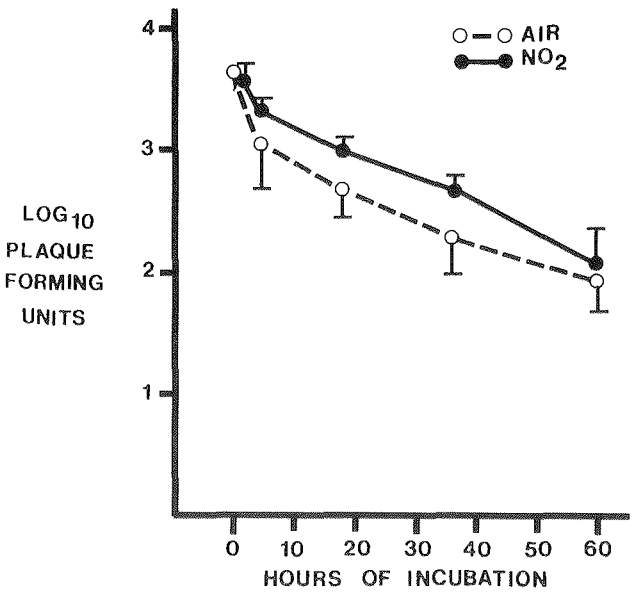

Figure 9. The 1.5 ppm NO_2 protocol, influenza virus inactivation. Cells obtained by bronchoalveolar lavage 3.5 hours after exposure to air or NO_2 were incubated with influenza virus, divided into five aliquots, incubated from 4 to 60 hours at 37°C, and subsequently assayed for infectious virus. Control samples of virus in medium without cells are shown in Figure 8. Results are expressed as means ± SEM. Means and standard errors for these data are presented in Table 8.

creased titers in three subjects, and no change in one subject.

To determine whether or not NO_2 alters the course of alveolar macrophage infection with influenza virus, techniques were developed during Phase 3 to measure NO_2 effects on viral protein expression in lavaged cells. Alveolar macrophages were exposed to virus or medium in vitro, incubated up to 24 hours to allow infection to occur, and then pulsed with ^{35}S-methionine. Lysates of alveolar macrophages were subjected to SDS-PAGE, followed by autoradiography. Peripheral blood monocytes from the same subjects, also virus-infected, were analyzed simultaneously for comparison. Autoradiographs of virus-infected peripheral blood monocytes suggested synthesis of new proteins not observed in sham-exposed cells. Figure 10 shows a representative autoradiograph of lysates of cells pulsed four to six hours after infection.

Infected peripheral blood monocytes contained new protein bands, which had previously been shown by immunoprecipitation (Mock et al. 1987) to correspond to influenza hemagglutinin (85 kDa), to neuraminidase and nucleoprotein (60 kDa), which comigrate, and to matrix protein (23 kDa). Autoradiographs of infected alveolar macrophages appeared to demonstrate minimal or no synthesis of proteins corresponding to neuraminidase and nucleoprotein, matrix protein, or hemagglutinin.

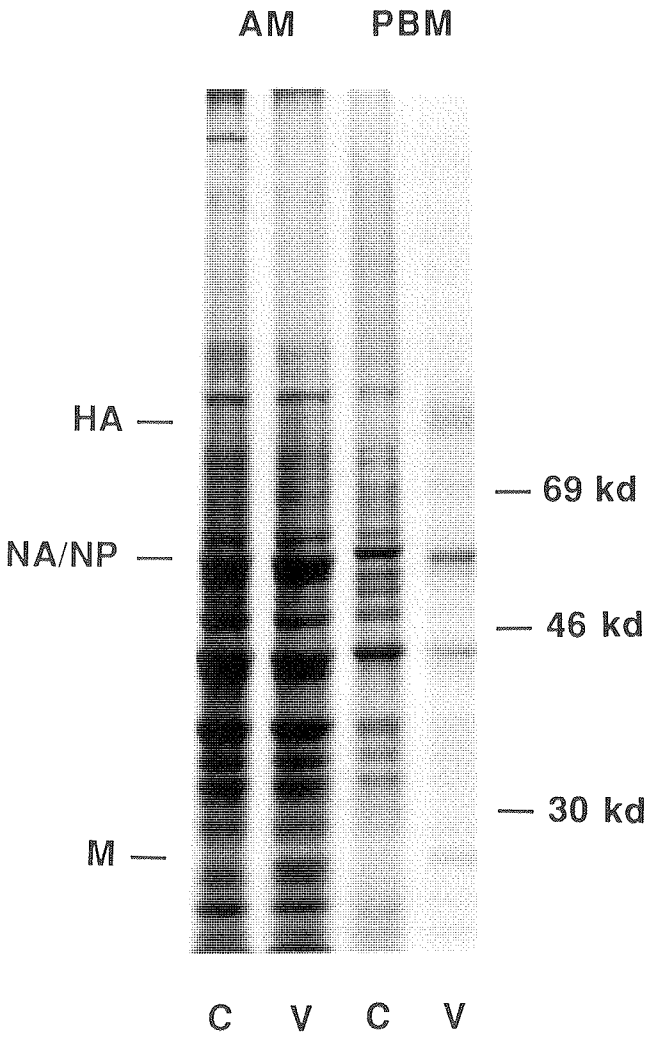

Figure 10. Protein synthesis in alveolar macrophages (AM) and 24-hour-adhered peripheral blood macrophages (PBM) exposed to influenza virus. Alveolar macrophages and PBM from the same subject were concomitantly exposed (V) or sham-exposed (C) to influenza virus in vitro at a MOI of 3. Results using cells from one subject are shown with cells pulse-labeled with ^{35}S for four to six hours after exposure. HA = influenza hemagglutinin; NA/NP = neuraminidase and nucleoprotein, which comigrate; M = matrix protein. Molecular weight values (30 kDa, 46 kDa, and 69 kDa) indicate the positions of standard proteins having the indicated molecular radius $\times 10^{-3}$.

Six subjects had adequate cell yields and sufficiently distinct protein bands after both air and NO_2 exposure to allow comparative quantification using laser densitometry. Bands representing protein synthesis appearing at the appropriate molecular weight for viral proteins, expressed as a percentage of total protein synthesis, showed no difference between air and NO_2 exposure for these six subjects. Considerable intersubject variability was evident (Table 9).

Interleukin-1 Secretion

In Phases 1 and 2, alveolar macrophage–derived supernatant fluids were analyzed for spontaneous and influenza virus–induced IL-1 biological activity using the standard mouse thymocyte comitogen assay. There was little baseline IL-1 secretion and a slight increase after influenza virus infection (Table 10). In general, no significant effect of NO_2 exposure on release of IL-1 by control or virus-infected macrophages was found. In Phase 3, IL-1 activity from supernatants of control cells obtained following NO_2 exposure was significantly less than that found after air exposure (Table 10, $p = 0.03$). However, this apparent change was most likely the result of contamination with lipopolysaccharide in some control samples in Phase 3, resulting in elevated IL-1 activity following air exposure. Examination of individual data (Appendix F)[6] did not reveal any correlation between virus inactivation responses and IL-1 secretion by alveolar macrophages.

For Phase 3 studies, IL-1 activity was measured using the mouse thymocyte comitogen assay and levels of IL-1β were measured immunologically by ELISA (Table 10, Appendix F)[6]. No significant differences in IL-1 secretion between air and NO_2 exposure were observed. Furthermore, no correlation between IL-1 secretion and inactivation of influenza virus was seen.

DISCUSSION

Despite years of laboratory, clinical, and epidemiologic research, the human health effects of NO_2 inhalation have not been fully characterized. The toxicological evidence has provided hypotheses to be tested in human populations, but limitations of the clinical and epidemiologic approaches have precluded definitive testing of these hypotheses. The EPA classifies NO_2 as a "criteria" pollutant and regulates it with a National Ambient Air Quality Standard. The scientific basis for justifying the current annual standard is inadequate, and no short-term standard exists. Furthermore, recent research has demonstrated that total exposures to NO_2 in the United States are, in large part, determined by indoor exposures, but the extent to which indoor exposures should be controlled is unclear. Gas stoves are used in homes of nearly half of the population, and kerosene space heaters are popular. Thus, there is a clear rationale for efforts to characterize better the effects of NO_2 exposures in both indoor and outdoor environments.

Morrow (1984), commenting on the inconsistency in hu-

[6] Appendix F is available on request from the Health Effects Institute.

Table 9. Viral Protein Synthesis by Alveolar Macrophages

	60 kDa (%)		23 kDa (%)		Total 60 kDa + 23 kDa (%)	
Subject	Air	1.5 ppm NO_2	Air	1.5 ppm NO_2	Air	1.5 ppm NO_2
1	14.73	14.56	6.20	6.33	20.92	20.89
2	4.50	14.10	7.24	0	11.74	14.10
3	6.65	4.55	1.76	2.04	8.41	6.59
4	9.36	3.38	2.06	2.59	11.43	5.97
5	7.08	9.93	4.73	4.71	11.81	14.64
6	2.20	0.24	2.40	3.14	4.60	3.38
Mean[a]	7.42	7.79	4.06	3.14	11.49	10.93
± SEM	± 1.77	± 2.43	± 0.95	± 0.89	± 2.21	± 2.73

[a] Air vs. NO_2, $p < 0.1$.

man experimental studies with NO_2, concluded that the measurement techniques in human studies often lack sensitivity and appropriateness for the presumed sites of NO_2 action. Nitrogen dioxide is known to injure the small airways, but pulmonary function tests are most sensitive at detecting changes in large-airway caliber. Alveolar assessments are more complex, but may be highly germane in the case of NO_2. With the development of bronchoalveolar lavage as a clinical investigative technique, we can, for the first time, extend human studies to assess oxidant-induced effects at the alveolar level. Although largely exploratory in nature, these studies present methodology for examining effects of pollutant exposure on airway inflammation, for assessing effects of pollutant inhalation on respiratory defense mechanisms, and for evaluating alterations in lung permeability of humans.

Previous studies utilizing bronchoalveolar lavage after oxidant exposure have revealed evidence of an inflammatory response. Mohsenin and Gee (1987) reported a decrease in α_1-PI activity in bronchoalveolar lavage fluid, but no change in cell counts, from subjects exposed to 3.0 to 4.0 ppm NO_2 compared with air exposure. The present studies demonstrate that brief exposures to NO_2 at concentrations up to 1.5 ppm neither alter epithelial permeability as assessed by protein concentrations nor cause an influx of inflammatory cells into the alveoli of normal humans. These findings are in agreement with previous studies in humans (Mohsenin and Gee 1987; Frampton et al. 1989a). Although more prolonged exposures in animal studies at levels as low as 0.40 ppm NO_2 have resulted in increased epithelial permeability to proteins (Sherwin and Carlson 1973; Sherwin and Layfield 1976), it is likely that higher levels are required to cause permeability changes after very brief exposures. For example, Bhalla and colleagues (1987) demonstrated al-

Table 10. Interleukin-1 Secretion by Control and Virus-Infected Alveolar Macrophages[a]

	Air		NO_2	
	Control	Virus	Control	Virus
Peaks protocol (n = 15)	4,407 ± 1,714	7,091 ± 2,310	4,519[b] ± 1,425	5,821[c] ± 1,549
Continuous 0.60 ppm NO_2 (n = 7)	4,820 ± 1,979	5,558 ± 1,017	7,359[b] ± 3,218	9,934[c] ± 3,630
Continuous 1.5 ppm NO_2[d] (n = 14)	8,725 ± 1,775	7,866 ± 1,786	4,714[e] ± 862	7,196[c] ± 2,900

[a] Culture supernatants from alveolar macrophages were assayed using mouse thymocyte comitogen assay. Data are expressed as maximum increase in counts per minute of tritiated-thymidine incorporation at any dilution of the supernatant, above that seen with PHA and thymocytes alone, means ± SEM.

[b] Air vs. NO_2 for control macrophages, $p > 0.1$.

[c] Air vs. NO_2 for virus-exposed macrophages, $p > 0.1$.

[d] Results of ELISA are given in Appendix F, which is available on request from Health Effects Institute.

[e] Air vs. NO_2 for control macrophages, $p = 0.03$.

tered permeability to radiolabeled diethylenetriaminepentaacetate (DTPA) and bovine serum albumin in the lungs of rats after two-hour exposures to 12 ppm NO_2, but not after exposures to 6 ppm NO_2. Evidence for a cellular inflammatory response is also lacking after brief low-level exposures to NO_2 in animals (Mochitate et al. 1986; Schlesinger 1987b).

Human responses to O_3 have been assessed using bronchoalveolar lavage and provide a striking contrast to the present studies. Seltzer and associates (1986) found an increase in neutrophils in lavage fluid three hours after exposure to 0.40 or 0.60 ppm O_3. Koren and coworkers (1989) showed that the neutrophil influx is present 18 hours after a two-hour exposure to 0.40 ppm O_3 and is accompanied by evidence of increased alveolar-capillary permeability to proteins. The absence of inflammation in response to exposure to NO_2 in the present study may simply reflect the decreased potential for tissue oxidation of NO_2 relative to O_3. However, it is also possible that the NO_2 effects differ in mechanism as well as degree.

NO_2 exposure, unlike O_3, did not provide evidence for a generalized influx of plasma proteins into the alveolar spaces; however, Frampton and colleagues (1989a) reported a 47 percent increase in levels of α_2-macroglobulin in lavage fluid obtained three hours after a three-hour exposure to 0.60 ppm NO_2. In the absence of other high molecular weight proteins, it was suggested that the increase in α_2-macroglobulin might reflect local secretion in response to alteration in the protease-antiprotease balance induced by the pollutant exposure. In contrast to our observations after continuous exposure to 0.60 ppm NO_2, no effect on α_2-macroglobulin levels was observed after exposure to intermittent 2.0 ppm peaks of NO_2, although total intake (concentration × time × minute ventilation) was 1.5-fold greater for the peaks protocol (Table 1). This suggests that intermittent peak exposures may produce less alteration in alveolar homeostasis, supporting our previous observations regarding NO_2 effects on alveolar macrophage interaction with influenza virus (Frampton et al. 1989c). No alteration in α_2-macroglobulin levels was observed when bronchoalveolar lavage was performed 18 hours after a continuous exposure to 0.60 ppm NO_2, suggesting that the changes were transient.

No significant increase in α_2-macroglobulin in lavage fluid was observed after continuous exposure to 1.5 ppm NO_2. This raises the possibility that, despite statistical significance at the traditional level of $p < 0.05$, the increase in α_2-macroglobulin observed in our previous protocol (0.60 ppm followed by lavage 3.5 hours later) may have been a chance observation (Frampton et al. 1989a). Alternatively, it is possible that changes in α_2-macroglobulin in response to NO_2 exposure do not demonstrate clear exposure-response relationships and that the observed effects with exposure at 0.60 ppm are extinguished at higher levels by other, yet unknown, effects of the pollutant. Such a phenomenon has been observed in studies of alveolar macrophage mobility and phagocytic capacity in rabbits exposed to low levels of NO_2 (Schlesinger 1987a,b). Furthermore, for asthmatic individuals, the absence of a definite exposure-response relationship for pulmonary function changes produced by NO_2 exposure has been noted (Utell 1989). Finally, with these low exposure levels and small subject groups, it is possible that we are dealing with "responders" and "nonresponders," which would complicate interpretation of any exposure-response relationship (Frampton et al. 1989c).

In 1988, the National Center for Health Statistics reported that respiratory infections remained an important cause of morbidity and mortality. An estimated 80.1 per 100 persons in the United States experienced acute respiratory conditions. Of this total, 38.2 were attributed to influenza, 25.9 to the common cold, and 4.6 to acute bronchitis and pneumonia (Schoenborn and Marano 1988). In 1985, acute respiratory infections and influenza directly caused more than 68,000 deaths and undoubtedly contributed indirectly to many others. The economic costs of respiratory infections are enormous (McConnochie et al. 1988).

Identification of risk factors for respiratory infection would provide an opportunity for intervention to reduce the large burden of morbidity and mortality associated with these common conditions. The present studies examined the effects of controlled in vivo exposures to low levels of NO_2 on responses of alveolar macrophages, obtained by bronchoalveolar lavage after exposure, to challenge with influenza virus in vitro. Exposure protocols and pollutant levels were designed to approximate ambient conditions, and the use of influenza virus as a probe of alveolar macrophage function provided a model relevant to important human disease.

At the 1.5-ppm level, there was evidence that NO_2 impairs the ability of alveolar macrophages to inactivate influenza virus in vitro. This tendency of in vivo NO_2 exposure to impair the ability of alveolar macrophages to inactivate influenza virus in vitro was also noted by Frampton and associates (1989c). Altered inactivation was not observed with alveolar macrophages from normal subjects who were exposed to intermittent peaks of NO_2 or for whom the lavage was delayed for 18 hours. To assess the possible magnitude of the differences between control (air) and NO_2 exposures, confidence intervals were calculated for the difference (air – NO_2) in the (log transformed) titer of infectious virus remaining on day 2 of incubation. The

endpoints of the confidence intervals were transformed back to the original scale, and the results, therefore, are given as the ratio of infectious virus remaining after air exposure to infectious virus remaining after NO_2 exposure. For the 0.6 ppm NO_2 study (Phase 1), the 95 percent confidence interval is (0.22, 4.85). In other words, we are 95 percent confident that the difference lies between the extremes of a fivefold increase or a fivefold decrease in virus titers after NO_2 exposure compared to air exposure. The two exposures are not significantly different at the 5 percent level, because the confidence interval includes the null value of one. For the peaks protocol (Phase 2), the confidence interval is (0.29, 5.26). Therefore, the difference lies between a 3.5-fold decrease in virus titers after NO_2 exposure and a 5.3-fold increase after air exposure. For the 1.5 ppm NO_2 study (Phase 3), the confidence interval is (0.105, 1.99). Therefore, there is a 95 percent chance that the result lies between a 9.5-fold increase after NO_2 exposure and a 2-fold decrease after air exposure.

We chose to measure IL-1 production by alveolar macrophages in these experiments, using in vitro challenge with influenza virus as a stimulus, for the following reasons: First, IL-1 is an essential cofactor for T-lymphocyte proliferation in response to antigen. The ability of monocytes and macrophages to secrete IL-1 in conjunction with the processing and presentation of viral antigens may be an important component of the antiviral immune response. Second, human peripheral blood monocytes, from which most alveolar macrophages are, presumably, derived, increase IL-1 secretion in response to challenge with influenza virus in vitro (Roberts et al. 1986). Third, IL-1 is chemotactic for lymphocytes (Hunninghake et al. 1987); alveolar macrophage production of IL-1 may mediate local lung recruitment of lymphocytes after inhalation injury or viral challenge.

Some studies (Wewers et al. 1984; Frampton et al. 1987) have shown that alveolar macrophages produce less IL-1 than blood monocytes produce; an alteration of patterns of IL-1 secretion by NO_2 exposure might indicate a shift in lavaged cell populations to more recently recruited cells. Such a shift may not be detected by standard morphological examination. Preliminary analysis of cells obtained by lavage after NO_2 exposure and characterized by fluorescence-activated flow cytometry reveals such a shift in the relative size distribution of alveolar macrophage subpopulations (Frampton et al. 1989b). After exposure to 1.5 ppm NO_2 for three hours, smaller cells suggestive of monocytes migrating from peripheral blood were identified, despite the absence of an increase in total cells in the lavage fluid. Efforts to characterize further such shifts in lavage cell populations in response to oxidant exposure are clearly warranted.

A previous report (Morrow and Utell 1989) reviewed the strengths and limitations of controlled human studies utilizing pulmonary function as an endpoint. The important question of whether or not ambient levels of NO_2 alter pulmonary function or airway reactivity remains unresolved, partly because of the difficulty in comparing studies in which varying levels of exposure have been administered under varying conditions to subjects chosen with different screening criteria. In these studies, normal subjects were rigorously selected for absence of airway reactivity. However, we observed that after exposure to 1.5 ppm NO_2 for three hours, airway reactivity to carbachol was increased when compared with the air exposure. The changes in airway reactivity were generally small and seemingly not clinically significant, although one subject showed a fall in FEV_1 of approximately 20 percent after NO_2 exposure; this would indicate a potential for important clinical effects in some individuals. In contrast, exposure to intermittent peaks of 2.0 ppm NO_2 or continuous exposure to 0.60 ppm NO_2 for three hours (Frampton et al. 1989c) had no effect on reactivity. There was no correlation between change in airway reactivity and ability to detect the pollutant (Table 4); similarly, there was no relation between altered airway reactivity and the presence of effector cells in lavage, because cell differentials were unchanged after the pollutant exposure. Mohsenin (1987) reported that exposure of normal subjects to 2.0 ppm NO_2 for one hour, without exercise, increased airway sensitivity to methacholine challenge. Because of the absence of exercise, total intake of NO_2 in Mohsenin's studies was less than 25 percent of that in the 1.5 ppm NO_2 protocol and well below that for either the peaks or 0.60 ppm NO_2 exposures. Perhaps the methodology incorporating measurements of partial flow rates was more sensitive for detecting small changes in airway reactivity in subjects with low sensitivity to bronchoconstricting agents.

The dosimetric approach used in this study was designed mainly to describe better the NO_2 exposure. Although the mean data for total dose for the three phases vary from 3,440 to 8,123 μg NO_2, there was considerable variability in dose within each group. This results from variations in minute ventilation despite our successful effort to maintain the exposure concentrations precisely and our attempt to calibrate each subject's workload during exercise in order to produce a more predictable increase in minute ventilation. Although the protocols for Phase 1 and Phase 2 were proposed to achieve nearly equivalent exposures based on C × T products, the influence of exercise is apparent from the calculations of total NO_2 deposition (5,634 μg in Phase 1 vs. 3,440 μg in Phase 2; see Appendix D). However, despite the

nearly 1.7-fold variation between the exposures, our findings do not support the suggestion that peak levels are more important than dose or duration. With the exception of the endpoint of airway hyperreactivity, there was little evidence of an exposure-response relationship; however, this does not preclude such findings in future studies, especially if a wider range of NO_2 doses were intentionally used.

On the basis of experimental studies, Miller and associates (1982) developed a dosimetric model for NO_2 in the human tracheobronchial airways that suggests the lower airways receive a predictable dose on the respiratory surface (approximately 10^{-8} μg NO_2/cm^2 for each μg NO_2/m^3 input) down to the transitional airways, where the surface dose increases 3- to 4-fold, and the alveolar region, where it decreases 10- to 100-fold. According to their model, increasing the tidal volume from 500 to 1,500 mL is expected to increase the lung uptake of NO_2 from 60 to 90 percent. This increase in dose is due almost entirely to the increased NO_2 uptake in alveolar areas. Unless there is an important differential sensitivity to NO_2 of effectors in the large airways, transitional airways, and alveoli, one must conclude that the transitional airways are the site of greatest NO_2 dose and response irrespective of the respiratory mode or breathing pattern. This model supports our belief that the alveolar region is a more appropriate site for detecting NO_2 effects than the large airways.

In conclusion, we acknowledge that this series of exploratory studies yielded few definitive findings. Furthermore, we were unable to confirm the observations of Mohsenin and Gee (1987) that low-level NO_2 exposure reduces the activity of α_1-PI using both functional and immunological assays (Johnson et al. 1990). Although our studies were not conceived to fuel the regulatory process, it is evident that controlled clinical studies have been, and will continue to be, key elements in the process of setting standards for criteria pollutants. Given the admitted exploratory nature of these studies, the generally negative findings should not be overinterpreted in the regulatory process. We would reemphasize that all of these studies were carried out with healthy volunteers; however, one must recall that the Clean Air Act requires that the National Ambient Air Quality Standards protect even the most sensitive and susceptible groups in the general population. For some pollutants, susceptible individuals are distinguished by certain medical characteristics; for example, those with asthma are especially susceptible to alterations in pulmonary function by sulfur dioxide exposure. For other pollutants, such as NO_2, the physiological or pathological characteristics determining susceptibility are not readily identified with the methodologies used to date. The application of the methodologies in these studies presents an investigative dilemma, because they cannot be easily applied to susceptible populations. However, it is conceivable that limited application of bronchoalveolar lavage to susceptible groups such as the elderly or populations with underlying lung disease may prove feasible and permit a more comprehensive understanding of NO_2 effects on the respiratory tract. We remain concerned that future efforts to promulgate an NO_2 standard will be surrounded by controversy; as with so many studies, our findings raise as many questions as they resolve. We believe, however, that efforts to characterize the influence of NO_2 inhalation, including repeated exposures, on respiratory defense mechanisms are warranted if a more scientifically credible data base is desired for the standard-setting process.

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APPENDIX A. Research Team

PERSONNEL

M. J. Utell, Professor of Medicine and of Toxicology, coprincipal investigator

P. E. Morrow, Emeritus Professor of Toxicology, coprincipal investigator

M. W. Frampton, Assistant Professor of Medicine, coinvestigator

N. J. Roberts Jr., Associate Professor of Medicine, coinvestigator

J. N. Finkelstein, Associate Professor of Pediatrics, coinvestigator

C. Cox, Associate Professor of Biostatistics, consultant in statistics

F. R. Gibb, Associate in Biophysics, investigator

D. M. Speers, Pulmonary Research Technician in Medicine

H. E. Beiter, Laboratory Research Technician in Biophysics

J. Nichols, Technical Associate in Medicine

M. Heeney, Laboratory Technician in Pediatrics

H. Lisman, Technical Associate (electronics) in Biophysics

DESIGNATED RESPONSIBILITIES

Exposure, analytical, and physical plant team: Morrow and Gibb

Clinical evaluations and analyses of lavage fluid: Utell, Frampton, Roberts, Finkelstein, Speers, Nichols, and Heeney

Physical plant engineering: Gibb

Statistical analyses: Cox and Speers

Quality assurance: Beiter and Morrow

Electronics and instrumentation maintenance: Lisman and Gibb

APPENDIX B. Symptomatology Questionnaires

BASE-LINE QUESTIONNAIRE

This questionnaire was used immediately and 24 hours after exposure to air or NO_2 for three hours. Note that questions 1 through 54 were subject history queries answered at the initial interview. That portion of the questionnaire is Appendix G, which is available upon request from the Health Effects Institute.

55. Does anyone in your immediate family (parents, brothers, sisters, or children) have asthma now or a history of asthma in the past?
    1. Yes
    2. No
56. In the past six weeks have you had any symptoms of a cold, "flu," or any other respiratory infection?
    1. Yes
    2. No
57. What is your occupation?
    1. Student
    2. Other: Please describe briefly.
58. Are you routinely exposed to any fumes or dusts that make you cough, wheeze, or short of breath?
    1. Yes
    2. No
59. Are you presently taking any medications?
    1. Yes
    2. No
60. If you answered Yes to number 59, list medications and dosage.
61. If you have asthma and are not currently taking medication, what medication have you taken in the past five years?
62. If you have asthma, when did your last attack occur?
63. Do you take any vitamin C or vitamin E (including what might be in a multivitamin)?
    1. Yes
    2. No
64. If the answer to number 63 is yes, please write the doses of vitamin C and vitamin E you take and how often you take them. If you take a multivitamin, give the brand name and the dose of vitamin C and vitamin E in it. (If you do not know them offhand, please find them out and get back to us.)
65. Are there any gas-burning appliances (stoves, unvented heaters, etc.) in use in your home on a regular basis or that have been in use in the past few weeks?
    1. Yes
    2. No
66. Has there been a kerosene heater in use in your home in the past few weeks?
    1. Yes, frequently used
    2. Yes, occasionally used
    3. If yes, how long ago was it used (days or weeks)?
    4. No
67. Are you getting desensitization injections?
    1. Yes
    2. No

Have you ever had shots?
1. Yes
2. No
3. If yes, when?

68. Have you ever had formal skin testing?
1. Yes
2. No
3. If yes, when?

69. Do you now smoke marijuana regularly, occasionally, or never?
1. Regularly
2. Occasionally (usually less than once each week)
3. Never

70. If you do not smoke marijuana now, did you ever smoke it regularly or occasionally?
1. Regularly
2. Occasionally (usually less than once each week)
3. Never smoked marijuana

71. Do you presently reside with anyone who smokes cigarettes regularly?
1. Yes
2. No

POSTEXPOSURE QUESTIONNAIRE

1. During the exposure period, did you develop any of the following symptoms?

a. Cough	0 1 2 3 4 5
b. Sputum production	0 1 2 3 4 5
c. Shortness of breath	0 1 2 3 4 5
d. Substernal irritation	0 1 2 3 4 5
e. Throat irritation	0 1 2 3 4 5
f. Nasal congestion or discharge	0 1 2 3 4 5
g. Headache	0 1 2 3 4 5
h. Fatigue (beyond that attributable to exercise)	0 1 2 3 4 5
i. Nausea	0 1 2 3 4 5
j. Wheeze	0 1 2 3 4 5
k. Chest tightness	0 1 2 3 4 5
l. Eye irritation	0 1 2 3 4 5

0 = not present
1 = minimal—not noticeable unless specifically asked about
2 = mild—noticeable but not annoying
3 = moderate—noticeable and annoying
4 = severe—limits activity or performance
5 = incapacitating—causes ongoing activity to be discontinued

2. Could you smell or taste anything unusual about the air you were breathing?

Yes No

3. General comments:

APPENDIX C. Protocols

PROTOCOL: PHASE 1 (PEAKS)

Day 1

Questionnaire
History and physical examination
Base-line pulmonary function tests
Carbachol: Propylene glycol, 2.5 and 5.0 mg/mL
Repeat pulmonary function tests after each dose

Day 2

Exposure: Air or 0.05 ppm NO_2 with 3- to 15-minute peaks of 2 ppm NO_2

0:00–0:20	Rest
0:20–0:30	Exercise 1 with $\dot{V}_E$ and O_2 saturation
0:30–0:40	Pulmonary function tests
0:40–0:50	Rest
0:45–1:00	First peak
0:50–1:00	Exercise 2 with $\dot{V}_E$ and O_2 saturation
1:00–1:10	Pulmonary function tests
1:10–1:30	Rest
1:25–1:40	Second peak
1:30–1:40	Exercise 3 with $\dot{V}_E$ and O_2 saturation
1:40–1:50	Pulmonary function tests
1:50–2:10	Rest
2:05–2:20	Third peak
2:10–2:20	Exercise 4 with $\dot{V}_E$ and O_2 saturation
2:20–2:40	Rest
2:40–2:50	Exercise 5 with $\dot{V}_E$ and O_2 saturation
2:50–3:00	Pulmonary function tests
	Exposure complete; start postexposure tests
	Questionnaire
3:00–3:30	Waiting period
3:30–4:15	Pulmonary function tests
	Carbachol: 2.5, 5.0, and 10.0 mg/mL
	Repeat pulmonary function tests after each dose
4:15–6:00	Waiting period
6:00–6:30	Prepare for lavage:
	Start intravenous line, phlebotomy, electrocardiogram
	Premedicate with atropine and lidocaine

6:30–7:00	Bronchoalveolar lavage
7:30–8:30	Observation period
8:30	End of study day for subject

Day 3 (same as day 2)

PROTOCOL: PHASE 2 (CONTINUOUS 0.60 PPM NITROGEN DIOXIDE, DELAYED LAVAGE)

Day 1

Questionnaire
History and physical examination
Base-line pulmonary function tests
Carbachol: Propylene glycol, 2.5 and 5.0 mg/mL
Repeat pulmonary function tests after each dose

Day 2

Exposure: Air or 0.60 ppm NO_2

0:00–0:20	Rest
0:20–0:30	Exercise 1 with $\dot{V}_E$ and O_2 saturation
0:30–0:40	Pulmonary function tests
0:40–0:50	Rest
0:50–1:00	Exercise 2 with $\dot{V}_E$ and O_2
1:00–1:10	Pulmonary function tests
1:10–1:30	Rest
1:30–1:40	Exercise 3 with $\dot{V}_E$ and O_2 saturation
1:40–1:50	Pulmonary function tests
1:50–2:10	Rest
2:10–2:20	Exercise 4 with $\dot{V}_E$ and O_2 saturation
2:20–2:40	Rest
2:40–2:50	Exercise 5 with $\dot{V}_E$ and O_2 saturation
2:50–3:00	Pulmonary function tests
	Exposure complete
	Questionnaire
	End of study day for subject

Day 3

Return to study

20:00–20:30	Prepare for lavage:
	Start intravenous line, phlebotomy, electrocardiogram
	Premedicate with atropine and lidocaine
21:00–21:30	Bronchoalveolar lavage
21:30–22:30	Observation period
22:30	End of study day for subject

Day 4 (same as day 2)

Day 5 (same as day 3)

PROTOCOL: PHASE 3 (CONTINUOUS 1.5 PPM NITROGEN DIOXIDE)

Day 1

Questionnaire
History and physical examination
Base-line pulmonary function tests
Carbachol: Propylene glycol, 2.5 and 5.0 mg/mL
Repeat pulmonary function tests after each dose

Day 2

Exposure: Air or 1.5 ppm NO_2

0:00–0:20	Rest
0:20–0:30	Exercise 1 with $\dot{V}_E$ and O_2 saturation
0:30–0:40	Pulmonary function tests
0:40–0:50	Rest
0:50–1:00	Exercise 2 with $\dot{V}_E$ and O_2 saturation
1:00–1:10	Pulmonary function tests
1:10–1:30	Rest
1:30–1:40	Exercise 3 with $\dot{V}_E$ and O_2 saturation
1:40–1:50	Pulmonary function tests
1:50–2:10	Rest
2:10–2:20	Exercise 4 with $\dot{V}_E$ and O_2 saturation
2:20–2:40	Rest
2:40–2:50	Exercise 5 with $\dot{V}_E$ and O_2 saturation
2:50–3:00	Pulmonary function tests
	Exposure complete; start postexposure tests
	Questionnaire
3:00–3:30	Waiting period
3:30–4:15	Pulmonary function tests
	Carbachol: 2.5, 5.0, and 10.0 mg/mL
	Repeat pulmonary function tests after each dose
4:15–6:00	Waiting period
6:00–6:30	Prepare for lavage:
	Start intravenous line, phlebotomy, electrocardiogram
	Premedicate with atropine and lidocaine
6:30–7:00	Bronchoalveolar lavage
7:30–8:30	Observation period
8:30	End of study day for subject

Day 3 (same as day 2)

APPENDIX D. Dosimetry

Table D.1. Nitrogen Dioxide Deposited in Peaks Protocol (Phase 1)[a]

Subject	Total	Rest Background	Rest Peaks	Exercise Background	Exercise Peaks
kp117	4,996	62	355	75	4,504
jr128	4,903	76	412	61	4,354
dm129	4,708	62	355	76	4,215
gg131	5,821	69	378	87	5,287
cg133	5,972	89	485	78	5,320
db134	5,022	74	400	77	4,471
jb136	6,085	75	406	80	5,524
ts105	6,679	92	502	99	5,986
rl122	4,143	62	355	56	3,670
rs109	6,497	97	530	87	5,783
rr130	5,823	67	366	88	5,302
jk125	4,175	61	333	66	3,715
rg132	6,212	99	541	86	5,486
mo135	5,020	58	316	81	4,565
sg137	5,252	60	327	79	4,786
Mean	5,415	74	404	78	4,865
SD	772	14	75	11	723

[a] Values are given in micrograms.

Table D.2. Nitrogen Dioxide Deposited in 0.60 ppm Nitrogen Dioxide Protocol (Phase 2)[a]

Subject	Total	Rest	Exercise
sg127	3,166	940	2,226
ts105	3,399	1,396	2,003
sm138	2,755	735	2,020
np141	4,422	1,205	3,217
mf142	3,285	881	2,404
id143	3,729	1,072	2,657
rs109	3,662	1,351	2,311
vk139	3,102	1,014	2,088
Mean	3,440	1,074	2,366
SD	504	230	407

[a] Values are given in micrograms.

Table D.3. Nitrogen Dioxide Deposited in 1.5 ppm Nitrogen Dioxide Protocol (Phase 3)[a]

Subject	Total	Rest	Exercise
dz144	7,188	2,420	4,768
rs109	7,975	2,640	5,335
rd146	8,612	2,823	5,789
sm147	8,189	2,456	5,733
dg150	8,110	2,273	5,837
rw151	8,206[b]	2,566[b]	5,640[b]
mo135	9,241	2,456	6,785
pm140	6,001	2,163	3,838
lp145	6,963	1,833	5,130
dp148	9,162	3,263	5,899
es149	7,027	2,236	4,791
wc152	8,663	2,786	5,877
kk153	8,480	2,530	5,950
es154	9,543	3,263	6,580
mk155	8,491	2,090	6,401
Mean	8,123	2,520	5,624
SD	967	399	763

[a] Values are given in micrograms.

[b] Estimated minute ventilation, inductive plethysmograph malfunction.

APPENDICES AVAILABLE ON REQUEST

The following appendices may be obtained by writing to the Health Effects Institute, 141 Portland Street, Cambridge, MA 02139. Please provide both the Investigators' Report title and the appendix title.

E. Consent Forms (Three Protocols)

F. Raw Data

1. Pulmonary Functions Tests and Airway Reactivity
2. Cell Recovery
3. Lavage Proteins and Enzymes
4. Virus Inactivation
5. Interleukin-1 Secretion

G. Subject History Questionnaire

ABOUT THE AUTHORS

Mark J. Utell is Professor of Medicine and Toxicology and Director of the Pulmonary and Critical Care Unit at the University of Rochester Medical Center. He received his M.D. degree from Tufts University School of Medicine in 1972. His research interests have centered on the effects of environmental pollutants on the human respiratory tract.

Mark W. Frampton is Assistant Professor of Medicine at the University of Rochester Medical Center. He received his M.D. degree from the New York University School of Medicine in 1973. After several years in the practice of internal medicine, he obtained training in pulmonary medicine at the University of Rochester from 1985 to 1988. His primary research interest is the effects of inhaled pollutants on respiratory defense against infection.

Norbert J. Roberts Jr., is Associate Professor of Medicine in the Infectious Diseases Unit of the University of Rochester School of Medicine. He received his M.D. degree from the New York University School of Medicine in 1971. His research interests are in human immunoregulation, with studies concentrating on antiviral defenses.

Jacob N. Finkelstein received his Ph.D. in biochemistry in 1976 from Northwestern University in Chicago, Illinois. He received postdoctoral training at the University of Rochester (1976 to 1978) in pulmonary biology and inhalation toxicology. He currently is Associate Professor of Pediatrics and Toxicology at the University of Rochester Medical Center. His research interests have centered on the cellular and molecular responses of the pulmonary epithelium during lung injury and repair.

Christopher Cox is Associate Professor of Biostatistics and Toxicology. He also holds an appointment in the University of Rochester Center for Biomedical Ultrasound. He received his Ph.D. degree in mathematics from the University of Illinois in 1972. He collaborates actively with a number of research groups at the University of Rochester Medical Center. His research interests are in the area of exponential family regression models.

Paul E. Morrow received his B.S. and M.S. degrees in chemistry from the University of Georgia and his Ph.D. in pharmacology from the University of Rochester in 1951. He received postdoctoral training at the University of Göttingen (1959) and the University of Zurich (1960) and spent sabbatical leaves with the M.R.C. Toxicology Unit, Carshalton, England, and the Comitato Nacionale Energie Nucleare (Casaccia), Rome, Italy, in 1968 and 1969. He served on the University of Rochester faculty from 1952 to 1985, at which time he retired as Emeritus Professor of Toxicology in Biophysics. Dr. Morrow's primary research interest is the pulmonary toxicology of inhaled substances.

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ABBREVIATIONS

ANOVA	analysis of variance
BAL	bronchoalveolar lavage
BTPS	body temperature and pressure, saturated with water vapor
DTPA	diethylenetriaminepentaacetate
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
IL-1	interleukin-1
IL-1β	interleukin-1β
MDCK	Madin-Darby canine kidney
MEM	minimal essential medium
MOI	multiplicity of infection
NAAQS	National Ambient Air Quality Standard
NO	nitric oxide
NO_2	nitrogen dioxide
NO_x	oxides of nitrogen
O_3	ozone
PBS	phosphate-buffered saline
PHA	phytohemagglutinin
ppm	parts per million
^{35}S	sulfur-35
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SG_{aw}	specific airway conductance
σ_g	geometric standard deviation
$TCID_{50}$	50 percent tissue culture infectious dose
$\dot{V}_E$	expired volume of ventilation per minute (minute ventilation)

INTRODUCTION

In the summer of 1984, the Health Effects Institute (HEI) issued a Request for Applications (RFA 84–3) that solicited proposals for studies on "Mechanisms of Oxidant Toxicity." In response to this RFA, Drs. Mark J. Utell and Paul E. Morrow, from the University of Rochester School of Medicine and Dentistry, submitted a proposal entitled "Mechanisms of Nitrogen Dioxide Toxicity in Humans." The HEI Research Committee approved the three-year study, which began in July 1985. Total expenditures were $504,766. The Investigators' Report for the study was received at the HEI in December 1989 and was accepted by the Health Review Committee in October 1990. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of the HEI and to the public. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Health Review Committee's Commentary.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h) and Sections 207(c)(4) through (6) impose specific requirements for reduction in motor vehicle emission of certain oxidants (and other pollutants). Section 202(i) calls for the Administrator to study whether or not additional reductions in light-duty vehicle and light-duty truck emissions should be undertaken.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS)[1] to protect the public health. The current primary NAAQS standard for nitrogen dioxide is 0.053 parts per million (ppm) as an annual arithmetic mean concentration. Because the determination of the appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the health risks that they present, research into the health effects of oxides of nitrogen in studies such as this one is essential to the informed regulatory decision-making required by the Clean Air Act.

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

SCIENTIFIC BACKGROUND

Nitrogen dioxide (NO_2) is a reactive gas that is present in the smog in many metropolitan areas. A significant question regarding NO_2 is what adverse human health effects may be produced by acute and long-term exposures to ambient concentrations of this gas. Much of the current scientific information concerning the deleterious human health effects of NO_2 is based on epidemiologic evidence. These findings suggest that alterations in lung function and increased susceptibility to respiratory infections in children and adults are related to NO_2 inhalation (Speizer et al. 1980; Samet et al. 1987). It is important to understand whether or not inhalation of ambient levels of NO_2 by healthy human subjects produces changes in respiratory function parameters and affects respiratory defense mechanisms.

The current NAAQS for NO_2 is 0.053 ppm (100 $\mu g/m^3$) as an annual average (U.S. Environmental Protection Agency 1982). Although the average background level of NO_2 is less than 0.005 ppm, ambient outdoor air concentrations can exceed this standard and can sometimes reach peak concentrations of 0.5 ppm. Nitrogen dioxide is also recognized as an important indoor air pollutant. Peaks of 0.2 to 0.5 ppm NO_2 can be produced indoors when gas stoves or space heaters fueled by gas or kerosene are used (Speizer et al. 1980; Samet et al. 1987). It is, therefore, noteworthy that the health risks associated with chronic NO_2 inhalation stem not only from outdoor exposures, but also from exposures within the home.

The air we breathe contains a background level of NO_2 that is produced by natural sources, such as bacterial metabolism, lightning, and forest fires. The higher levels achieved in the urban atmosphere are primarily attributable to the combustion of fossil fuels by motor vehicles, electric utilities, and a variety of industrial and agricultural operations (National Research Council 1977; Morrow 1984; Finlayson-Pitts and Pitts 1986). During the combustion process, chemical reactions between oxygen in the air and nitrogen in the fuel and in the surrounding heated air predominately produce nitric oxide. Nitric oxide itself exerts little biological toxicity at ambient concentrations, but

it can be oxidized readily to the more toxic NO_2. Nitrogen dioxide is also the acid anhydride of nitrous and nitric acids, and reactions between NO_2 and atmospheric constituents can produce both of these acids.

Meaningful interpretation of the epidemiologic data regarding the health effects of NO_2 in humans has been complicated by several factors (Samet and Utell 1990). These include inadequate or inaccurate personal exposure data for the study subjects, short duration of the exposure period for the studies, and the impact of confounding effects that occur when the influence of one variable has not been separated from the effects of other variables (Samet et al. 1987). In epidemiologic studies on NO_2, confounding variables for the study subjects include a history of smoking, occupational exposure to NO_2, socioeconomic status, and whether or not the subjects have asthma. Much remains to be learned about the human health consequences of long-term exposures to low levels of this oxidant gas (Morrow 1984).

LUNG INJURY CAUSED BY NITROGEN DIOXIDE

Experiments with animals have been essential for understanding the structural and functional alterations produced in the lungs by NO_2 inhalation and how these effects may relate to those produced in humans. The toxic effects produced by NO_2 are related to the gas's chemical properties as a free radical, a compound that has an odd number of electrons (Mustafa and Tierney 1978). As a reactive gas, NO_2 can interact with and damage a variety of cell components (Freeman and Crapo 1982; Pryor et al. 1982).

Earlier investigators, using NO_2 concentrations ranging from 0.8 to 25 ppm, reported that the deleterious effects of NO_2 inhalation included some acute inflammatory changes and damage to the ciliated cells that line all of the airways, including the smallest ones, the terminal and respiratory bronchioles (Freeman et al. 1968a; Evans et al. 1972; Stephens et al. 1971, 1972). These studies demonstrated that, across a broad range of concentrations, injury caused by NO_2 is particularly prevalent in the bronchoalveolar duct region, which is the intersection between the terminal and respiratory bronchioles and their adjacent alveoli. Inflammation and cell injury at this important intersection of the smallest airways and the area where gas exchange first occurs may affect distribution of air and gas exchange in the more distal regions of the lungs. This is also the site where centrilobular emphysema develops. Even at concentrations near ambient levels, dosimetric models (Miller et al. 1982) have predicted that the maximum tissue dose of NO_2 is achieved at the bronchoalveolar duct junction. Morphometric studies of rats exposed for six weeks to 0.5 or 2.0 ppm NO_2 with daily two-hour peaks at three times these concentration levels have corroborated injury in this region (Chang et al. 1986). Freeman and colleagues (1968b) reported that chronic exposure of rats to 2 ppm NO_2 for up to two years produced alterations in this region that may contribute to the pathogenesis of emphysema. However, the relationship between the effects of NO_2 injury and the pathogenesis of chronic lung diseases in humans, such as emphysema, is not yet clearly defined.

EFFECTS OF NITROGEN DIOXIDE INHALATION ON PULMONARY INFECTIVITY

There is some evidence that NO_2 exposure is linked with increased rates of pulmonary infections in humans (Speizer et al. 1980; Samet et al. 1987). A review of the results of animal studies indicates that NO_2 exposure produces alterations in mucociliary clearance and in pulmonary macrophage function (Graham and Miller 1985). The observed decrements in these respiratory defense mechanisms, which normally serve to keep the lungs sterile and free of infection, may account for the decreased resistance to disease noted in humans. Morphologic studies with animals indicate that NO_2 exposure damages the cilia on the ciliated cells that line the airways of the lungs (Evans et al. 1972). This damage can impair the ability of these cells to sweep mucus, inhaled particles, and infectious organisms, such as bacteria and viruses, up and out of the lungs. An increased retention time of these organisms due to ciliary impairment is one credible mechanism for the observed increased infectivity in animals and humans.

Pulmonary alveolar and airway macrophages also are compromised after NO_2 inhalation (Gardner 1984). These cells are best known for their phagocytic function of ingesting particles, infectious organisms, including bacteria and viruses, and debris, as well as for their role in inflammatory reactions (Brain et al. 1977). In a recent study, Schlesinger (1987) reported that macrophages lavaged from rabbits exposed to 0.3 or 1.0 ppm NO_2 exhibited some decreases in phagocytic ability and random mobility. Impaired removal, increased residence time, and decreased killing efficiency of infectious organisms by macrophages, as observed in animal studies, may also relate to the increased infection rates reported in human epidemiologic studies.

Pulmonary macrophages also produce and release a wide variety of factors, including some that serve important antiviral and antibacterial functions, and others that are prominent in the orchestration of the inflammatory response (Fels and Cohn 1986). Nitrogen dioxide inhalation may affect production of these factors, and these changes can be monitored by assays of bronchoalveolar lavage fluid after exposure. With this technique, constituents of the airways and

alveolar spaces, such as the macrophages, inflammatory cells, and other components, can be washed from the lungs and evaluated for changes produced by an NO_2 exposure. For example, the release of interleukin-1 is a signal for recruitment, differentiation, and activation of lymphocyte subsets in the lungs after inhalation injury or viral challenge. Synthesis of this factor may serve as one marker in the lavage fluid of the antiviral immune response by macrophages following an NO_2 exposure.

The mouse infectivity model has been used frequently to evaluate alterations in pulmonary defenses caused by NO_2 inhalation (Goldstein et al. 1973; Gardner 1979; Graham et al. 1987; Jakab 1988). In this model, mice are treated with bacterial pathogens either before or after an NO_2 exposure. The effects of the exposure on bacteriocidal activity and clearance mechanisms in the lungs then can be evaluated. Because of the similarity between human and rodent antibacterial defense systems (Green 1984), Goldstein (1984) supported the use of these infectivity models to predict the response to infections that may occur in humans. The drawbacks of the mouse infectivity model are that its results are not readily reproducible in other rodents, such as rats, and that it is still unclear if the findings for this model are relevant to the effects produced by NO_2 in humans. Furthermore, the human epidemiological findings most likely reflect viral, not bacterial, infections, and at present it is unclear whether or not NO_2 interferes with human lung antiviral defense mechanisms.

Until recently, the majority of animal studies have focused on the effects of NO_2 on antibacterial rather than antiviral defenses (Graham and Miller 1985). Data from Rose and colleagues (1989) provided evidence that mice exposed to NO_2 before and after intratracheal inoculation with murine virus were more susceptible to viral infections and subsequent reinfection by the virus 30 days later. Clinical studies with human subjects who were exposed to NO_2 and then treated intranasally with live, attenuated influenza virus provided equivocal results (Kulle and Clements 1988). These ambiguous findings may have been the result of a small sample size, the selection of young healthy subjects for the study, and the use of an attenuated virus that could not infect the lower respiratory tract. Since respiratory infections induced by viruses are a common and significant human health problem, more definitive studies on the relationship between exposure to NO_2 and susceptibility to viral infections are needed.

The inclusion of peaks of higher NO_2 concentration during a continuous low-level background concentration is a feature of exposure protocols that more closely mimics the fluctuations in NO_2 levels in the ambient environment. Findings from animal studies suggest that the presence of these concentration peaks during an exposure is an important component of the effects produced by NO_2 (Miller et al. 1987). Gardner and colleagues (1979) reported that, compared to animals continuously exposed to a fixed concentration of NO_2, the frequency and amplitude of exposure peaks during intermittent exposures were significant factors for the increased mortality rates observed with the mouse infectivity model. They suggested that, although the mathematical products of the concentration and the duration of different exposure regimens may be identical, concentration had a greater influence on mortality rates than the duration of the exposure. They also suggested that the exposure peaks were an important factor contributing to compromised lung antibacterial host defenses and to altered pulmonary function test parameters.

To resolve some of the questions related to NO_2 exposure and human susceptibility to infections, results from clinical studies that include exposure peaks may help determine the applicability of experiments with animals to the effects produced in humans. Such studies can, in turn, provide meaningful data for risk assessment regarding NO_2 exposures and the enhanced potential for respiratory infections.

HUMAN STUDIES WITH NITROGEN DIOXIDE

Results of clinical exposure studies with human subjects may contribute to the understanding of acute effects and the potential long-term health consequences of exposure to NO_2. Pulmonary function tests are a noninvasive clinical approach that characterize the responses produced in humans by inhalation of NO_2 (Samet and Utell 1990). In healthy human subjects, the principal response to NO_2 measured by pulmonary function tests has been an increased resistance to airflow in the subjects' airways. This effect has been observed only at concentrations that exceed approximately 2 ppm NO_2, which is much higher than levels normally encountered in the ambient environment. Although the evidence is somewhat controversial, it has been reported that certain susceptible populations, such as persons with asthma, may incur a greater increase in airway resistance after NO_2 inhalation than humans without asthma exposed to the same concentration (Orehek et al. 1976; Bauer et al. 1986; Mohsenin 1987). In a review article, Utell (1989) noted that some investigators have reported enhanced bronchial responsiveness in persons with asthma exposed to NO_2 concentrations less than 0.5 ppm. However, others have found few or no such effects.

Bronchoalveolar lavage is another useful clinical approach that can be used alone or in conjunction with pulmonary function tests to understand the effects of NO_2 in-

halation. Data from lavage samples can be correlated with the findings of the somewhat less sensitive, but noninvasive, pulmonary function tests performed during and after an exposure. In this way, both lung function and cellular responses to a pollutant challenge can be compared.

Clinical exposures in controlled settings with human subjects present an opportunity to evaluate the specific adverse health effects produced by NO_2 (Utell 1988). As previously discussed, numerous studies with laboratory animals have been used to evaluate the functional and structural changes produced by NO_2, but the mechanisms of NO_2 toxicity, particularly in humans, are not yet clearly understood (Morrow 1984). Consequently, the use of models to extrapolate the adverse effects of NO_2 from animals to humans is a valuable approach. However, it is also a difficult and complex undertaking.

Clinical studies with human exposures to NO_2 can validate the findings observed in animals and may also detect findings that are unique to humans. In a clinical study, an investigator defines specific exposure conditions and concentration levels, such as the inclusion of pollutant peaks during the exposure period, and selects test subjects according to specified criteria. In the clinical setting, physiological measurements of exposure response can be obtained after specific periods of rest and exercise. In addition, bronchoalveolar lavage can be performed in the subjects to determine the presence of inflammatory cells and specific factors in the lungs after the exposures (Koren et al. 1989). The results derived from these assays then can be correlated with those from pulmonary function studies to obtain a broader perspective of the effects of NO_2 inhalation.

There are drawbacks to clinical exposures that are not found in laboratory animal studies. These include limitations in the number of subjects that can be studied and relatively short exposure periods. A common disadvantage of both animal and human studies is that investigators can study the effects of only one or a few combined pollutants within a given exposure, rather than the complex mixture of pollutants that occurs in the natural environment.

Despite their drawbacks, clinical studies can provide valuable scientific data that cannot be obtained from epidemiologic studies alone. As previously discussed, numerous confounding factors complicate interpretation of the currently available epidemiologic data regarding the adverse human health effects of ambient exposures to NO_2. Clinical exposures can help explain the short- and long-term human health effects of NO_2. Information from such studies may contribute to our understanding of the mechanisms of toxicity of NO_2 at concentrations that occur in ambient atmospheres both outdoors and inside the home.

JUSTIFICATION FOR THE STUDY

Understanding the mechanisms of oxidant toxicity and their implication for lung disease in humans is critical for establishing relevant ambient air quality standards for these pollutants. Through RFA 84–3, the Health Effects Institute wanted to support studies to understand the relationship between lung injury induced by inhalation of oxidants and the development of chronic lung disease. The HEI was particularly interested in studies that would examine changes produced by oxidants at concentrations comparable to those common in urban areas.

A principal impetus for the two main objectives of the clinical study proposed by Utell and his colleagues was the alteration in lung function and the increased susceptibility to respiratory infections in children and adults attributed to NO_2 inhalation that had been reported in epidemiologic studies (Speizer et al. 1980; Samet et al. 1987). Utell and his coworkers proposed to evaluate whether or not inhalation of ambient levels of NO_2 by healthy human subjects produced changes in respiratory function parameters and affected respiratory defense mechanisms. They proposed to expose humans to low levels of NO_2 in environmental chambers, perform pulmonary function tests on these subjects, and obtain bronchoalveolar lavage samples after completion of the exposures. With these lavage samples, they would determine whether or not NO_2 exposure modified cell function, particularly pulmonary macrophage function, and whether or not the lavage fluid contained cells and protein markers indicative of an inflammatory response resulting from the exposures.

The Health Research Committee thought that the proposed study was responsive to the goals of the RFA and that it was a well-balanced combination of in vivo and in vitro approaches. Data from such a study could contribute to a better understanding of the mechanisms of toxicity caused by inhalation of low levels of NO_2 in humans.

OBJECTIVES OF THE STUDY

The major aim of these studies was to assess the respiratory and clinical effects of short-term exposures to NO_2 in humans at levels relevant to those present in the environment. The authors defined three specific goals to identify the potential mechanisms of toxicity of this reactive gas in their selected subjects. These goals were: (1) to evaluate the changes in pulmonary function and airway reactivity, as well as any symptoms produced by the exposure; (2) to

quantify changes in cells and marker proteins obtained from bronchoalveolar lavage samples; and (3) to examine whether or not NO_2 exposure potentiated susceptibility to viral infections by modifying pulmonary defense mechanisms.

The investigators also wanted to determine whether or not exposure to a low-level background with spikes of NO_2 concentration was more likely to produce pulmonary alterations than continuous exposure to a fixed NO_2 concentration. In addition, a key reason for the use of bronchoalveolar lavage was to evaluate the effectiveness of this technique for measuring NO_2 toxicity.

STUDY DESIGN

The basic study approach was a double-blind crossover design in which healthy volunteers were exposed to controlled levels of NO_2 or filtered air for three hours in an environmental chamber. On exposure days, subjects exercised intermittently and underwent pulmonary function testing before, during, and immediately after exposure. To compare the changes in airway reactivity caused by an NO_2 exposure, the subjects were treated both before and after NO_2 exposure with carbachol, a drug that causes airway constriction. After the subjects inhaled the aerosolized carbachol, which mimicked the neural action of parasympathetic bronchoconstriction, changes in the resistance to airflow in their airways were measured. After exposure, the subjects were questioned about their symptoms and then underwent bronchoalveolar lavage procedures either 3.5 or 18 hours after exposure.

There were three phases in the experimental design. In Phase 1, subjects were exposed to three 15-minute peaks of 2.0 ppm NO_2 during a three-hour exposure to a base-level concentration of 0.05 ppm NO_2. Bronchoalveolar lavage was performed 3.5 hours after the exposure. In Phase 2, there was a continuous three-hour exposure to 0.60 ppm NO_2, and lavage was performed 18 hours later. In Phase 3, the NO_2 concentration for the three-hour exposure was increased to 1.5 ppm, and lavage was performed 3.5 hours after the exposure.

The lavage samples were examined for changes in cell populations that would indicate an inflammatory response. The lavage fluid was also tested for total protein and albumin concentrations, activities of selected enzymes, and the functional activity of α_2-macroglobulin. In a collaborative study with Dr. David Johnson that was also supported by the HEI (Johnson et al. 1990), lavage samples were sent to Dr. Johnson for analysis of α_1-protease inhibitor. Alterations in pulmonary defense mechanisms were evaluated by two in vitro methods: one tested the potential of cells from pooled lavage to inactivate influenza virus, and the other examined the production of interleukin-1 by lavaged pulmonary macrophages.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

This study incorporated a complex, multifaceted approach to examine the effects of NO_2 in human subjects. Because the experimental design of these studies was exploratory in nature, the extent to which the original objectives were met is best evaluated in that light. Overall, the proposed objectives were achieved. The investigators aptly demonstrated the feasibility of performing a series of controlled human exposures to NO_2 using a variety of dose patterns. Their results included a broad range of pulmonary function tests, including bronchoconstrictive challenge tests and analyses of bronchoalveolar lavage fluid and cells.

The lavage samples were obtained by employing excellent technical approaches. These samples were used in a variety of assays to measure alterations in the composition of the lavage and to explore a number of different aspects of the toxicology of NO_2. This study also examined whether or not NO_2 compromises particular pulmonary macrophage functions that are important in the defense against respiratory tract viral infections.

METHODS AND STUDY DESIGN

A drawback of the multifaceted, complex design of this study was that its three phases were not interfaced to allow meaningful comparisons among all of the data from the different phases. For example, Phase 2 involved a three-hour, continuous exposure to 0.6 ppm NO_2 with lavage 18 hours after exposure. Similarly, Phase 3 involved a three-hour continuous exposure that differed only in that 1.5 ppm NO_2 was used. In this phase, however, bronchoalveolar lavage was performed 3.5 hours after the exposure, hence limiting useful comparisons of the lavage data with Phase 2. Phases 1 and 2 were cleverly designed to have nearly equivalent total exposure doses based upon the product of the NO_2 concentration and the duration of exposure. This planning produced data useful for comparisons between the pulmonary function data, but, because of differences in the timing of the lavage, equivalent comparisons among the various lavage endpoints could not be made.

With few exceptions, the authors were clear about the fact that their experimental design permitted them to address

only partially many of their specific aims. A serious flaw resulting from this design was that none of the basic investigative parameters was fully explored in a systematic manner.

RESULTS AND INTERPRETATION

The Investigators' Report provides an exceptionally lucid presentation of a complex series of experiments. The authors reported no significant changes in the pulmonary function test results after any NO_2 exposure, except for a small but significant increase in airway reactivity after exposure to the highest NO_2 concentration. Similarly, they found no significant changes in the cells and markers of the bronchoalveolar lavage fluid or in the capacity of pulmonary macrophages to resist infection after any of the exposures.

The authors made great efforts throughout the text to explain their points clearly and to use tables effectively to illustrate their findings. The statistical methods were described clearly and the statistical procedures and data analyses were handled properly. The authors are also to be commended for their detailed discussion of quality assurance procedures and for their dosimetric approach to the NO_2 exposures.

The dosimetric approach chosen by the investigators provided data useful for evaluating both mean uptake of NO_2 in micrograms and mean delivery rates of NO_2 at rest and during exercise. Dosimetry is a valuable method for determining NO_2 uptake in individual subjects and improves the likelihood of more accurate determinations of airway reactivity and the variability of response. This approach allowed direct comparisons of the pulmonary function test results from the different protocols in the study.

An excellent example of the usefulness of this dosimetric approach is the interpretation of the findings in Phase 1, in which short peaks of 2.0 ppm of NO_2 were imposed on a low background, and in Phase 2, in which there was a continuous exposure to 0.6 ppm of NO_2. Although the two phases were designed to provide approximately the same dose, exercise in Phase 1 resulted in a calculated 70 percent greater total mean intake of NO_2 than in the Phase 2 study. An absence of differences in the pulmonary function findings between these two phases provided compelling evidence that short-term peak exposures may not produce greater effects in a pulmonary function test than continuous exposure to a fixed NO_2 concentration. This conclusion appears valid, at least for the exposure levels in these studies, which in themselves produced minimal, if any, functional impairments.

As stated by the authors, the subjects for their studies were rigorously selected for an absence of airway reactivity in response to carbachol treatment. This criterion may have resulted in the selection of a group of nonresponsive subjects for these experiments and may be a key factor accounting for the largely negative findings of the pulmonary function test results in this study.

Although there were no statistically significant changes in pulmonary function tests or symptoms reported as a direct response to NO_2 alone, evidence of increased airway reactivity was noted with carbachol treatment after an NO_2 exposure. A statistically significant effect was found after inhalation of the highest dose of carbachol following exposure to 1.5 ppm NO_2. In general, as pointed out by the authors, the extent of changes in airway responsiveness was small and seemingly insignificant. The exception was one individual in whom a 20 percent fall in forced expiratory volume in one second was observed. Although this finding implied increased susceptibility in a given individual, the overall findings in the present study are in conflict with those of Mohsenin (1987). He reported that normal subjects exposed to 2.0 ppm NO_2 for one hour without exercise exhibited an increase in airway responsiveness after treatment with methacholine, another bronchoconstrictive agent.

Utell and associates previously reported that pulmonary leukocytes lavaged from humans experimentally exposed to 0.6 ppm NO_2 were deficient in their ability to inactivate influenza virus (Frampton et al. 1989). However, those effects were noted in cells from bronchoalveolar lavage samples obtained 3.5 hours after exposure to 0.6 ppm NO_2. In the present study, the lavage samples were obtained 18 hours after a 0.6 ppm NO_2 exposure. This delay could account for the return to control values for viral inactivation capacity by the macrophages. In addition, interpretation of the inactivation data was complicated by the possibility that virus inactivation was due simply to virus attrition during the in vitro incubation period. This fact, however, would not appear to account for the lack of difference between cells obtained from NO_2 and air exposures in the Phase 1 and Phase 2 studies. For the Phase 3 studies, in which some adjustments were made in the assay conditions, there was a tendency toward higher infectious virus titers after NO_2 exposures than after air exposures, but the findings were not statistically significant.

The possibility that it was easier to detect an effect on antibacterial defenses when lavage was performed 3.5 rather than 18 hours after the cessation of exposure is consistent with the mouse infectivity model (Graham et al. 1987). In this model, inhalation of a bacterial aerosol immediately after pollutant exposure resulted in the potentiation of the in-

fection, whereas delayed administration of the bacterial aerosol diminished or even reversed the effect. Based on animal data, it would not be surprising if exposure to NO_2 resulted in a wave of pulmonary macrophage responses, with the response during exposure differing from later responses. One obvious difference is that leukocytes either present or recruited into the airways during pollutant exposure are subjected to the direct effects of the pollutant. In contrast, leukocytes recruited into the airways after the exposure may have different initial characteristics and do not undergo exposure to the pollutant.

As a prelude to future studies, the investigators devised a novel approach by which they assessed whether or not NO_2 exposure altered the viral protein synthesis by lavaged macrophages that were subsequently infected in vitro. No significant differences were observed, but this may be attributable to a wide variability among the data from the subjects. These results suggest that this assay is not yet ready for routine use as a probe for subtle alterations in viral defense mechanisms.

Using an immunological assay, the investigators were unable to demonstrate significant alterations in the amounts of α_2-macroglobulin in the bronchoalveolar lavage fluid after any NO_2 exposure. This glycoprotein can form complexes with many proteases and may act locally in the lungs as an antiprotease (Hovi et al. 1977; Fels and Cohn 1986). Similarly, Johnson and colleagues (1990) reported no differences in the functional activity or immunological concentration of the antiprotease α_1-protease inhibitor, measured in lavage samples from Phases 1 and 3 of this study. In contrast, Mohsenin and Gee (1987) reported a 45 percent decrease in the functional activity of α_1-protease inhibitor in lavage samples from human subjects after exposure to 3 or 4 ppm NO_2. The outcome in the present study is an important negative finding. Much attention has been placed on the hypothesis that NO_2 and other pulmonary inflammatory agents capable of causing chronic lung disease do so by inactivating inhibitors of lung leukocyte proteolytic enzymes (Janoff 1985). Because the data obtained to test this theory have been conflicting, a critical reassessment of this hypothesis is needed.

Finally, the protocol of this study also included an assay for arylsulfatase. Release of this enzyme may be an indicator of macrophage activation, but no change in its activity was observed in the lavage samples. Similarly, there was no difference in the total protein content of the lavage fluid. This finding indicates that the concentrations of NO_2 used in this study did not produce pulmonary edema, at least not at levels detectable by the bronchoalveolar lavage techniques.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The type of study conducted by Utell and his colleagues with normal, healthy subjects should be extended to more sensitive individuals to respond to the mandates of the Clean Air Act and to understand better the effects of air pollutants on susceptible populations. These populations include persons with asthma, those with other preexisting lung conditions, the elderly, and people who exhibit increased airway reactivity. A balance between scientific questions and any potential risks to subjects' health from pollutant exposure should be a primary consideration for these studies. As illustrated by the present study, the selection of subjects who are either nonresponsive or potentially very responsive to a given exposure condition must be carefully considered during the study design. An alternative approach suggested by the results of the present report is to repeat these same studies with healthy subjects using higher concentrations of NO_2.

The exemplary efforts of these authors with regard to quality assurance procedures highlight the need to improve this component in all air pollution health research. Enhanced attention to this area is particularly important for those studies evaluating the health consequences of ambient pollutants that are of direct regulatory concern.

Although Phases 1 and 2 of this study were designed so that the subjects would receive comparable doses of NO_2, no significant effects were observed in these phases. Questions, therefore, remain unanswered regarding whether or not pulmonary function parameters, responsiveness to bronchoconstrictive challenge, and pulmonary macrophage function in humans will respond differently to short-term peak exposures than they do to continuous exposures. Studies of lung leukocyte cell function after different exposures of NO_2 regimens should be extended to measures of antimicrobial defenses, including phagocytosis and the production of superoxide anion radicals. These studies should also include a more comprehensive evaluation of the possible effects of the release of potentially harmful macrophage products on the lung cells and extracellular matrix. This evaluation is particularly important with regard to the implied potential of NO_2 to induce chronic lung disease.

The present studies suggest, but do not prove, that effects on pulmonary macrophage viral defense mechanisms may occur when lavage is performed 3.5 hours, but not 18 hours, after the cessation of NO_2 exposure. These equivocal results call for a systematic approach to evaluate the effects of varying the interval between exposure cessation and bron-

choalveolar lavage. In addition, more studies using recent advances in molecular biology techniques to detect, for example, alterations in production or turnover of a nucleic acid message for specific cell products in response to a pollutant exposure, would be welcomed. In this way, the impact of pollutants on pulmonary macrophages, including modulations in defenses against infectious agents, could be documented more clearly.

Human studies, such as those described in the present report, may produce data that are essential for regulatory decisions regarding air quality standards. Unfortunately, these studies are time consuming and resource intensive. In addition, the availability of the facilities and skilled personnel needed to conduct such controlled exposure experiments and then combine them with the pulmonary function testing and bronchoalveolar lavage procedures is limited. To maximize the information obtained from such a study, research support organizations could facilitate cooperative interactions among investigators interested in this important research area. This type of interaction was demonstrated by the collaborative studies with Johnson and his colleagues (1990).

CONCLUSIONS

The experimental design, data analyses, and interpretation of the findings of this study focused on the important question of the effects of NO_2 exposure in humans. Using three different exposure regimens, the authors found few significant effects produced by their NO_2 exposures. In itself, however, this was a very valuable and productive series of studies. The studies provide ample justification for a research approach that combines pulmonary function testing with bronchoalveolar lavage to examine the effects of NO_2 exposure in humans. The experimental design of these studies was exploratory in nature because the effects of low concentrations of NO_2 exposure in humans were not known. It is understandable that a study such as the present one may not produce many substantive findings because of its exploratory nature.

The Clean Air Act clearly specifies that standards for primary air quality pollutants, such as NO_2, must be set to protect sensitive populations. Classically, individuals with reactive airways, such as persons with asthma, have been considered one of the populations at particular risk to pulmonary irritants, such as NO_2. A data base exists suggesting that persons with asthma are at increased risk of the bronchoconstrictive effects of NO_2. This effect has been either noted as a direct reaction to the gas or measured as an increase in airway reactivity in response to a bronchoconstrictive drug. Because carbachol-sensitive individuals were rigorously excluded from this study, it is unlikely that the subjects and data obtained in this study are representative of populations at particular risk to the bronchoconstrictive effects of NO_2.

In summary, this study can be viewed as a series of exploratory investigations that provide guidance for future studies. Because these experimental acute exposures to ambient and higher levels of NO_2 did not alter pulmonary function and lung defenses in humans, the question regarding the health effects of chronic exposure to NO_2 remains unresolved.

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Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
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Report No.	Title	Principal Investigator	Publication Date
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8	Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung	J.L. Mauderly	May 1987
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13	Effects of Nitrogen Dioxide on Alveolar Epithelial Barrier Properties	E.D. Crandall	October 1987
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18	Respiratory Infections in Coal Miners Exposed to Nitrogen Oxides	M. Jacobsen	July 1988
19	Factors Affecting Possible Carcinogenicity of Inhaled Nitropyrene Aerosols	R.K. Wolff	August 1988
20	Modulation of Pulmonary Defense Mechanisms Against Viral and Bacterial Infections by Acute Exposures to Nitrogen Dioxide	G.J. Jakab	October 1988
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Research Reports

Report No.	Title	Principal Investigator	Publication Date
40	Retention Modeling of Diesel Exhaust Particles in Rats and Humans	C.P. Yu	May 1991
41	Effects of 4 Percent and 6 Percent Carboxyhemoglobin on Arrhythmia Production in Patients with Coronary Artery Disease	D.S. Sheps	May 1991
42	Effects of Methanol on Human Neurobehavioral Measures	M.R. Cook	July 1991

Copies of these reports can be obtained by writing to the Health Effects Institute, 141 Portland Street, Suite 7300, Cambridge, MA 02139.

The Health Effects Institute (HEI) is an independent nonprofit corporation that is "organized and operated . . . 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

The Institute is governed by a four-member Board of Directors whose members are Archibald Cox (Chairman of the Board), Carl M. Loeb University Professor (Emeritus) at Harvard University; William O. Baker, Chairman (Emeritus) of Bell Laboratories and Chairman of the Board of Rockefeller University; Donald Kennedy, President of Stanford University; and Walter A. Rosenblith, Institute Professor (Emeritus), Massachusetts Institute of Technology.

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-eight domestic and foreign manufacturers of vehicles or engines contribute to the Institute's budget in shares proportionate to the number of vehicles or engines that they sell in the United States.

THE HEI RESEARCH PROCESS

The Institute 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 multidisciplinary 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 HEI's mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, who review the merits of the study as well as the procedures, independence, and quality of the selection process.

THE HEI REVIEW PROCESS

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

Each Investigator's Report is peer-reviewed, generally by a biostatistician and three outside technical reviewers chosen by the Review Committee. At one of its regularly scheduled meetings, the Review Committee discusses the Investigator's Report. The comments of the Committee and the peer reviewers are sent to the investigator, and he or she is asked to respond to those comments and, if necessary, revise the report. The Review Committee then prepares its Commentary, which includes a general background on the study, a technical evaluation of the work, a discussion of the remaining uncertainties and areas for future research, and implications of the findings for public health. After evaluation by the HEI Board of Directors, the HEI Research Report, which includes the Investigator's Report and the Review Committee's Commentary, is published in monograph form. The Research Reports are made available to the sponsors, the public, and many scientific and medical libraries, and are registered with NTIS, MEDLINE, and Chemical Abstracts.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing of the release of an HEI Research Report is tailored to ensure that it does not interfere with the journal publication process.

HEI HEALTH EFFECTS INSTITUTE 141 Portland Street, Cambridge, MA 02139 [617] 621-0266

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