HEI HEALTH EFFECTS INSTITUTE

Altered Susceptibility to Viral Respiratory Infection During Short-Term Exposure to Nitrogen Dioxide

Richard M. Rose, Paula Pinkston, William A. Skornik
New England Deaconess Hospital, Harvard Medical School, Harvard School of Public Health, Boston, MA

Includes the Report of the Institute's Health Review Committee

Research Report Number 24

HEALTH EFFECTS INSTITUTE

HEI

The Health Effects Institute (HEI) is a nonprofit corporation founded in 1980 to assure that objective, credible, high-quality scientific studies are conducted on the potential human health effects of motor vehicle emissions. Funded equally by the U.S. Environmental Protection Agency (EPA) and 27 automotive manufacturers or marketers in the United States, HEI is independently governed. Its research projects are selected, conducted, and evaluated according to a careful public process, including a rigorous peer review process, to assure both credibility and high scientific standards. HEI makes no recommendations on regulatory and social policy. Its goal, as stated by former EPA Administrator William D. Ruckelshaus, is "simply to gain acceptance by all parties of the data that may be necessary for future regulations."

Research Agreement No. 84-12

Printed at Capital City Press, Montpelier, VT

TABLE OF CONTENTS

Research Report No. 24

Altered Susceptibility to Viral Respiratory Infection During Short-Term Exposure to Nitrogen Dioxide

INVESTIGATORS' REPORT Richard M. Rose, Paula Pinkston, William A. Skornik

HEALTH REVIEW COMMITTEE'S REPORT Health Effects Institute

ABBREVIATIONS

ED_{50}	effective dose of NO_2 that results in infection of 50 percent of animals
ELISA	enzyme-linked immunosorbent assay
F	F statistic in an F-test
^3H	tritium
HEPA filter	high-efficiency particulate air filter
ID_{50}	inoculum dose resulting in infection of 50 percent of animals
MCMV	murine cytomegalovirus
NADH	reduced nicotinamide adenine dinucleotide
NO_2	nitrogen dioxide
pfu	plaque-forming units
ppm	parts per million
t	t statistic in a T-test

Altered Susceptibility to Viral Respiratory Infection During Short-Term Exposure to Nitrogen Dioxide

Richard M. Rose, Paula Pinkston, William A. Skornik

ABSTRACT

The studies reported here focus on the relation of nitrogen dioxide exposure to susceptibility to viral respiratory infection in a murine model of pneumonia, created by intratracheal inoculation of an endogenous murine pathogen, mouse cytomegalovirus. The purpose of this work is to clarify the potential role of nitrogen dioxide exposure in the pathogenesis of viral infection of the lower respiratory tract.

Previous human epidemiologic studies have presented conflicting information about the relationship of nitrogen dioxide to acute, self-limited episodes of respiratory illness, which are characteristic of viral respiratory infection. Some studies have found an association between exposure to elevated ambient levels of nitrogen dioxide and increased occurrence of acute respiratory illness. In one study this association was found to be strongest in children in the first two years of life. However, other epidemiologic studies have failed to observe this relation. To determine if there is scientific evidence for the possible relation of nitrogen dioxide exposure to human respiratory infection, our studies were performed to assess the impact of nitrogen dioxide on respiratory tract susceptibility to initial, or primary, infection, as well as to recurrent infection, or reinfection, with the identical virus. The latter mechanism of viral respiratory infection is of particular interest, since reinfection is a common method for the development of infection of the lower respiratory tract during early childhood.

Outbred CD-1 mice were exposed to either air or nitrogen dioxide for six hours a day on two consecutive days prior to inoculation with murine cytomegalovirus, and then were reexposed to the same level of nitrogen dioxide for six hours a day on four consecutive days, beginning the day after viral inoculation. Susceptibility to primary infection was determined by inoculating animals with an amount of virus (10^2 plaque-forming units) that is too small to produce viral infection in the lungs of normal animals. Mice exposed to 5 parts per million (ppm) nitrogen dioxide routinely developed viral replication in the lung and histologic evidence of pneumonitis after inoculation with this amount of virus, whereas air-exposed animals did not. Most importantly, animals exposed to 5 ppm nitrogen dioxide could be infected with a viral inoculum that was 100-fold smaller than that required to consistently produce viral infection in air-exposed mice. Enhanced susceptibility to infection was found after exposure to 5 ppm nitrogen dioxide, but was not observed with exposure to 2.5 or 1 ppm nitrogen dioxide. Susceptibility of the lower respiratory tract to primary murine cytomegalovirus infection was associated with alterations in pulmonary macrophage function in vivo. This includes diminished endocytosis of ^{198}Au-colloidal-gold particles and diminished ability to eradicate murine cytomegalovirus.

To assess the impact of nitrogen dioxide exposure on susceptibility to reinfection, animals were inoculated with a quantity of virus sufficient to produce active infection in both air- and nitrogen-dioxide-exposed mice (10^4 plaque-forming units). Animals were exposed to either air only or nitrogen dioxide only during this initial contact with the virus. Thirty days later, animals were reinoculated with 10^5 plaque-forming units of the identical virus. Animals exposed to 5 ppm, but not to 2.5 ppm, nitrogen dioxide during the initial viral infection were susceptible to reinfection with this virus, while air-exposed animals were generally resistant to reinfection. Susceptibility to reinfection with murine cytomegalovirus was associated with diminished macrophage expression of the Ia epitope (a marker for antigen-presenting cells) and reduced splenic lymphocyte blastogenic response to murine cytomegalovirus antigen. No difference between air- and nitrogen-dioxide-exposed animals was observed with respect to serum levels of murine cytomegalovirus antibody after the initial infection.

The exposure threshold for these effects on viral susceptibility was 5 ppm nitrogen dioxide. No consistent alteration in respiratory susceptibility to murine cytomegalovirus was seen after exposure to 2.5 or 1 ppm nitrogen dioxide. Exposure of mice to 5 ppm nitrogen dioxide alone did not produce histologic evidence of acute lung injury. Cellular and biochemical analysis of bronchoalveolar lavage fluid from animals exposed to this concentration of nitrogen dioxide revealed only a mild increase in albumin concentration. Therefore, exposure of mice to a concentration of nitrogen dioxide (5 ppm) that does not produce evidence of major lung injury is nevertheless capable of enhancing the susceptibility of the lower respiratory tract to viral infection.

Dr. Richard M. Rose, Division of Pulmonary Medicine, New England Deaconess Hospital, 185 Pilgrim Road, Boston, MA 02215.

INTRODUCTION

Recent epidemiologic studies in humans have raised concerns that ambient nitrogen dioxide (NO_2), both indoors and outdoors, may have adverse respiratory effects. In a study of Chattanooga, TN, schoolchildren, Shy and colleagues (1970) reported a greater frequency of acute respiratory illness in children who resided in a neighborhood near an industrial point source of NO_2 than in children who resided in an area with lower NO_2 levels. A subsequent study by Pearlman and coworkers (1971) also demonstrated increased frequency of bronchial symptoms in children who were exposed to increased levels of NO_2. However, reanalysis of the study design and the methods used for NO_2 measurement has called into question the conclusion of these studies (Ferris 1978).

Ambient NO_2 is produced in the home environment during gas cooking or heating, and may lead to average 24-hour levels that are four to seven times greater than those in homes with electric stoves (Melia et al. 1978). Two studies have reported that children residing in homes with gas stoves had greater rates of acute respiratory illness than children in homes where electric stoves were used (Melia et al. 1977; Speizer et al. 1980). In these studies, peak NO_2 concentrations of 0.25 to 1 ppm were often measured in the kitchen or nearby rooms in houses with gas stoves. These levels exceed the annual National Ambient Air Quality Standard for NO_2 of 0.053 ppm, but are comparable to one-hour peak averages that reach as high as 0.5 ppm NO_2 in some urban areas of the United States (U.S. Environmental Protection Agency 1982).

Studies linking NO_2 to increased rates of acute respiratory illness are, in most instances, unable to exclude a potential role for other environmental factors, particularly coexisting air pollutants. Also, most of these studies do not precisely delineate the nature of the acute respiratory illnesses being assessed. The most commonly noted symptoms associated with exposure to increased levels of NO_2 are coryza, sore throat, and cough without sputum. This symptom complex could be the result of the toxic effects of inhaled NO_2 on the respiratory tract, or could be caused by intercurrent episodes of respiratory infection. If the self-limited episodes of respiratory illness associated with NO_2 exposure in epidemiologic studies are infectious in origin, they are probably caused by respiratory viruses, which are the most common etiologic agents associated with this clinical syndrome.

In spite of the suggestion from human epidemiologic studies that excessive exposure to NO_2 may be linked to increased frequency of viral respiratory infection, the majority of previous investigations into the effects of NO_2 on altered respiratory tract susceptibility to infection have focused on bacterial pathogens. In general, these studies show impaired killing of bacteria by lung phagocytes obtained from NO_2-exposed animals, and enhanced infectivity of bacterial pathogens for the lower respiratory tract of NO_2-exposed animals (reviewed by Jakab 1980). For instance, a brief four-hour exposure of mice to 7 ppm NO_2 diminished intrapulmonary killing of *Staphylococcus aureus* (Goldstein et al. 1973). Jakab (1987) has extended this observation by demonstrating that the effect of NO_2 exposure on pulmonary antibacterial defenses depends on both the exposure protocol (for example, whether NO_2 exposure occurs before or after the bacterial challenge) and the bacterial species tested. Infectivity studies in which survival from bacterial challenge is assessed also suggest that NO_2 exposure has an adverse effect on pulmonary responses to infection. In studies performed by Ehrlich (1966), two-hour exposures to more than 3.5 ppm NO_2 were associated with increased mortality from challenge with *Klebsiella pneumoniae*. This effect was reversible, and could be demonstrated during chronic exposure to as little as 0.5 ppm NO_2 (Ehrlich and Henry 1968).

In contrast to the numerous studies that document an adverse effect of NO_2 exposure on host responses to bacterial lung infection, far fewer studies have examined any possible interaction between NO_2 and viral lung infection. Moreover, previous work in this area has yielded inconclusive results. In a variety of animal models employing influenza viruses, exposure to NO_2 either had no effect (Lefkowitz et al. 1986) or it enhanced mortality (Henry et al. 1970). Neither bacterial infectivity studies nor work with experimental viral infection has adequately addressed the issue raised by human epidemiologic studies: the possible role of NO_2 exposure in altered susceptibility to respiratory infection.

In an effort to clarify the possible role of excessive NO_2 exposure on viral infectivity, exploratory experiments were performed to determine if exposure to NO_2 alters the susceptibility to viral infection of the respiratory tract in the murine system. The model used in these studies is ideal for this purpose, because it utilizes an endogenous murine pathogen that produces infection in a dose-dependent manner after intratracheal inoculation (Rose et al. 1982). Furthermore, since previous work with this experimental infection has clarified many of the specific antiviral defense mechanisms that play a role in the host response to both primary infection and reinfection, this model system can be used to relate the potential effects of NO_2 on viral susceptibility to alterations in discrete host defense mechanisms.

SPECIFIC AIMS

The hypothesis tested in these studies is that exposure to NO_2 enhances the susceptibility of the lower respiratory tract to viral infection. This potential interaction was assessed during initial, or primary, infection and during reinfection of animals that had been previously inoculated with the test virus. Related experiments were performed to identify the possible effect of NO_2 exposure on relevant antiviral defense mechanisms of the lower respiratory tract. The overall intention was to provide a scientific basis for the potential association between NO_2 exposure and increased frequency of viral respiratory illness that is suggested by some human epidemiologic studies.

METHODS

GENERAL DESIGN OF STUDIES

These experiments were planned to be of an exploratory nature, to determine where important new findings might reside. It was anticipated that, on the basis of these observations, a more detailed analysis of pathogenic mechanisms would then be undertaken.

Efforts were made in the conduct of these studies to minimize intrinsic sources of bias. At the time of their arrival to the animal care facility, animals were randomized to the various experimental study conditions by assignment to individual cages (four to six animals per cage) dedicated to the separate experimental conditions. This procedure was necessary to limit the possible spread of infection between animals from different study conditions that would occur if they were housed in the same cage. All animals, regardless of cage assignment, were treated identically with respect to ambient air temperature and the frequency with which food, water, and bedding were replenished. Although air-exposed animals were placed in an exposure chamber different from that in which animals were exposed to NO_2, the exposures occurred at the same time of day, and at the same relative humidity and temperature.

A major, unanticipated obstacle was the difficulty in reliably obtaining very small viral inocula for use in the animal studies relating to viral susceptibility. Unlike other microbes, such as bacteria, viruses are not stable at room temperature, and must be stored at low temperatures to preserve viability. In the process of thawing the stored virus for use in these studies, it was found that the proportion of the "prefreeze" viral inoculum retrieved was, in fact, variable. Since the actual amount of virus present could not be determined with precision prior to the inoculation of the animals (a plaque assay for quantifying the viral inoculum takes five to seven days), the amount of virus given to the animals often varied by more than 10 percent from the desired amount. When this occurred, the experiments were not included in the analysis because of the potential confounding effect of this variability in the viral inoculum size on an important endpoint of study, the development of infection. In general, this difficulty nullified the results of approximately a third of all our animal susceptibility studies, and it occasionally resulted in variation in the number of animals reported for the various study conditions.

ANIMALS

Four- to six-week-old barrier-maintained CD-1 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) for use in these studies. This strain of mouse has been previously shown to be susceptible to the test virus. All animals were used in experiments within one week of delivery. To prevent accidental infection during experiments, animals were housed in Micro-Isolator cages (Lab Products Inc., Haywood, NJ). To further ensure quality control of test animals in terms of coexistent and previous viral infection, sera from two to four animals per delivery were screened by Microbiological Associates (Bethesda, MD) for the presence of antibody to a panel of murine viruses, including murine cytomegalovirus (MCMV), Sendai virus, and mouse hepatitis virus. Groups of animals with serologic evidence of infection with any of these agents were excluded from further study.

EXPOSURE TO NITROGEN DIOXIDE

Animals were exposed to NO_2 for six hours a day on two consecutive days prior to inoculation with the virus, and were reexposed to the same level of NO_2 for six hours a day on four consecutive days beginning the day after viral inoculation. Exposure to NO_2 occurred only during initial, or primary, infection with the virus. Reinfection studies were performed on air-exposed animals that had been exposed to air or NO_2 during the primary infection. This mode of NO_2 exposure was chosen because test animals were exposed before and after viral instillation, a pattern that may be a more realistic model for human NO_2 exposure than one in which exposure occurs either before or after the introduction of the virus.

Animals were exposed in individual, open-mesh, stainless-steel cages within a 170-l stainless-steel and Lucite environmental exposure chamber (NYU-type inhalation

chamber) to either clean filtered air or NO_2. The animals were without food and water during the exposure period. After exposure, they were returned to standard cages with free access to food and water.

Nitrogen dioxide was generated from nitrogen tetroxide (N_2O_2dimer) that was placed in a specially designed vapor generator and was cooled to a temperature between −15°C and 10°C, which determines the concentration of NO_2 produced. The vapor above the nitrogen tetroxide was carried to the inlet airstream of the exposure chamber, with nitrogen as the carrier gas. The nitrogen flow rate was controlled with a mass flow controller. Final gas concentration was achieved by regulating inlet nitrogen gas flow with air diluted through a high-efficiency particulate air (HEPA) filter.

The NO_2 concentration in the chamber was continuously monitored with an oxides-of-nitrogen (NO, NO_2, NO_x) analyzer (Model 14B/E, Thermo Electron Corp., Waltham, MA) and recorded on a strip-chart recorder. Variation in the NO_2 concentration was less than 2 percent during exposures (Figure 1). Small, low-speed fans were positioned within the exposure chamber to ensure adequate mixing. Chamber temperature and relative humidity were monitored, and ranged between 20° and 25°C and 46 and 74 percent, respectively. This variability in exposure conditions reflects experiment-to-experiment differences; during individual exposures, the conditions for air and NO_2 exposure were similar.

ESTABLISHMENT AND EVALUATION OF INFECTION

The test strain of virus, MCMV, was newly obtained from the American Type Culture Collection (Rockville, MD). To obtain a virulent, animal-passaged virus for use in these studies, virus was derived from the partially purified salivary gland homogenates of infected animals, and was stored at −70°C in RPMI medium containing 10 percent fetal calf serum and 10 percent glycerol, as described previously (Rose et al. 1982). To ascertain the viral titer used in individual experiments, each inoculum was separately quantified in a plaque-forming assay. Only inocula that varied by less than 10 percent from the desired amount were used.

Respiratory tract infection with MCMV was established by intratracheal inoculation of animals, as previously described (Rose et al. 1982). The amount of virus in the lung homogenates was determined by a plaque-forming assay on the primary cultures of mouse fibroblasts at intervals after viral instillation. Recovery of MCMV from the lung homogenates at day 5 or days 10 to 12 after inoculation was considered to represent evidence of viral infection in the lower respiratory tract. In some animals, but not all, this was confirmed by histopathologic findings.

In studies of primary viral infection, MCMV was instilled after the animals had been exposed to air or NO_2 for two days; exposure was resumed on the day after viral inoculation. In the reinfection experiments, initial infection and inhalation exposure occurred in the same manner as described for primary infection. Upon the completion of exposure on day 5 after initial viral inoculation, both NO_2- and air-exposed animals were maintained in standard cages for 30 days. At this time, both groups of animals were rechallenged with MCMV. Therefore, the inhalation exposures in both the primary and reinfection studies occurred only during the initial phase of primary infection.

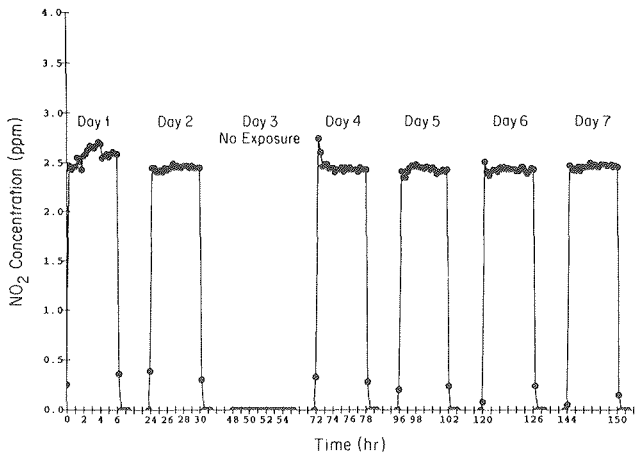

Figure 1. Measurements of variation in NO_2 concentration during a representative study (2.5 ppm NO_2 exposure). —●— = NO_2 level.

HISTOPATHOLOGY

Lung histopathology was assessed by light microscopy. Excised lungs were inflated, via the trachea, with 10 percent buffered formalin, 2.5 percent glutaldehyde, at a transpulmonary pressure of 20 cm of water. Blocks from these specimens were embedded in paraffin, and multiple transverse sections were cut, from apex to base, at 5-μm thickness. All specimens were evaluated in a blinded fashion by the principal investigator.

ANALYSIS OF PULMONARY LAVAGE CELLS AND FLUID

Bronchoalveolar lavage was performed by insertion of a 22-gauge plastic catheter into the trachea, and consisted of six 1-ml washes with sterile phosphate-buffered saline, as described previously (Rose et al. 1982). A consistent proportion of lavage fluid (approximately 80 percent) was recovered from animals in all exposure groups. Lavage cells

were isolated by centrifugation at 100 × g for 10 minutes at 20°C, and were then counted in a hemocytometer. Morphology of cells was characterized with the use of a modified Wright's stain (Meltzer 1981), and cell viability was assessed by ability to exclude trypan blue.

Cell-free lavage fluid was analyzed for biochemical indices of lung injury by the methods described by Beck and coworkers (1982). The lysosomal enzyme peroxidase was measured by spectrophotometry of the enzymatic modification of guaiacol at 470 nm. The intracellular enzyme lactate dehydrogenase was assayed by following the oxidation of reduced nicotinamide adenine dinucleotide (NADH) in the presence of pyruvate at 340 nm. Albumin was determined by measuring the binding of bromcresol green at 630 nm, with bovine serum albumin as a standard.

MACROPHAGE STUDIES

In vivo phagocytosis by lung phagocytes was assessed by measuring the fraction of ^{198}Au-colloidal gold ingested by these cells after intratracheal instillation, as described by Brain and Corkery (1977).

In vitro infection of pulmonary macrophages with MCMV was accomplished by plating pulmonary lavage cells (10^5 cells/well) on 6.4-mm wells of a flat-bottomed microtiter dish (Costar Tissue Culture Cluster, Cambridge, MA). Cells still adherent after a three-hour incubation were routinely greater than 98 percent viable and more than 95 percent phagocytosed 1.1-μm latex beads. Phagocytic capacity was assessed by incubating 5 × 10^4 pulmonary lavage cells suspended in RPMI medium with 10 percent fetal calf serum with between 5 × 10^5 and 7 × 10^5 latex beads for 20 minutes, at 37°C, with frequent gentle shaking. The percentage of cells that ingested at least one bead was determined by light microscopy after the cells were washed three times in phosphate-buffered saline to remove noningested beads. Adherent cells were infected with MCMV (0.1 ml), at a multiplicity of infection of 5 virions per cell, for one hour. Unabsorbed virus was then washed off three times, and cell cultures were replaced in RPMI medium 1640 supplemented with 10 percent fetal calf serum and 50 μg/ml gentamicin. The supernatant from duplicate wells at intervals after infection was assayed for virus, as described above.

The amount of virus associated with macrophages during in vivo infection was assessed in cells obtained by bronchoalveolar lavage. After extensive washing, adherent cells were removed by gentle scraping with a rubber spatula, counted in a hemocytometer, and freeze-thawed once at −70°C to release intracellular virus. The amount of virus was then determined by plaque assay on primary lung fibroblasts.

Macrophage expression of the Ia epitope, a membrane marker associated with antigen-presenting ability, was determined in lung lavage cells by indirect immunofluorescence, as described by Beller and colleagues (1980). In these studies, cells adherent to coverslips were first incubated with rat antimurine Ia antibody, and then fluorescein-labeled goat antirat IgG (Boehringer Mannheim, Indianapolis, IN). The percentage of cells staining positively was then determined by evaluating at least 200 cells. All experiments included a positive control stained with either mouse antileukocyte common antigen antibody (Boehringer Mannheim) or rat antimurine hydrogen monotypic antibody (Boehringer Mannheim). The negative control for these studies consisted of cells incubated with saline prior to fluorescein-labeled anti-IgG. In all studies, antibody preparations were diluted in phosphate-buffered saline with 2 percent w/v bovine serum albumin and 0.02 percent w/v sodium azide adjusted to pH 7.2.

LYMPHOCYTE STUDIES

The proliferative responses of splenic lymphocytes were measured by culturing 5 × 10^5 cells from each animal in triplicate wells for every condition studied. Cells were then incubated for 48 hours with either lectin (concanavalin A, 1 μg/ml; or phytohemagglutinin, 5 μg/ml) or various concentrations of MCMV antigen. Viral antigen was prepared by exposing a stock solution of virus in an uncovered plastic dish to ultraviolet light for 60 minutes at a distance of 8 inches. Plaque assay of the antigen preparation was then performed to ensure that no viable virus was present. In preliminary studies, it was found that 48 hours was the optimal duration of exposure to either lectin or viral antigen for maximal stimulation of lymphocyte blastogenesis. Lymphocyte proliferative responses were determined at this time by an overnight pulse of cell cultures with ^{3}H-thymidine (Amersham International, UK; specific activity, 2 Ci/ml; dose, 0.5 mCi/well). A stimulation index was determined for each culture by calculating the ratio of cpm/ml in stimulated (that is, lectin- or antigen-exposed) cultures to cpm/ml in unstimulated cultures.

SEROLOGIC STUDIES

Murine cytomegalovirus antibody determination was performed on sera obtained by direct cardiac puncture of animals between 30 and 45 days after initial inoculation with virus. These studies were performed at the Charles River Breeding Laboratories (Wilmington, MA) by enzyme-linked immunosorbent assay (ELISA).

STATISTICAL METHODS

Statistical methods were chosen to analyze data sets that often contained multiple variables, with relatively few observations pertaining to individual variables. For many of these low-"n value" studies, the use of statistical analysis is meant to be a guide to potentially meaningful associations that can be validated or refuted by future experiments that employ a greater number of observations. Where appropriate, single pairwise comparisons were made by Student's T-test. Chi-squared analysis was performed in order to determine the strength of the association of NO_2 exposure with susceptibility to infection, compared to air exposure. In order to clarify data relating the concentration of NO_2 to an observed effect (for example, dose-response data), an estimation of the 50 percent endpoint for a given parameter was made by the method of Reed and Muench (1938). The effect of graded NO_2 exposure on viral recovery in the lung was analyzed by a one-way analysis of variance. Two-way analysis of variance was performed on data such as the bronchoalveolar lavage cellular samples, in order to account for the independent, and potentially interactive, effects of NO_2 exposure and viral infection.

RESULTS

SUSCEPTIBILITY TO PRIMARY INFECTION WITH SMALL VIRAL INOCULA

To determine whether or not NO_2 alters susceptibility to primary MCMV infection, animals were exposed to varying concentrations of NO_2 for six hours on two consecutive days, and then inoculated with an amount of virus, 10^2 plaque-forming units (pfu), that previously had been found to be too small to produce evidence of infection in normal (air-exposed) animals. Starting the day after inoculation, animals were again exposed to the same concentration of NO_2 for six hours on four more consecutive days. As depicted in Table 1 and Appendix A, virus was recovered from the lung of only one of 30 animals exposed to either air or 1 ppm NO_2 at any time after inoculation. In contrast, two of 10 animals exposed to 2.5 ppm, and the majority of animals exposed to 5 ppm, and of those exposed to 10 ppm NO_2, were found to have virus present in their lung homogenates. This differing susceptibility to viral infection between animals exposed to 5 and 10 ppm NO_2, and those exposed to air, was statistically significant ($\chi^2 = 31.9$; $p < 0.001$). Estimation of the effective concentration of NO_2 resulting in infection of 50 percent of the animals (ED_{50}) was 3.9 ppm.

To delineate the smallest viral inoculum capable of producing infection after intratracheal instillation in animals exposed to 5 ppm NO_2, animals were inoculated with varying quantities of virus during exposure (Table 2). The lowest inoculum capable of reliably producing infection was only 10^2 pfu for NO_2-exposed mice, but had to be 100 times greater (10^4 pfu) for air-exposed mice. Animals exposed to 5 ppm NO_2 were significantly more likely to become infected than were air-exposed animals after instillation of viral inocula ranging from 10 to 10^3 pfu ($\chi^2 = 9.82$; $p < 0.005$). The viral inoculum estimated to be capable of infecting 50 percent of air-exposed animals (ID_{50}) was 7×10^3 pfu; the ID_{50} for animals exposed to 5 ppm NO_2 was 5×10 pfu. In addition, viral recovery in the lung was greater after inoculation with 10^4 pfu in animals exposed to 5 ppm NO_2 than in those exposed to air ($t = 3.12$; $p = 0.036$).

Further experiments were performed to clarify the impact of NO_2 exposure on viral replication in the lung as well as on mortality. In these studies, an inoculum of virus was used that consistently produced infection, but was too small to produce mortality, in air-exposed animals (10^5 pfu). Mortality was observed in 4 of 20 uninfected animals exposed to 15 ppm NO_2 and in 28 of 30 uninfected animals exposed to 27 ppm NO_2. In infected animals, mortality occurred in eight of nine animals exposed to 15 ppm NO_2, but was not observed in animals exposed to lower concentrations. Analysis of mortality as a function of NO_2 exposure demonstrated a significant difference between infected animals exposed to 15 ppm NO_2 and those exposed to lower concentrations ($\chi^2 = 21.8$; $p < 0.001$). Viral recovery in the lung was also greater in animals exposed to increasing con-

Table 1. Pulmonary Responses to a Small Viral Inoculum[a]

Exposure	Ratio of Infected to Inoculated Animals[b]	Peak Viral Titer in Lung[c]
Air	1/24	1.25×10^3
NO_2		
1 ppm	0/6	None recovered
2.5 ppm	2/10	$1.25 \pm 0.05 \times 10^3$
5 ppm	11/15[d]	$1.49 \pm 1.04 \times 10^3$
10 ppm	5/6[e]	$1.31 \pm 0.45 \times 10^3$

[a] 10^2 pfu.

[b] Animals were sacrificed at 5 or 10 to 12 days after viral inoculation, and the lungs were harvested for virologic and histologic study. Infection was considered to be present if virus was recoverable from the lung five days after inoculation.

[c] This represents the highest mean viral titer $\pm$ 1 SD (pfu/g lung) present in animals with recoverable virus at day 5 after inoculation, when peak viral growth was observed.

[d] $\chi^2 = 31.9$; $p < 0.001$ compared to air exposure (χ^2 analysis with one degree of freedom).

[e] $\chi^2 = 36.68$; $p < 0.001$ compared to air exposure.

Table 2. Inoculum Size and the Development of Pneumonitis

Exposure	Viral Inoculum[a]			
	10 pfu	10^2 pfu	10^3 pfu	10^4 pfu
Air	0/4	0/6	0/5	7/10
Peak viral titer in lung[b]	—[c]	—	—	$4.51 \pm 3.56 \times 10^3$
5 ppm NO_2	1/6	4/5[d]	6/6[e]	5/5
Peak viral titer in lung[b]	1.20×10^2	$5.75 \pm 0.49 \times 10^2$	$2.02 \pm 2.02 \times 10^4$	$2.0 \pm 2.1 \times 10^4$[f]

[a] Ratio of infected to inoculated animals (See Table 1 for experimental details).

[b] Mean viral titer ± 1 SD (pfu/g lung) in lung homogenates at peak viral replication (day 5) in animals with recoverable virus.

[c] — = no recoverable virus in any animals tested.

[d] $\chi^2 = 9.82$; $p < 0.005$ compared to air exposure (χ^2 analysis with one degree of freedom).

[e] $\chi^2 = 11.02$; $p < 0.001$ compared to air exposure.

[f] $t = 3.12$; $p = 0.036$ compared to peak viral titer in air-exposed animals receiving 10^4 pfu (Student's T-test for unpaired samples).

centrations of NO_2. A one-way analysis of variance demonstrated a significant difference in peak viral titer according to level of NO_2 exposure ($F = 4.01$; $p < 0.05$).

SUSCEPTIBILITY TO REINFECTION

The aim of these studies was to investigate the impact of NO_2 exposure during initial infection on the development of protective immunity for the test virus. Animals were initially infected with an inoculum of virus capable of producing infection in both air- and NO_2-exposed animals (10^4 pfu). Thirty days after the initial instillation of virus, animals were reinoculated with 10^5 pfu of the identical virus. In these studies, exposure to NO_2 occurred only during the period of initial viral infection. As shown in Table 3 and Appendix A, 11 of 20 animals exposed to 5 ppm NO_2 during the primary infection had virus recoverable from the lung after reinoculation with the test virus, whereas animals exposed to 1 or 2.5 ppm NO_2, or to air, routinely had no virus present. Susceptibility to reinfection of animals exposed to 5 ppm NO_2 was significantly different from the susceptibility of animals exposed to air, 1 ppm NO_2, or 2.5 ppm NO_2 ($\chi^2 = 17.6$; $p < 0.001$).

CHARACTERIZATION OF PRIMARY VIRAL INFECTION ASSOCIATED WITH EXPOSURE TO 5 PPM NITROGEN DIOXIDE AND INOCULATION OF 10^2 PFU MURINE CYTOMEGALOVIRUS

Histopathology

Light microscopy revealed no consistent abnormalities in the lung specimens obtained from uninfected animals exposed to either air or 5 ppm NO_2 (Appendix B). Air-exposed animals infected with a small viral inoculum (10^2 pfu) also exhibited no histologic abnormalities. In contrast, the lungs of some animals exposed to 5 ppm NO_2 and infected with 10^2 pfu showed signs of acute pneumonitis (Figure 2). Of 16 NO_2-exposed animals studied five days after inoculation with virus, seven exhibited no histologic abnormality, six demonstrated focal areas of alveolar consolidation, and three had focal areas of increased interstitial cellularity. Twelve animals were examined at day 10 after initiation of infection. In this group, five had normal histology, five had a focal alveolar pattern, and two had a focal interstitial pattern. In three of 28 infected animals exposed to 5 ppm NO_2, the alveolar and interstitial pattern was present at day 5 or day 10 in the same specimen. The interstitial pattern appeared to have no anatomic predilection, while the alveolar pattern was often found to be contiguous to medium and small airways in the basal lung segments.

Table 3. Reinfection of Animals Previously Inoculated with Murine Cytomegalovirus[a]

Exposure	Ratio of Infected to Inoculated Animals[b]	Viral Titer in Lung[c]
Air	1/22	1.35×10^3
NO_2		
1 ppm	0/7	None recovered
2.5 ppm	1/7	1.05×10^2
5 ppm	11/20[d]	$1.70 \pm 2.98 \times 10^3$

[a] Animals were exposed to NO_2 or air during primary infection with an inoculum of MCMV (10^4 pfu), which produced infection in all animals. Thirty to 40 days after initial instillation of virus, animals were reinoculated with MCMV (10^5 pfu).

[b] Infection was considered to be present if virus could be recovered from the lung either five or 12 days after reinoculation.

[c] Mean ± SD of virus recovered from all animals at either five or 12 days after reinoculation.

[d] $\chi^2 = 17.6$; $p < 0.001$ compared to the proportion of reinfected animals exposed to 1 and 2.5 ppm NO_2 (χ^2 analysis with one degree of freedom).

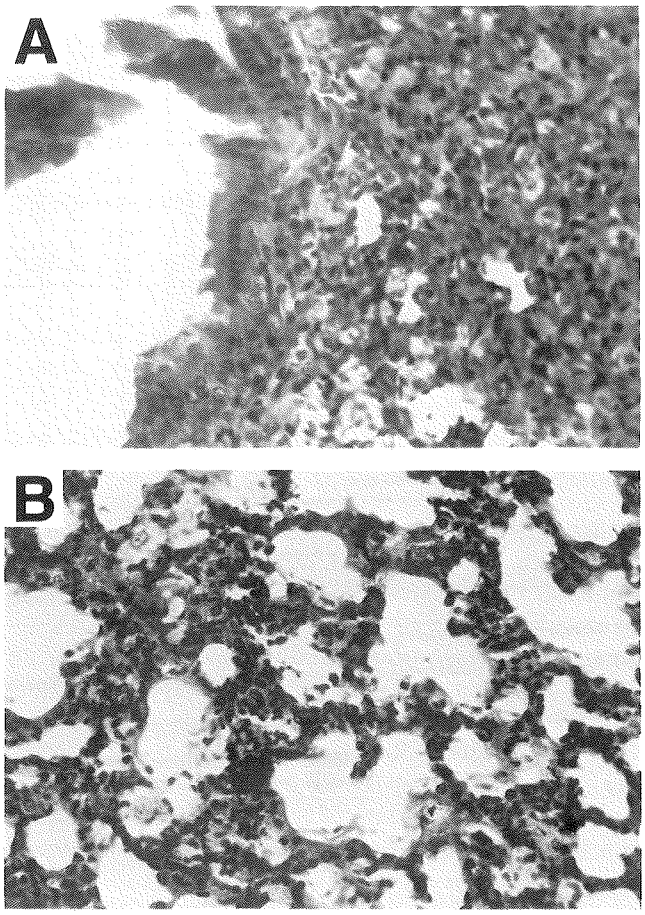

Figure 2. Lung histopathology in animals exposed to 5 ppm NO_2 during primary infection with 10^2 pfu virus. A: pattern of alveolar consolidation in close proximity to a medium-size airway (original magnification × 312.5). B: pattern of interstitial pneumonitis (original magnification × 312.5).

This pneumonitis appeared to heal without pathologic sequelae, since all six animals examined 15 days after initiation of infection had normal lung histology.

Bronchoalveolar Lavage

To further characterize the combined impact of 5 ppm NO_2 and infection with 10^2 pfu on the lower respiratory tract, the cellular and biochemical composition of bronchoalveolar lavage fluid was analyzed in animals studied within one hour of viral instillation (day 0), and was compared with fluid obtained from animals studied six days later (Figure 3 and Appendix C). Analysis of lavage cells demonstrated the presence of increased numbers of neutrophils in both air- and NO_2-exposed animals inoculated with the test virus. Analysis of biochemical parameters indicative of lung injury showed a modest rise in albumin concentration in lavage fluid obtained from infected and

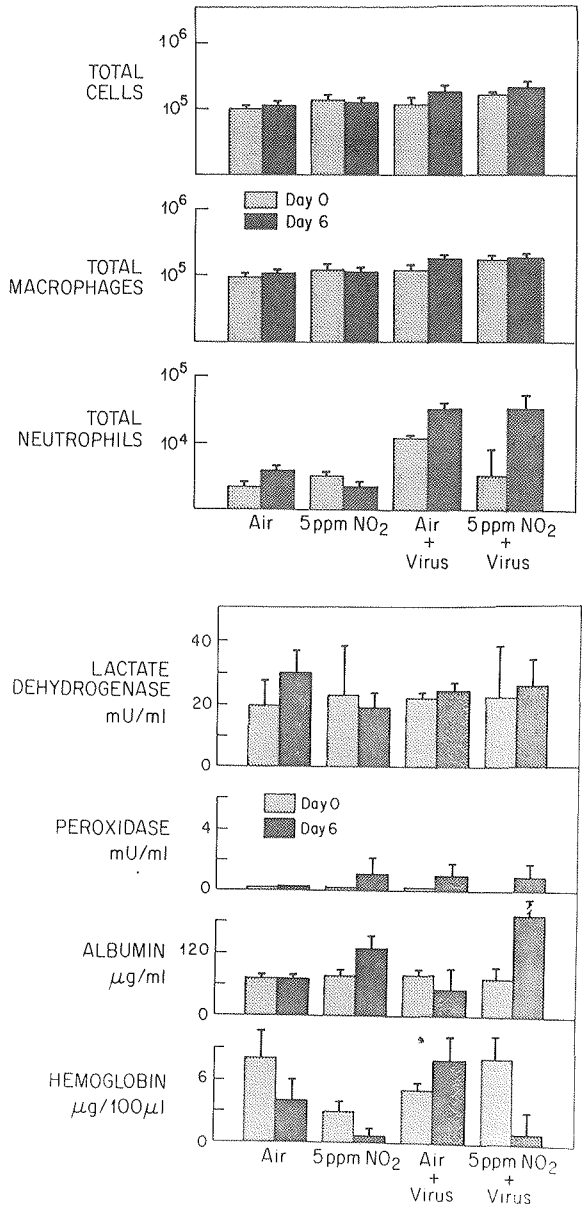

Figure 3. Bronchoalveolar lavage analysis in animals studied within one hour of inoculation with 10^2 pfu MCMV (NO_2- or air-exposed infected animals), or prior to initiation of exposure in uninfected animals, compared with findings from animals studied six days later. Each data point represents the mean ± SD for at least three animals studied separately. A: cellular analysis. Total number of cells retrieved in the pooled bronchoalveolar lavage is shown in the ordinate; the different exposure groups are shown on the abscissa. These data were analyzed by a two-way analysis of variance to test the independent and interactive effects of NO_2 exposure and virus inoculation on cell number at day 0 and day 6. A significant association was observed between these time points in the number of lavage fluid neutrophils present in air- and NO_2-exposed animals inoculated with virus ($F = 8.14$; $p < 0.05$). B: biochemical analysis. Biochemical indices, expressed as either concentration unit per ml of total bronchoalveolar lavage fluid recovered or μl of lysed cell pellet (hemoglobulin), are shown on the ordinate; the different exposure groups are shown on the abscissa. This data set was analyzed by a series of pairwise comparisons to determine the significance of differences between day 0 and day 6 within a given group. A significant difference was observed for the change in albumin concentration in NO_2-exposed, virus-infected animals ($t = 4.34$; $p = 0.049$).

uninfected animals exposed to 5 ppm NO_2. This implies the presence of a mild degree of injury to the pulmonary capillary membrane. A small amount of peroxidase was found in fluid obtained on day 6 from air-exposed infected animals, as well as from uninfected animals and infected animals exposed to NO_2. This reflects the release of this lysosomal enzyme from phagocytic cells. No consistent changes over time between groups were noted with respect to the concentration of lactate dehydrogenase (an intracellular enzyme of lung cells that is a marker of lung cell injury) or hemoglobin (a marker for the presence of pulmonary hemorrhage).

The lung histopathology and bronchoalveolar lavage analysis suggest that MCMV infection, produced here by combined exposure to 5 ppm NO_2 and inoculation with an amount of virus that is normally subinfectious for air-exposed animals, results in a mild pneumonitis that is not associated with evidence of substantial pulmonary parenchymal injury. Exposure to 5 ppm NO_2 alone for six days resulted in an increase in lavage fluid albumin, but no histopathologic evidence of inflammation or injury.

INVESTIGATION OF THE PATHOPHYSIOLOGY OF ENHANCED SUSCEPTIBILITY TO VIRAL INFECTION ASSOCIATED WITH NITROGEN DIOXIDE EXPOSURE

Macrophage Studies

To assess pulmonary macrophage phagocytic capacity in vivo, animals were exposed to either NO_2 or air for six hours on two consecutive days and were then inoculated intratracheally with ^{198}Au-colloid. The fraction of gold ingested by lung phagocytes in situ during a 90-minute incubation period was 0.85 ± 0.05 (mean ± SD) for four air-exposed animals, 0.74 ± 0.08 for four animals exposed to 1 ppm NO_2, and 0.55 ± 0.1 for five animals exposed to 5 ppm NO_2 ($p < 0.01$ for 5 ppm NO_2 compared to air, by Student's T-test for unpaired samples). Exposure to 15 ppm NO_2 resulted in a mean value of 0.72 ± 0.2 in five animals. This apparent improvement in pulmonary phagocyte capacity after exposure to a greater concentration of NO_2 may be related to the presence of additional phagocytic cells in the lower respiratory tract, as a consequence of an inflammatory response to 15 ppm NO_2. To account for the effect of lung phagocyte number on the fraction of ^{198}Au-colloid ingested in situ, this fraction can be expressed per 10^6 lung lavage cells: control, 1.566 ± 0.21; 1 ppm NO_2, 1.59 ± 0.29; 5 ppm NO_2, 0.97 ± 0.27; 15 ppm NO_2, 0.93 ± 0.23. Thus, a dose-dependent effect of NO_2 exposure on phagocytic capacity is observed in the range of 1 to 5 ppm; exposure to 15 ppm does not appear to cause further suppression of phagocytic function.

In spite of a diminished rate of ingesting ^{198}Au-colloidal gold, pulmonary macrophages are nevertheless capable of ingesting MCMV in vivo. As shown in Table 4, virus was found to be associated with macrophages retrieved from animals exposed to 5 ppm NO_2 at intervals ranging from two hours to 12 days after viral instillation. Macrophage-associated virus was not detected in any air-exposed animal that was given the same viral inoculum.

To clarify the relationship of this observation to macrophage antiviral capacity, the infectivity of MCMV for macrophages obtained from air-exposed and 5-ppm NO_2-exposed animals was assessed during in vitro infection of cells (Figure 4 and Appendix D). No difference in viral replication was observed between macrophages obtained from air- and NO_2-exposed animals. This suggests that any effect of NO_2 exposure on the intrinsic antiviral capacity of macrophages is either reversible or depends on factors present in the alveolar microenvironment. It is also possible that the design of this experiment, which assessed viral replication in adherent cells, selected "healthier" macrophages capable of adherence to plastic, and may not have tested those cells whose ability to attach to surfaces may have been impaired as a result of NO_2 exposure.

Macrophage expression of the Ia epitope, a marker for antigen-presenting capacity, was assessed in cells harvested by bronchoalveolar lavage during primary infection, as well as reinfection, with the virus (Table 5 and Appendix E). No difference was observed in macrophage expression of Ia during primary infection between animals exposed to 5 ppm NO_2 and animals exposed to air. However, a significant difference was found in Ia expression between NO_2- and air-exposed groups during reinfection with MCMV (day-1 $t = 4.2$, $p = 0.014$; day-5 $t = 4.42$, $p = 0.021$).

Lymphocyte Studies

The impact of NO_2 exposure on lymphocyte function was addressed by assessing the proliferative responses of

Table 4. Macrophage-Associated Virus After Instillation of a Large Viral Inoculum[a]

	Days After Inoculation[b]		
Exposure	0	5	12
Air	0/2	0/3	0/3
5 ppm NO_2	4/4 $4.3 \pm 2.0 \times 10^3$	4/4 $7.5 \pm 3.4 \times 10^2$	2/4 $4 \pm 1 \times 10$

[a] 10^5 pfu.

[b] Values given are the ratio of animals whose pulmonary macrophages had recoverable virus to the total number of animals studied, and mean ± SD viral pfu/10^5 macrophages.

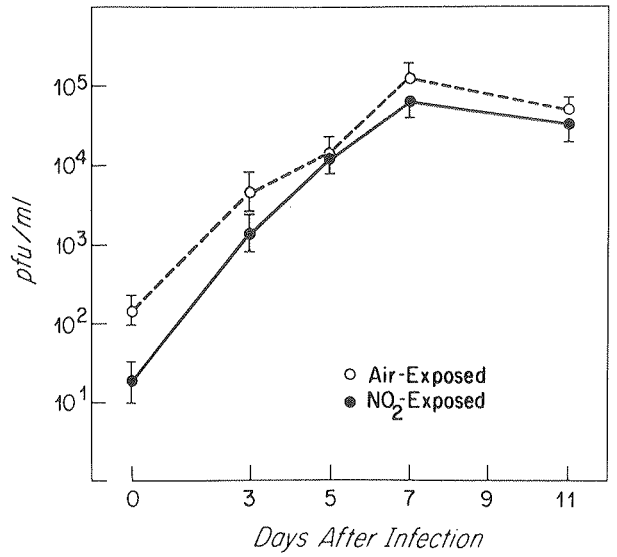

Figure 4. In vitro infection of pulmonary macrophages obtained from animals exposed to air or 5 ppm NO_2. Plaque-forming units of virus in culture supernatants are shown on the vertical axis; time after infection is shown on the horizontal axis.

Table 5. Pulmonary Macrophage Expression of the Ia Epitope

Exposure[a]	Days After Inoculation[b] 1	5	9
Primary Infection			
Air	4.01 ± 1.96 (n = 8)	3.34 ± 1.46 (n = 4)	2.63 ± 1.10 (n = 3)
5 ppm NO_2	4.84 ± 2.22 (n = 10)	2.07 ± 1.38 (n = 5)	2.98 ± 1.32 (n = 4)
Comparison of air to NO_2	NS[c]	NS	NS
Reinfection			
Air	5.5 ± 0.79 (n = 4)	10.02 ± 1.81 (n = 3)	
5 ppm NO_2	1.88 ± 1.52 (n = 4)	3.08 ± 2.02 (n = 3)	
Comparison of air to NO_2	t = 4.2 p = 0.014	t = 4.42 p = 0.021	

[a] Animals were exposed to 5 ppm NO_2 or air during primary infection with 10^4 pfu MCMV and were rechallenged with 10^5 pfu MCMV 30 days later (reinfection).

[b] Mean percent ± SD of pulmonary macrophages, lavaged from the lung at various days after viral instillation, that stain positively for Ia by indirect immunofluorescence. Uninfected air- or NO_2-exposed animals routinely had less than 2 percent Ia-positive cells.

[c] NS = not significant.

splenic lymphocytes, since the small numbers of this cell type normally present in the lung limit the availability of lung lymphocytes for study in the murine system. Splenic lymphocyte proliferative responses were studied after cells were exposed to lectins or MCMV antigen (Table 6 and Appendix F). Active viral infection depressed lectin-induced blastogenesis to similar degrees in air- and NO_2-exposed animals. Air-exposed immune animals (for example, those inoculated with virus 30 days before study) had evidence of a secondary or anamnestic response to the viral antigen, but this was not apparent in cells harvested from animals exposed to 5 ppm NO_2 during initial infection.

Humoral immune responses were studied by determining the level of virus-specific antibody present in the serum of animals studied between 30 and 45 days after primary infection. In all 11 air-exposed animals infected with 10^4 pfu virus, the serum antibody titer was 1:32 or greater. Similarly, all 12 animals exposed to 5 ppm NO_2 during primary infection and infected with the same amount of virus also had a titer of 1:32 or greater.

DISCUSSION

Our studies of mice indicate that short-term exposure to 5 ppm NO_2 increases the susceptibility of the lower respiratory tract to primary infection with a virus. In this experimental system, NO_2 appears to influence susceptibility to both primary infection and reinfection with the test virus.

During primary infection, differing susceptibility between NO_2- and air-exposed animals was manifested by a substantial difference in the size of the viral inoculum required to produce either virologic or pathologic evidence of lower respiratory tract infection. The inoculum that consistently produced evidence of viral infection during exposure to 5 ppm NO_2 was more than 100-fold lower than that needed to infect air-exposed animals. This finding suggests one potential mechanism to account for the increased frequency, reported in some studies, of acute respiratory illness in humans exposed to elevated levels of ambient NO_2: There may be an increased likelihood of developing infection from viral inocula that are too small to produce infection in individuals who are either unexposed to NO_2 or exposed to lower concentrations of NO_2.

Characterization of the infection produced by the combined effects of NO_2 and a normally subinfectious viral inoculum (10^2 pfu) demonstrated viral replication in the lung that was not associated with biochemical evidence of injury (Figure 3). Histologically, mild pneumonitis was observed in some animals. Shanley and Pesanti (1985) demonstrated that MCMV replication in the lung, per se, is usually not

Table 6. Splenic Lymphocyte Responses After Primary Infection[a]

	Phyto-hemag-glutinin[b] (5 μg/ml)	MCMV Antigen[b] 1:50	MCMV Antigen[b] 1:200
Air-Exposed			
Uninfected	6.60 ± 1.91	2.24 ± 0.16	1.06 ± 0.50
Infected	4.33 ± 2.18	1.90 ± 2.02	2.71 ± 4.36
Immune	3.39 ± 1.83	4.60 ± 2.44	3.57 ± 1.92
NO_2-Exposed[c]			
Uninfected	10.57 ± 2.46	1.12 ± 0.56	0.95 ± 0.34
Infected	5.89 ± 1.73	0.97 ± 0.59	1.23 ± 0.80
Immune	4.86 ± 2.17	0.97 ± 0.33[d]	0.93 ± 0.39[d]

[a] Lymphocytes were harvested from either uninfected animals (two days after exposure to NO_2 or air) or infected animals five days ("infected") or 30 days ("immune") after inoculation with virus. Cells from each animal (5×10^5) were incubated in triplicate wells with lectin or viral antigen for 48 hours prior to an overnight pulse with ^{3}H-thymidine. These conditions result in maximal lymphocyte stimulation in control cells.

[b] Mean ± SD stimulation index (ratio of cpm/ml in stimulated cultures to cpm/ml in unstimulated cultures). Each group represents pooled data for four to six animals.

[c] Nitrogen dioxide exposure occurred for six days during primary infection.

[d] $p < 0.01$ compared to air-exposed immune lymphocytes (two-way analysis of variance).

associated with pathologic evidence of lung damage; however, the combination of viral replication and host immunosuppression can lead to the development of interstitial pneumonitis. In the current studies, NO_2 exposure during the course of viral replication was sufficient to produce pathologic changes that resulted in pneumonia.

Exposure to NO_2 during primary viral infection also enhances susceptibility to reinfection with the same virus. This finding suggests that NO_2 exposure can modify the development of protective immunity that follows primary viral infection, and in so doing, may cause a predisposition to recurrent respiratory infection. Reinfection is a common mechanism for the development of upper and lower respiratory tract infection in early childhood (Henderson et al. 1979). The potential impact of NO_2 exposure on childhood reinfection with respiratory viruses could be another factor underlying the epidemiologic observations linking NO_2 exposure to increased frequency of acute respiratory illness. This possible relationship between NO_2 and susceptibility to reinfection is particularly intriguing in light of some human studies (Shy et al. 1970) that suggest that the influence of NO_2 exposure on the frequency of acute respiratory illness is greatest in the first few years of life—the period when reinfection with viruses is also seen.

The exposure threshold for the observed increased susceptibility to primary infection with the test virus was 5 ppm NO_2, and the ED_{50} was estimated to be 3.9 ppm NO_2. It is also possible that effects would be found at even smaller concentrations of NO_2 if the number of animals studied at these exposure levels were larger. Increased mortality from the combined effects of NO_2 and viral infection was seen at 15 ppm NO_2. These findings indicate that exposure to increasing concentrations of NO_2 has incremental effects on respiratory tract defenses against viruses. Lower-level exposure enhances susceptibility to mild pneumonitis, and exposure to higher concentrations leads to serious infection and mortality. If human responses to NO_2 are similar to those found in mice, these results predict that exposure to NO_2 near the suggested 24-hour air quality standard of 0.05 ppm (U.S. Environmental Protection Agency 1982) would either have no effect or would enhance susceptibility to episodes of mild viral infection. However, exposure to NO_2 at these levels seems unlikely to predispose immunocompetent humans at rest to serious infection. This hypothesis is consistent with epidemiologic data that link NO_2 exposure to increased frequency of self-limited respiratory illness. However, factors that affect the competence of local lung defenses, such as immunosuppressive drugs, or conditions that increase the pulmonary dose of NO_2, such as exercise (Bauer et al. 1986), could act in concert with exposure to ambient levels of NO_2 to produce more serious viral infections.

Can the NO_2 levels that in these studies altered susceptibility to viral infection in mice be directly extrapolated to predict human health risks? Anatomic and physiologic differences between the murine and human respiratory tracts may influence the local concentration of NO_2 at the alveolar level. Nevertheless, previous work with a related oxidant pollutant, ozone, demonstrated that these morphologic differences do not appear to influence the predicted tissue-dose deposition curves for the lung (Miller et al. 1978). This finding supports the validity of extrapolating from animal studies to humans for oxidant pollutants like NO_2.

Potential differences between the time course of NO_2 exposure employed in these studies and that associated with naturally occurring human exposure may be important. Human exposure typically occurs in brief peaks, while the levels used here were uniform and consistent over time. Recent work by Graham and associates (1987) and Miller and coworkers (1987) demonstrates that NO_2 peaks contribute substantially to the development of altered lung defenses against infection and to abnormal pulmonary function in exposed mice.

The exposure threshold for the increased susceptibility to virus observed in these studies, 5 ppm NO_2, is near the

range of peak NO_2 concentrations recorded in some home environments with gas stoves (Melia et al. 1978; Speizer et al. 1980). It is also noteworthy that exposure to this level of NO_2 produced few biochemical or cellular indicators of lung injury and inflammation in bronchoalveolar lavage fluid (Figure 3), and was not associated with histologic alterations under light microscopy (Figure 2). These findings suggest that altered respiratory tract susceptibility to viral infection is one of the most sensitive measures of exposure to NO_2.

Although previous animal studies have demonstrated a clear relationship between NO_2 exposure and bacterial infectivity (Jakab 1980), the relation of NO_2 exposure to viral infectivity is not well established. Earlier work that used influenza viruses to infect in a variety of animal species exposed acutely or chronically to NO_2 has shown either no effect (Lefkowitz et al. 1986), or enhanced mortality (Henry et al. 1970). Discrepancies between our studies and previous ones may result from differences in exposure patterns (the present study is one of the few to expose animals before and after the introduction of virus), in the viral agent used (the current study used an endogenous murine pathogen), and in the species of animal under study.

Our study indicates that alterations in pulmonary macrophage function may play a role in the enhanced susceptibility of NO_2-exposed animals to primary viral infection. The pulmonary macrophage is an important aspect of the lung's defense against primary infection with viruses. Previous work has confirmed the importance of this cell in the pathogenesis of MCMV infection in mice (Brautigam et al. 1979; Rose et al. 1982; Selgrade et al. 1982). For this reason, the impact of NO_2 exposure on pulmonary macrophages was investgated. Exposure to 5 ppm NO_2 depressed in vivo phagocytosis of ^{198}Au-colloid by macrophages and inhibited the ability of macrophages to eradicate the test virus after intratracheal inoculation (Table 4). This apparent virucidal defect could not, however, be demonstrated during in vitro infection of cells obtained by bronchoalveolar lavage from animals exposed to 5 ppm NO_2 (Figure 4). This suggests that any NO_2-induced alteration in macrophage virucidal capacity may depend on factors in the lung microenvironment, and is reversible.

These observations extend our understanding of the relation between NO_2 exposure and macrophage virucidal function. Valand and coworkers (1970) demonstrated that a three-hour exposure to 25 ppm NO_2 diminished the ability of rabbit macrophages to secrete interferon in response to a viral challenge. Similarly, parainfluenza-virus-infected rabbits exposed to 15 ppm NO_2 for three hours exhibited less resistance to subsequent inoculation with rabbitpox virus than did air-exposed animals (Acton and Myrvik 1972). Taken together with these studies, the current work suggests that NO_2 exposure may adversely affect some antiviral functions of the pulmonary macrophage. This does not exclude the possibility that NO_2 affects susceptibility to primary viral infection by alternative mechanisms. Natural killer cells have been shown to be an important host defense against systemic infection with MCMV (Selgrade et al. 1982). It may be that NO_2 exposure impairs the antiviral activity of natural killer cells in the respiratory tract; however, this hypothesis has not yet been addressed.

The ability to reinfect animals exposed to NO_2 during primary infection implies a deficiency in the development of virus-specific immunity. This does not appear to be attributable to alteration in humoral immunity, since serum MCMV antibody levels were comparable in NO_2- and air-exposed animals. This is consistent with previous work (Fenters et al. 1971) that demonstrated no effect of NO_2 exposure on the development of virus-specific antibody after influenza virus infection. Two key findings suggest that abnormal cellular immune function may be responsible for the lack of protective immunity in NO_2-exposed animals. First, and most importantly, splenic lymphocytes obtained from NO_2-exposed animals 30 days after primary infection failed to proliferate in response to MCMV antigen, whereas lymphocytes from air-exposed animals were fully capable of blastogenesis under these conditions (Table 6). This suggests that in spite of the known immunosuppressive effect of MCMV infection on cellular immunity (Loh and Hudson 1980), the effects of the virus itself could not be implicated as the sole cause of impaired lymphocyte responses in NO_2-exposed animals, since these responses were appropriate in the group of infected animals that were exposed to air alone.

Although splenic lymphocyte responses have been observed to be altered by NO_2 exposure in a variety of assay systems (Holt et al. 1979; Lefkowitz et al. 1986), these studies have not used microbial antigens to which control animals exhibit an immune response. For instance, Holt and colleagues (1979) found that lectin-induced blastogenesis by splenic lymphocytes was depressed during acute and chronic exposures to 10 ppm NO_2. On the other hand, exposure to considerably higher concentrations of NO_2 (26 ppm) actually increased antigen-specific lymphocyte stimulation after immunization with sheep red blood cells (Hillam et al. 1983). This may have resulted from increased antigen translocation through respiratory epithelium damaged by oxidant exposure (Schnizlein et al. 1980).

The ability of lymphocytes to proliferate after exposure to a "recall" antigen is an appropriate anamnestic response of the cellular immune system. This response is critical to the production of lymphocyte-derived antiviral defenses,

such as interferon-δ and cytotoxic T-cells. The failure of lymphocytes from NO_2-exposed animals to develop this virus-specific anamnestic response indicates that other parameters of cellular immunity may also be deficient. This was verified by the demonstration that macrophage expression of the Ia epitope is diminished in NO_2-exposed animals, compared to air-exposed animals, during reinfection with the test virus (Table 5). Since interferon-δ is the only known inducer of this epitope (Unanue and Allen 1987), it appears likely that local pulmonary production of interferon-δ is deficient during reexposure to MCMV. Studies are in progress to measure pulmonary levels of interferon directly to confirm this supposition.

In summary, these experiments demonstrate that, in mice, short-term exposure to 5 ppm NO_2 enhances the susceptibility of the lower respiratory tract to both primary infection and reinfection with a virus. This susceptibility is based, at least in part, on NO_2-associated alterations in pulmonary macrophage function and lymphocyte anamnestic responses to the viral antigen. Although the exposure threshold necessary to produce these effects on viral susceptibility is considerably greater than ambient levels of NO_2, exposure to this level of NO_2 alone produced no biochemical or histologic indications of major pulmonary injury. Therefore, exposure to a concentration of NO_2 that has no observable effects on lung structure is nevertheless capable of predisposing the respiratory tract to infection with a virus.

ACKNOWLEDGMENT

The authors gratefully acknowledge the help and support of Professor Joseph D. Brain.

REFERENCES

Acton JD, Myrvik QN. 1972. Nitrogen dioxide effects on alveolar macrophages. Arch Environ Health 24:48–52.

Bauer MA, Utell MJ, Morrow PE, Speers DM, Gibb FR. 1986. Inhalation of 0.30 ppm nitrogen dioxide potentiates exercise-induced bronchospasm in asthmatics. Am Rev Respir Dis 134:1203–1208.

Beck BD, Brain JD, Bohannon DE. 1982. An in vivo hamster bioassay to assess the toxicity of particulates for the lungs. Toxicol Appl Pharmacol 66:9-29.

Beller DI, Kiely JM, Unanue ER. 1980. Regulation of macrophage populations: I. Preferential induction of Ia-rich peritoneal exudates by immunological stimuli. J Immunol 124:1426–1430.

Brain JD, Corkery GC. 1977. The effect of increased particles on the endocytosis of radiocolloids by pulmonary macrophages in vivo: Competitive and toxic effects. In: Inhaled Particles IV (Walton WH, ed.). Pergamon Press, Oxford, England.

Brautigam AR, Dutko FJ, Olding LB, Oldstone MBA. 1979. Pathogenesis of MCMV infection: The macrophage as a permissive cell for cytomegalovirus infection, replication, and latency. J Gen Virol 44:349–359.

Ehrlich R. 1966. Effect of nitrogen dioxide on resistance to respiratory infection. Bacteriol Rev 30:604–614.

Ehrlich R, Henry MC. 1968. Chronic toxicity of nitrogen dioxide: I. Effect on resistance to bacterial pneumonia. Arch Environ Health 17:860–865.

Fenters JD, Ehrlich R, Findlay J, Spangler J, Tolkacz V. 1971. Serologic response in squirrel monkeys exposed to nitrogen dioxide and influenza virus. Am Rev Respir Dis 104: 448–451.

Ferris BG. 1978. Health effects of exposure to low levels of regulated air pollutants: A critical review. J Air Pollut Control Assoc 28:482–497.

Goldstein E, Eagle C, Hoeprich P. 1973. Effect of nitrogen dioxide on pulmonary bacterial defense mechanisms. Arch Environ Health 26:202–204.

Graham JA, Gardner DE, Blommer EJ, House DE, Menache MG, Miller FJ. 1987. Influence of exposure patterns of nitrogen dioxide and modifications by ozone on susceptibility to bacterial infectious disease in mice. J Toxicol Environ Health 21:113–125.

Henderson FW, Collier AM, Clyde WA Jr, Denny FW. 1979. Respiratory syncytial virus infections, reinfections, and immunity: A prospective, longitudinal study in young children. N Engl J Med 300:530–537.

Henry MC, Findlay J, Spangler J, Ehrlich R. 1970. Chronic toxicity of NO_2 in squirrel monkeys: III. Effect on resistance to bacterial and viral infection. Arch Environ Health 20:566–570.

Hillam RP, Bice DE, Hahn FF, Schnizlein CT. 1983. Effects of acute nitrogen dioxide exposure on cellular immunity after lung immunization. Environ Res 31:201–222.

Holt PG, Finlay-Jones LM, Keast D, Papadimitrou JM. 1979.

Immunological function in mice chronically exposed to nitrogen oxides (NO_x). Environ Res 19:154–162.

Jakab GJ. 1980. Nitrogen-dioxide-induced susceptibility to acute respiratory illness: A perspective. Bull NY Acad Med 56:847–855.

Jakab GJ. 1987. Modulation of pulmonary defense mechanisms by acute exposures to nitrogen dioxide. Environ Res 42:215–228.

Lefkowitz SS, McGrain JJ, Lefkowitz DL. 1986. Effects of NO_2 on immune responses. J Toxicol Environ Health 17: 241–248.

Loh L, Hudson JB. 1980. Immunosuppressive effect of murine cytomegalovirus. Infect Immun 27(1):54–60.

Melia RJW, Florey C du V, Altman DS, Swan AV. 1977. Association between gas cooking and respiratory disease in children. Br Med J 2:149–152.

Melia RJW, Florey C du V, Darby SC, Palmes ED, Goldstein BD. 1978. Differences in NO_2 levels in kitchens with gas or electric cookers. Atmos Environ 12:1379–1381.

Meltzer MS. 1981. Use of Wright's stain and cytocentrifuge preparations. In: Methods for Studying Mononuclear Phagocytes (Adams D, Edelson PJ, Koren H, eds.) pp. 345–347. Academic Press, Orlando, FL.

Miller FJ, Graham JA, Raub JA, Illing JW. 1987. Evaluating the toxicity of urban patterns of oxidant gases: II. Effects in mice from chronic exposure to nitrogen dioxide. J Toxicol Environ Health 21:99–112.

Miller FJ, Menzel DB, Coffin DL. 1978. Similarity between man and laboratory animals in regional pulmonary distribution of ozone. Environ Res 17:84–101.

Pearlman ME, Finklea JF, Creason JP, Shy CM, Young MM, Horton RJM. 1971. Nitrogen dioxide and lower respiratory illness. Pediatrics 47:391–398.

Reed LJ, Muench H. 1938. A simple method for estimating fifty percent endpoints. Am J Hyg 27:493–497.

Rose RM, Crumpacker C, Waner JL, Brain JD. 1982. Murine cytomegalovirus pneumonia: Description of a model and investigation of pathogenesis. Am Rev Respir Dis 125: 568–573.

Schnizlein CT, Bice DE, Rebar AH, Wolff RK, Beethe RL. 1980. Effect of lung damage by acute exposure to nitrogen dioxide on lung immunity in the rat. Environ Res 23: 362–370.

Selgrade MK, Daniels MJ, Wu PC, Miller FJ, Graham JA. 1982. Effects of immunosuppression with cyclophosphamide on acute murine cytomegalovirus infection and virus-augmented natural killer cell activity. Infect Immun 38: 1046–1055.

Shanley JD, Pesanti EL. 1985. The relation of viral replication to interstitial pneumonitis in murine cytomegalovirus lung infection. J Infect Dis 151:454–458.

Shy CM, Creason JP, Pearlman ME, McClain KE, Benson FB, Young MM. 1970. The Chattanooga schoolchildren study: Effects of community exposure to nitrogen dioxide. II. Incidence of acute respiratory illness. J Air Pollut Control Assoc 20:582–588.

Speizer FE, Ferris BG Jr, Bishop YMM, Spengler J. 1980. Respiratory disease rates and pulmonary function in children associated with NO_2 exposure. Am Rev Respir Dis 121:3-10.

Unanue ER, Allen PM. 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 236:551–557.

U.S. Environmental Protection Agency. 1982. Air Quality Criteria for Oxides of Nitrogen. EPA-600/18–82–026. Environmental Criteria and Assessment Office, Research Triangle Park, NC.

Valand SB, Acton JD, Myrvik QN. 1970. Nitrogen dioxide inhibition of viral-induced resistance in alveolar macrophages. Arch Environ Health 20:303–309.

APPENDIX A. Nitrogen Dioxide Susceptibility Studies

Table A.1. Virus Recovered in Lung Homogenates During Primary Infection[a]

Exposure	Viral Titer in Lung (pfu/g)
10^2 pfu Inoculum	
Air	
Day 5	1.25×10^3
	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
Day 12	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
1 ppm NO_2	
Day 5	0
	0
	0
Day 12	0
	0
	0
2.5 ppm NO_2	
Day 5	1.30×10^3
	2.00×10^3
	0
	0
	0
Day 10	0
	0
	0
	0
	0

(Table continues next column.)

Table A.1. (*continued*)

Exposure	Viral Titer in Lung (pfu/g)
5 ppm NO_2	
Day 6	1.85×10^3
	1.25×10^3
	0
	1.50×10^3
	1.66×10^3
	7.69×10^2
	4.87×10^2
	3.41×10^2
	4.10×10^3
	1.50×10^3
Day 12	7.40×10^2
	9.09×10^2
	0
	0
	0
10 ppm NO_2	
Day 5	1.81×10^3
	1.42×10^3
	7.14×10^2
Day 12	3.50×10^3
	2.85×10^3
	0
10^5 pfu Inoculum	
Air	
Day 5	6.00×10^3
	1.89×10^3
	2.30×10^3
	3.00×10^3
	1.00×10^3
1 ppm NO_2	
Day 5	1.50×10^2
	4.80×10^2
	3.50×10^3
	1.20×10^3
5 ppm NO_2	
Day 5	3.30×10^4
	9.50×10^4
	1.20×10^5
	3.60×10^5
	1.10×10^5
10 ppm NO_2	
Day 5	3.10×10^5
	6.45×10^5
	8.10×10^4
	6.89×10^4
15 ppm NO_2	
Day 5	6.90×10^5
	4.00×10^5
	3.70×10^5
	4.54×10^4

[a] Each data point represents the results obtained from the study of a single animal.

Table A.2. Exposure to 5 Parts per Million Nitrogen Dioxide or Air and Infection with Varying Quantities of Murine Cytomegalovirus[a]

Exposure	Viral Titer in Lung (pfu/g)
1.2×10 pfu Inoculum	
Air	
Day 5	0
	0
	0
Day 12	0
	0
	0
NO_2	
Day 5	1.00×10^2
	0
	0
Day 12	0
	0
	0
1.2×10^2 pfu Inoculum	
Air	
Day 5	0
	0
	0
	0
Day 12	0
	0
NO_2	
Day 5	6.25×10^2
	0
	5.26×10^2
Day 12	1.40×10^2
	8.00×10^2
	1.20×10^2
1 to 1.75×10^3 pfu Inoculum	
Air	
Day 5	0
	0
	0
Day 12	0
	0
	0
NO_2	
Day 5	5.50×10^4
	1.60×10^4
	1.60×10^3
	8.50×10^3
Day 12	1.30×10^3
	2.80×10^3
1.0 to 2.0×10^4 pfu Inoculum	
Air	
Day 5	0
	3.15×10^2
	1.50×10^3
	0
	7.69×10^2
	0
	4.20×10^3
	6.50×10^3
	1.00×10^4
	8.30×10^3
NO_2	
Day 5	8.54×10^4
	7.77×10^4
	5.26×10^3
	1.20×10^5
	3.80×10^4

[a] Each data point represents the results obtained from the study of a single animal.

Table A.3. Virus Recovered in Lung Homogenates During Reinfection[a]

Exposure	Viral Titer in Lung (pfu/g)
Air	
Day 5	0
	0
	0
	0
	0
	0
	0
	0
Day 12	1.35×10^3
	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
NO_2	
1 ppm	
Day 5	0
	0
	0
	0
Day 11	0
	0
	0

(*Table continues next column.*)

Table A.3. (*continued*)

Exposure	Viral Titer in Lung (pfu/g)
2.5 ppm	
Day 5	1.05×10^2
	0
	0
	0
Day 11	0
	0
	0
5 ppm	
Day 5	1.50×10^3
	1.00×10^4
	1.20×10^2
	1.40×10^2
	1.00×10^2
	1.00×10^2
	0
	8.50×10^2
	1.50×10^3
	1.00×10^3
Day 12	0
	0
	0
	0
	0
	0
	0
	0
	1.40×10^2
	3.00×10^2

[a] Each data point represents the results obtained from the study of a single animal.

APPENDIX B. Histology

Exposure	Total Number of Animals Examined	Predominant Pattern[a]		
		Normal	Focal Alveolar	Focal Interstitial
Air				
Uninfected	12			
Day 5		4		
Day 10		4		
Day 15		4		
Infected with 10^2 pfu MCMV	14			
Day 5		5		
Day 10		6		
Day 15		3		
NO_2				
Uninfected	19			
5 ppm				
Day 5		5		
Day 10		5		1
Day 15		7	1	
Infected with 10^2 pfu MCMV	13			
2.5 ppm				
Day 5		5		
Day 10		4		1
Day 15		3		
5 ppm	34			
Day 5		7	6	3
Day 10		5	5	2
Day 15		6	0	0

[a] The predominant histologic pattern was determined by examining multiple, horizontally cut sections from the apex to base of either the right or left lung from each animal. A focal alveolar pattern represents the accumulation of acute or chronic inflammatory cells, or both, in the alveolar spaces (Figure 2a). A focal interstitial pattern represents the presence of increased cellularity or thickness, or both, of the interstitial space in a focal distribution (Figure 2b). A diffuse interstitial process represents increased cellularity or thickness, or both, of the interstitial space with a diffuse pattern. Between five and 10 sections were examined for each animal. Each animal was assessed and recorded separately.

APPENDIX C. Bronchoalveolar Lavage Analysis

Table C.1. Cellular Analysis[a]

Exposure	Total Cells ($\times 10^5$)	Total Macrophages ($\times 10^5$)	Total Neutrophils ($\times 10^5$)
Uninfected			
Air			
Day 0[b]	1.00	0.95	0.05
	1.20	1.16	0.04
	0.83	0.825	0.05
	0.90	0.90	0.00
	(0.98 ± 0.13)[c]	(0.95 ± 0.12)	(0.035 ± 0.02)
Day 6	1.80	1.68	0.12
	0.83	0.82	0.01
	0.73	0.70	0.03
	1.20	1.10	0.10
	(1.14 ± 0.42)	(1.075 ± 0.37)	(0.065 ± 0.04)
5 ppm NO_2			
Day 0	3.60	3.60	0.00
	0.88	0.72	0.16
	0.92	0.92	0.00
	1.45	1.39	0.06
	(1.70 ± 1.10)	(1.65 ± 1.10)	(0.055 ± 0.06)
Day 6	0.89	0.78	0.11
	1.65	1.00	0.65
	2.50	1.85	0.65
	1.40	1.30	0.10
	(1.61 ± 0.58)	(1.23 ± 0.40)	(0.37 ± 0.27)
Infected with 10^2 pfu MCMV			
Air			
Day 0	1.80	1.80	0
	2.40	2.20	0.20
	1.40	1.20	0.20
	0.90	0.85	0.05
	(1.625 ± 0.54)	(1.50 ± 0.52)	(0.11 ± 0.08)
Day 6	1.16	0.76	0.39
	3.60	2.80	0.80
	4.00	3.60	0.40
	2.50	2.00	0.50
	(2.81 ± 1.10)	(2.29 ± 1.04)	(0.52 ± 0.16)
5 ppm NO_2			
Day 0	2.00	1.90	0.10
	1.20	1.10	0.10
	3.37	3.37	0
	1.50	1.50	0
	(2.01 ± 0.83)	(1.96 ± 0.85)	(0.54 ± 0.05)
Day 6	3.40	2.70	0.70
	2.27	1.50	0.77
	1.00	0.70	0.30
	4.80	4.40	0.40
	(2.86 ± 1.40)	(2.32 ± 1.39)	(0.54 ± 0.19)

[a] Each data point represents the results obtained from the study of a single animal.

[b] Day 0 refers to animals studied just prior to initiation of NO_2 exposure or viral inoculation. Day 6 refers to animals studied six days after initiation of NO_2 exposure alone, or six days after viral inoculation for infected animals exposed to either air or NO_2.

[c] Numbers in parentheses show mean ± SD.

Table C.2. Biochemical Analysis[a]

Exposure	Lactate Dehydrogenase (mU/ml)	Peroxidase (mU/ml)	Albumin (μg/ml)	Hemoglobin (μg/100 μg)
Uninfected				
Air				
Day 0[b]	24.40	0	79.00	6.10
	6.40	0	68.20	18.80
	29.64	0	56.08	0
	(20.14 ± 9.90)[c]	(0)	(67.76 ± 9.36)	(8.30 ± 7.83)
Day 6	27.90	0	62.00	0
	21.20	0	62.00	4.90
	41.90	0	83.00	6.20
	(3.033 ± 8.60)	(0)	(69.00 ± 9.80)	(3.70 ± 2.60)
5 ppm NO_2				
Day 0	44.61	0	78.80	4.67
	12.00	0	67.28	1.50
	12.50	0	83.23	2.80
	(23.03 ± 15.25)	(0)	(76.43 ± 6.72)	(2.99 ± 1.30)
Day 6	12.80	0.63	75.20	0
	23.00	0	110.60	0
	24.00	0	181.00	1.90
	17.40	4.10	ND[d]	0
	(19.30 ± 4.50)	(1.18 ± 1.70)	(122.26 ± 43.90)	(0.475 ± 0.82)
Infected with 10^2 pfu MCMV				
Air				
Day 0	21.05	0	85.40	4.87
	24.00	0	68.50	6.20
	21.00	0	70.00	4.90
	(22.01 ± 1.40)	(0)	(74.63 ± 7.63)	(5.32 ± 0.62)
Day 6	27.00	2.00	70.00	14.30
	26.00	0	83.00	6.83
	22.00	0	62.00	5.60
	22.00	1.60	62.40	5.05
	(24.25 ± 2.27)	(1.15 ± 1.25)	(54.35 ± 30.90)	(7.93 ± 3.72)
5 ppm NO_2				
Day 0	51.80	0	86.40	12.29
	14.80	0	45.90	6.80
	11.70	0	60.80	
	(22.76 ± 21.21)	(0)	(64.36 ± 16.72)	(8.03 ± 3.10)
Day 6	37.90	0	181.60	0
	26.00	0.75	146.80	3.60
	16.30	2.00	235.00	0
	(26.73 ± 8.80)	(0.91 ± 0.82)	(187.80 ± 36.27)	(1.20 ± 1.69)

[a] Each data point represents the results obtained from the study of a single animal.

[b] Day 0 refers to animals studied just prior to initiation of NO_2 exposure or viral inoculation. Day 6 refers to animals studied six days after initiation of NO_2 exposure alone, or six days after viral inoculation for infected animals exposed to either air or NO_2.

[c] Numbers in parentheses show mean ± SD.

[d] ND = not detected.

APPENDIX D. Serologic Studies[a]

Exposure	Total Number of Animals Examined	Result[b] –	Result[b] +
Uninfected	3		
Air		3	
Infected with 10^2 pfu MCMV	10		
Air			5
5 ppm NO_2			5
Infected with 10^4 pfu MCMV	23		
Air			11
5 ppm NO_2			12

[a] These studies were performed on serum obtained from animals between 30 and 45 days after viral inoculation.

[b] Results of antibody studies performed by the ELISA method. A positive result corresponds to an antibody titer of 1:32 or greater. In all instances except one, a positive antibody study was confirmed by indirect immunofluorescence antibody staining. A negative result reflects the absence of a specific antibody in a serum sample.

APPENDIX E. Pulmonary Macrophage Expression of the Ia Epitope[a]

Exposure	Days After Inoculation 1	5	9
Air			
Uninfected	1.20	1.93	0.77
	1.35	0	1.20
	0	0	
	0		
Infected with 10^4 pfu MCMV	4.10	4.00	3.60
	7.30	4.63	1.44
	2.75	1.26	2.86
	5.60	3.45	
	4.80		
	2.53		
	1.00		
	4.00		
Reinfected with 10^4 to 10^5 pfu MCMV	6.60	10.57	
	5.20	8.00	
	5.40	11.50	
	4.80		
5 pm NO_2			
Uninfected	1.40	0	0.53
	0.50	3.14	0
	0	1.46	
Infected with 10^4 pfu MCMV	2.10	0.89	1.25
	5.50	2.00	4.30
	4.19	4.37	2.70
	3.06	1.96	3.65
	4.30	1.12	
	5.50		
	1.50		
	7.00		
	8.30		
	6.90		
Reinfected with 10^4 to 10^5 pfu MCMV	0	1.49	
	2.50	5.36	
	3.60	2.40	
	1.45		

[a] Data represent the percentage of cells staining positively for Ia out of 200 cells examined. Each data point represents the results from a single animal.

APPENDIX F. Lymphocyte Proliferative Responses

Table F.1. Primary Infection[a]

Exposure	Concanavalin A	Phytohemagglutinin		Mock Antigen[b]		MCMV Antigen				
	1 µg/ml	2.5 µg/ml	5 µg/ml	1:50	1:200	1:10	1:50	1:100	1:200	1:500
Air										
Uninfected	4.39	5.14	5.75	0.67	0.81	1.63	2.05	1.30	1.92	1.40
	7.58	8.10	9.57	0.91	0.60	1.53	1.07	0.90	0.67	0.52
	3.74	5.00	8.43	1.11	0.54	2.16	1.93	1.15	0.81	1.21
	3.80	3.67	5.05	1.53	0.75	5.42	2.05	1.44	1.09	1.12
	3.90	4.43	5.77	0.60	1.72	1.57	1.15	0.77	0.83	0.82
	6.74	5.42	5.07							
	(5.02 ± 1.69)[c]	(5.28 ± 1.50)	(6.60 ± 1.91)	(0.88 ± 0.55)	(0.88 ± 0.47)	(2.46 ± 1.67)	(2.24 ± 1.60)	(1.12 ± 0.27)	(1.06 ± 0.50)	(1.01 ± 0.34)
Infected	9.80	1.79	2.04	2.34	1.99	4.24	6.02	6.11	11.62	3.72
	17.86	4.20	4.49	0.79	0.55	2.99	1.22	0.92	0.87	0.96
	7.99	1.70	1.72	1.08	0.99	1.00	0.63	0.85	0.93	0.82
	11.70	3.56	4.29	1.22	1.06	1.30	1.12	1.49	1.18	1.17
	17.79	3.67	6.63	1.21	0.82	1.98	1.19	0.86	0.85	0.66
	27.00	5.94	6.86	1.65	0.92	1.47	1.24	1.16	0.83	0.92
	(15.36 ± 7.01)	(3.47 ± 1.58)	(4.33 ± 2.18)	(1.38 ± 0.54)	(1.05 ± 0.49)	(2.16 ± 1.23)	(1.90 ± 2.02)	(1.89 ± 2.07)	(2.71 ± 4.36)	(1.37 ± 1.16)
Immune	18.90	4.29	4.87	1.57	1.16	2.36	6.01	4.45	5.99	3.00
	48.44	2.56	4.60	0.96	0.75	1.36	7.04	1.68	3.99	2.96
	23.13	2.55	3.24	1.10	1.21	2.65	1.54	0.85	1.42	0.92
	14.86	2.83	3.86	0.78	0.98	2.58	3.84	3.05	2.89	1.98
	(23.83 ± 17.41)	(2.55 ± 1.41)	(3.39 ± 1.83)	(1.1 ± 0.33)	(1.025 ± 0.20)	(2.23 ± 0.59)	(4.60 ± 2.44)	(2.50 ± 1.58)	(3.57 ± 1.92)	(2.21 ± 0.98)
5 ppm NO_2										
Uninfected	15.49	8.92	9.36	1.19	0.69	1.28	0.65	0.96	1.01	0.88
	10.51	6.33	8.54	2.17	1.04	1.96	1.70	1.34	0.99	1.24
	6.57	5.45	12.30	0.50	0.39	0.63	0.41	0.42	0.33	0.32
	8.33	8.78	8.52	1.05	0.80	1.94	1.63	0.77	0.87	0.67
	7.40	11.61	14.73	1.06	0.60	3.08	1.54	1.10	1.34	1.34
	10.68	5.75	9.97	1.16	1.09	1.81	0.80	1.23	1.19	1.13
	(9.83 ± 3.22)	(7.80 ± 2.39)	(10.57 ± 2.46)	1.18 ± 0.54)	(0.76 ± 0.266)	(1.78 ± 0.81)	(1.12 ± 0.56)	(0.97 ± 0.33)	(0.95 ± 0.34)	(0.93 ± 0.38)
Infected		3.31	4.37	1.69	1.36	1.67	0.69	1.01	1.36	0.86
	14.41	3.37	3.93	1.00	0.83	0.68	2.09	2.55	2.77	2.02
	17.03	3.95	5.31	1.40	0.74	1.71	0.96	0.77	0.82	0.58
	19.10	5.53	8.56	1.38	0.85	1.13	0.95	1.07	1.11	0.64
	16.72	6.13	6.12	1.06	0.97	1.07	0.85	0.92	0.84	0.68
	24.86	6.66	7.06	1.17	0.72	1.32	0.31	0.54	0.53	0.37
	(17.53 ± 4.16)	(4.82 ± 1.46)	(5.89 ± 1.73)	(1.28 ± 0.25)	(0.91 ± 0.23)	(1.26 ± 0.59)	(0.97 ± 0.59)	(1.14 ± 0.71)	(1.23 ± 0.80)	(0.85 ± 0.59)
Immune	36.76	3.62	5.80	1.14	0.93	1.70	1.01	1.27	1.52	0.92
	50.00	2.33	2.92	0.88	0.34	0.79	0.66	1.02	0.76	0.69
	40.88	5.79	7.49	0.83	0.63	2.39	0.79	1.89	0.66	0.65
	26.40	2.80	3.24	0.75	0.64	1.89	1.43	1.18	0.78	0.60
	(36.51 ± 9.78)	(3.63 ± 1.53)	(4.86 ± 2.17)	(0.90 ± 0.16)	(0.63 ± 0.24)	(1.44 ± 0.75)	(0.97 ± 0.33)	(1.34 ± 0.38)	(0.93 ± 0.39)	(0.71 ± 0.14)

[a] Each data point represents the results obtained from the study of a single animal.

[b] Mock antigen was prepared in a fashion identical to that described for MCMV antigen, except uninfected mouse cells were used.

[c] Numbers in parentheses show mean ± SD.

Table F.2. Reinfection[a]

Exposure	Concanavalin A	Phytohemagglutinin		Mock Antigen		MCMV Antigen				
	1 μg/ml	2.5 μg/ml	5 μg/ml	1:50	1:200	1:10	1:50	1:100	1:200	1:500
Air										
Day 5	26.44	4.48	5.20	1.23	0.88	2.45	1.46	0.95	0.93	0.88
	8.88	0.81	1.46	1.24	1.38	1.21	0.61	0.84	1.07	0.42
	24.56	3.87	4.59	1.68	1.57	2.01	2.39	2.41	2.43	2.12
	(19.96 ± 9.64)[b]	(3.05 ± 1.96)	(3.75 ± 2.0)	(1.38 ± 0.25)	(1.27 ± 0.35)	(1.89 ± 0.62)	(1.48 ± 0.89)	(1.4 ± 0.87)	(1.47 ± 0.82)	(1.14 ± 0.87)
Day 11	12.52	2.88	3.60	0.98	1.32	1.36	1.23	1.29	1.30	1.81
	12.26	1.59	1.78	1.17	1.46	5.71	1.67	2.24	1.87	1.18
	50.27	3.32	5.09	2.34	3.04	32.29	19.47	30.34	17.80	9.90
	(25.01 ± 21.87)	(2.59 ± 0.89)	(3.49 ± 1.65)	(1.49 ± 0.73)	(1.94 ± 0.95)	(13.12 ± 16.74)	(7.45 ± 10.4)	(11.29 ± 16.50)	(6.99 ± 9.36)	(4.29 ± 4.86)
5 ppm NO_2										
Day 5	14.37	2.76	3.18	1.21	1.06	1.13	1.03	1.42	1.21	0.91
	13.00	3.51	3.59	1.15	1.12	1.09	1.63	1.23	1.00	0.86
	26.16	3.64	5.01	0.82	0.82	2.16	0.79	1.09	0.72	0.46
	(17.84 ± 7.23)	(3.30 ± 0.47)	(3.92 ± 0.96)	(1.06 ± 0.21)	(1.0 ± 0.15)	(1.46 ± 0.6)	(1.15 ± 0.43)	(1.24 ± 0.16)	(0.97 ± 0.24)	(0.74 ± 0.24)
Day 11	24.74	5.21	5.20	0.78	1.07	1.83	1.48	1.52	1.39	0.80
	18.20	2.65	2.64	0.78	0.98	5.18	2.12	2.09	2.17	1.46
	18.70	4.39	5.01	1.22	1.18	3.08	1.05	1.32	1.23	0.78
	(20.54 ± 3.64)	(4.08 ± 1.30)	(4.28 ± 1.42)	(0.92 ± 0.25)	(1.07 ± 0.1)	(3.36 ± 1.69)	(1.55 ± 0.53)	(1.64 ± 0.39)	(1.59 ± 0.50)	(1.01 ± 0.38)

[a] Each data point represents the results obtained from the study of a single animal.

[b] Numbers in parentheses show mean ± SD.

ABOUT THE AUTHORS

Richard M. Rose, M.D., is Director of the Pulmonary and Critical Care Medicine Division of the New England Deaconess Hospital in Boston, MA. He is currently an Assistant Professor of Medicine at Harvard Medical School. His research activities include studies on the role of cytokines and macrophages in viral pathogenesis, and the effects of oxidant gases on respiratory tract defenses against infectious agents.

Paula Pinkston, M.D., is a member of the Pulmonary and Critical Care Medicine Division of New England Deaconess Hospital and an Instructor in Medicine at Harvard Medical School. She has an active research interest in lung immunology, dating back to fellowship training in this area at the National Institutes of Health.

William A. Skornik, M.S., is a Research Associate in Physiology at the Harvard School of Public Health. He has a long-term interest in inhalation toxicology and has made many published contributions to this field.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Rose RM, Fuglestad JM, Skornik WA, Hammer SM, Wolfthal SF, Beck BD, Brain JD. 1988. The pathophysiology of enhanced susceptibility to murine cytomegalovirus respiratory infection during short-term exposure to 5 ppm nitrogen dioxide. Am Rev Respir Dis 137:912–917.

INTRODUCTION

In the summer of 1983, the HEI issued a Request for Applications (RFA 83-2) soliciting proposals on "Nitrogen Oxides and Susceptibility to Respiratory Infections." In September 1983, Dr. Richard M. Rose (Principal Investigator) of the New England Deaconess Hospital, Boston, MA, proposed a project entitled "Nitrogen Oxides and Susceptibility to Viral Respiratory Infection." The HEI asked Dr. Rose to modify his proposal and, in June 1984, approved the three-year project. Total expenditures of $469,955 were authorized. The project began in July 1984, and the final report was accepted by the Health Review Committee in April 1988. The Health Review Committee's Report is intended to place the Investigators' Report in perspective as an aid to the sponsors of the HEI and to the public.

THE CLEAN AIR ACT

The U.S. Environmental Protection Agency (EPA) sets standards for motor vehicle emissions of oxides of nitrogen (and other pollutants) under Section 202 of the Clean Air Act, and for ambient levels of nitrogen dioxide under Section 109.

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

The determination of the appropriate standards for emissions of oxides of nitrogen depends in part on an assessment of the risks to health that they present. Of the oxides of nitrogen, nitrogen dioxide has been of most concern, and its effect on health has been the focus of much research. A study of the effects of nitrogen dioxide on respiratory infection in an animal model can contribute knowledge useful in making the evaluations of probable health effects in humans that are an important part of informed regulatory decision-making under Section 202.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards. The current standards include primary and secondary standards for nitrogen dioxide. Those standards were last reviewed in 1985. Also, under Section 166 of the Act, the EPA, in February 1988, published regulations to prevent significant deterioration of air quality due to emissions of oxides of nitrogen. Research of the type described here can increase understanding of the effects of nitrogen dioxide on lung tissue, and thus contribute indirectly to the assessment of the appropriateness of the existing standards and of ongoing and future regulatory initiatives.

BACKGROUND

The nitrogen dioxide that is present in urban environments is derived largely from vehicular sources. Morning rush-hour traffic generates high concentrations of nitric oxide which, in the presence of oxygen and sunlight, are converted to nitrogen dioxide (Ehrlich 1980). The current one-hour National Ambient Air Quality Standard for nitrogen dioxide is 0.053 parts per million (ppm) averaged over one year. Although the nitrogen dioxide standard is being met generally, typical long-term ambient concentrations of nitrogen dioxide range from 0.001 ppm in isolated rural areas to hourly peaks in urban areas that can exceed 0.3 ppm (U.S. Environmental Protection Agency 1985). Nitrogen dioxide generated indoors by appliances such as gas stoves reaches average levels of 0.025 ppm, although peaks as high as 0.2 to 0.4 ppm may be reached (Samet et al. 1987). Nitrogen dioxide is known to cause serious biologic effects at high levels of exposure. There is also evidence to suggest that exposure to nitrogen dioxide leads to increased susceptibility to respiratory infections (see reviews by U.S. Environmental Protection Agency 1982; Morrow 1984; Samet et al. 1987).

The lung disposes of particulates and microorganisms through the cooperative efforts of various components of host defense mechanisms. Such mechanisms include transport mechanisms for clearance of foreign particles; phagocytic cell functions (alveolar macrophages and polymorphonuclear leukocytes); and lymphocyte cell functions (T-cells and B-cells) (Green et al. 1977; Green 1984). Each of these mechanisms is a complex and multicomponent process. Cilia-mediated transport physically removes particles, including bacteria and viruses, from the trachea and bronchi. In the nonciliated regions of the lung, phagocytic cells are the major line of defense. Alveolar macrophages have multiple roles—phagocytosis, antigen presentation, and T-cell stimulation. Lymphocytes participate in the disposal of deposited antigens and consist of two main types with different functions. Activated B-cells undergo rapid proliferation and secrete immunoglobulins. T-cells regulate cell-mediated responses (delayed hypersensitivity, graft rejec-

tion, cell destruction), and secrete cell function modulators (interleukines, interferons).

Oxidants, such as nitrogen dioxide, penetrate beyond the nasopharyngeal region, and can alter the normal functioning of the major pulmonary defenses (Gardner 1984). Data from animal studies suggest that such alterations of pulmonary defenses may make the lung vulnerable to secondary insults, such as infection (Gardner 1982; Pennington 1988).

Very few clinical studies on the effects of nitrogen dioxide on respiratory infections have been performed, primarily because of ethical and practical considerations (see, for example, Kulle and Clements 1988). A number of epidemiological studies have investigated this relationship; however, taken as a whole, the results of some epidemiological studies have suggested an association between nitrogen dioxide and respiratory illness, although others have not (see reviews by Samet 1987 and Pennington 1988). The relation between nitrogen dioxide exposure and respiratory infection has been explored in both in vivo and in vitro animal systems. The animal studies have shown that nitrogen dioxide does affect host defenses, although the mechanism of action is still not settled.

In vivo infectivity models have been developed that test the ability of oxidant gas exposures to affect susceptibility to respiratory infections. Such studies evaluate, among other endpoints, mortality and bactericidal activity (the capacity to kill bacteria in the lungs). Mortality studies in several animal species have documented decreased survival when animals were exposed to bacterial or viral strains in conjunction with an excess of 5 ppm nitrogen dioxide (Henry et al. 1970; Fenters et al. 1971; Ehrlich and Fenters 1973; McGrath and Oyervides 1985). Studies of bactericidal activity have shown impaired killing of bacteria by lung phagocytes in animals exposed to levels of nitrogen dioxide above 3.8 ppm (reviewed by Jakab 1980).

Specific components of the lung defense system have also been investigated to evaluate the effect of nitrogen dioxide on the course of infection (Pennington 1988). Much of the work has focused on macrophages, through evaluation of the physiological functions of macrophages, or measurement of levels of enzymes that are secreted either by macrophages directly or by macrophage-activated T-cells or B-cells.

Macrophages isolated from rabbits exposed for two hours daily to 0.3 or 1.0 ppm nitrogen dioxide over two weeks exhibited accelerated particle clearance, decreased phagocytic capacity, and decreased macrophage mobility compared to air-exposed animals (Vollmuth et al. 1986; Schlesinger 1987). Mochitate and colleagues (1986) noted both mildly enhanced activities of certain metabolic enzymes of the macrophage (such as glucose-6-phosphate dehydrogenase and glutathione peroxidase of the peroxidative metabolic pathway, and succinate-cytochrome c reductase of the mitochondrial respiratory system) and increased macrophage numbers when rats were exposed to 4 ppm nitrogen dioxide for 10 days. Twenty-four-hour exposure of rats to 10 ppm nitrogen dioxide did not affect subsequent phagocytosis by macrophages in culture, although exposures as high as 25 ppm were found to reduce phagocytosis (Katz and Laskin 1975). Amoruso and coworkers (1981) found dose-dependent decreases in superoxide anion radical production in alveolar macrophages isolated from rats exposed to 6.1 to 17.0 ppm nitrogen dioxide (for two to three hours). Superoxide anion radical production is implicated as playing a role in antibacterial activities.

Other investigators have focused on the effects of nitrogen dioxide on immunoglobulins and other biochemical immune factors. Mice exposed to 2 ppm nitrogen dioxide for 12 weeks showed a decrease in serum immunoglobulin A and a decrease in immunoglobulin G_1; IgM and IgG_2 were unaffected (Ehrlich et al. 1975). Lefkowitz and coworkers (1984) found no significant changes in interferon levels, a measure of immune system responsiveness, of mice after 5.0 ppm exposure for seven days. Thus, the experiments on defense mechanisms indicate that nitrogen dioxide affects specific components of the immunological system.

Unlike the animal models frequently used, however, the origin of human respiratory infections is most commonly viral. In addition, human respiratory infections generally result in morbidity, rather than mortality. Very little information is available on the combined effect of nitrogen dioxide exposure and viral infection. Such studies have generally been conducted on animals with mortality as an outcome, or have been conducted in vitro. For example, Valand and coworkers (1970) and Acton and Myrvik (1972) found that rabbits exposed to 15 and 25 ppm nitrogen dioxide for three hours yield macrophages that have lowered capacity to develop virus-induced resistance to reinfection and have inhibited phagocytosis. Studies of viral infection in conjunction with nitrogen dioxide exposures are important to fill the gap in our understanding of the effect of nitrogen dioxide on human health.

JUSTIFICATION FOR THE STUDY

Relatively high doses of nitrogen dioxide can cause serious toxic effects, but whether or not ambient levels of nitrogen dioxide exposure cause risks to health is uncertain. The HEI solicited proposals (RFA 83-2) that would help resolve the issue of whether or not nitrogen dioxide in or near the ambient exposure range increases the severity of, or en-

hances the susceptibility to, respiratory infections. Animal studies that included aspects of host defense mechanisms were particularly desirable.

In response to the RFA, Dr. Rose (Principal Investigator) proposed to study the putative effects of nitrogen dioxide on the course of respiratory viral infection in mice. Infectivity studies were planned to evaluate the influence of both acute and chronic exposure on the severity of pneumonitis induced by both mouse cytomegalovirus and Sendai virus. These proposed studies included determining the effect of nitrogen dioxide exposure on susceptibility to primary infection and reinfection, the effect of simultaneous exercise, the effect of route of infection (virus administered intranasally versus intratracheally), and the reversibility of nitrogen dioxide effects. In addition, studies were proposed to examine the mechanism of nitrogen dioxide effects, focusing on the antiviral functions of macrophages and lymphocytes. One of the strengths of the proposal was the potential usefulness of the immunological techniques for future investigations in humans.

GOALS AND OBJECTIVES

The objective of this research was to clarify the potential role of nitrogen dioxide exposure in the pathogenesis of viral infection of the lower respiratory tract. The study focused on infectious outcome as the endpoint in animals exposed to very low titers of virus. The specific goals of the authors, as stated in the Investigators' Report, were to examine the influence of exposure to inhaled nitrogen dioxide on the pathophysiology of viral respiratory infection in an established animal model (mice infected with the murine cytomegalovirus); to delineate specific alterations resulting from nitrogen dioxide exposure on host antimicrobial defenses, with particular emphasis on the antiviral mechanisms of the pulmonary macrophage and the function of T-lymphocytes; and to determine if nitrogen dioxide exposure results in immunological abnormalities detectable in peripheral lymphoid tissue.

The experiments undertaken included studies of:

1. The effect of nitrogen dioxide exposure on susceptibility to, and severity of, primary infection, using a subinfectious dose of murine cytomegalovirus;
2. The effect of nitrogen dioxide exposure on susceptibility to reinfection, using nitrogen dioxide exposure at the time of initial murine cytomegalovirus infection and again with subsequent reinfection with murine cytomegalovirus 30 days later;
3. The integrity of the macrophage plasma membrane, as measured by in vivo and in vitro particle uptake studies;
4. Systemic immune responses, by examining proliferation of splenic lymphocytes and virus-specific antibody production.

STUDY DESIGN

The general design of most of the experiments in this study called for exposing mice to varying concentrations of nitrogen dioxide for six hours per day on two consecutive days prior to inoculation with murine cytomegalovirus, and then reexposing them to the same level of nitrogen dioxide for six hours per day on four consecutive days, beginning the day after viral inoculation. The titer of the inoculum was selected to produce virologic or pathologic evidence of lower respiratory tract infection in exposed animals without producing evidence of infection in control animals. For reinfection studies, a second inoculation with virus was performed both on air-preexposed animals and on nitrogen-dioxide-preexposed animals that had been treated as described above.

Particle uptake was studied in animals that had been exposed to either nitrogen dioxide or air for six hours on two consecutive days. In vivo studies assessed macrophage phagocytic uptake of intratracheally instilled ^{198}Au-colloidal gold. In vitro studies determined the amount of gold taken up by lavaged cells in situ. Lymphocyte function was also assessed in vitro, after recovery of spleen cells from either nitrogen-dioxide- or air-exposed animals. Virus-specific antibody was measured in the serum of animals 30 to 45 days after primary infection.

SUMMARY OF INVESTIGATORS' CONCLUSIONS

The investigators report that relatively short-term exposures of mice to nitrogen dioxide, at concentrations of 5 ppm or greater, increased their susceptibility to infection of the lower respiratory tract with murine cytomegalovirus, as indicated by the substantially smaller (approximately one-hundredth) quantity of viral inoculum that was required to produce either virologic or pathologic evidence of infection. Exposure to nitrogen dioxide during the primary infection stage also appeared to result in an increased susceptibility to reinfection on challenge with the same virus, in the absence of nitrogen dioxide exposure, 30 days later.

Infection produced by the combined effects of nitrogen dioxide and virus was not associated with any biochemical evidence of injury. The nitrogen-dioxide-enhanced infection was associated with diminished macrophage phagocy-

tosis of ^{198}Au-colloidal gold in vivo, but not in vitro, and with reduced viral clearance from the lungs.

Finally, increased susceptibility to reinfection appeared to be associated with an interactive effect of nitrogen dioxide and the primary infection on the immune response. Macrophage Ia epitope expression was diminished, suggesting impairment of antigen-presenting capability. The animals also had a reduced memory T-cell proliferative response. There was no evidence of impairment of the humoral immune response.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators had originally submitted a proposal to the HEI with more ambitious experimental goals than those of the present study. In order to make the study more feasible and focused, the investigators agreed to limit the goals to those stated above (see Justification of the Study).

The investigators' goals of the study were largely met (see Goals and Objectives section); however, the results are of uncertain reliability, and need to be validated, as discussed below.

ASSESSMENT OF METHODS AND DATA ANALYSIS

In general, the experimental methods used in this study were appropriate for the conduct of research on experimental infections and immunologic reactions in laboratory animals.

The concentrations of nitrogen dioxide shown in the Investigators' Report (Table 1) ranged from 2.5 to 10 ppm, but a few experiments were conducted at nitrogen dioxide concentrations up to 27 ppm. Concentrations of 15 ppm and above were found to be lethal to some of the animals when viral infection was superimposed; therefore, they were not useful for studying the functional parameters intended in this study.

It appears that the animals were caged in groups after exposure to the virus. Because viral infection can spread in an uncontrolled fashion among animals in a cage, the practice of group caging raises questions about whether or not the total dose of virus received by the animals was adequately controlled. Furthermore, it also suggests that the treatment of each animal as a separate experimental unit for the purpose of data analysis may not have been appropriate.

The authors infected the animals through intratracheal inoculations. Although intratracheal instillation is more convenient to perform and requires fewer resources, it should be pointed out that intratracheal instillation bypasses the upper respiratory defense mechanisms and leads to uneven patterns of deposition that are different from those produced by inhaled virus (Brain et al. 1976). Intratracheal instillation with large doses over a short time period may also have overloaded specific defense mechanisms. It would have been preferable if the animals had been exposed by inhalation, as is typical of human exposure to virus.

Highly variable, and often small, numbers of animals were used in the reported experiments. The investigators encountered an unanticipated obstacle in obtaining reliable and reproducible small viral inocula for use in the animal studies. Inoculum titer variability of greater than 10 percent was designated inadequate; apparently over 30 percent of the experiments were discarded on this basis. However, a 10 percent variability in titers that range over several orders of magnitude does not appear to be large enough to warrant exclusion of data. The authors have also indicated that there was no evidence of infection in all the discarded experiments. Although the investigators believe this was because the viral titers administered were too low, they may have discarded valid data and, as a result, biased the results away from the null hypothesis (no effect due to nitrogen dioxide exposure).

The investigators have adopted the statistical procedure of estimating the 50-percent-response dosage (ED_{50}—the concentration that resulted in 50 percent infections with a standard viral dose) from their dose-response data, which in several cases had only one usable data point. This may have contributed to what appear to be inconsistent conclusions. Thus, it is reported that the 50-percent-response concentration of nitrogen dioxide was calculated to be 3.9 ppm. The report also states that the threshold for enhanced viral susceptibility from nitrogen dioxide exposure is at 5 ppm, apparently because 2.5 ppm did not result in any significant alteration of viral susceptibilities, but 5 ppm did. However, no doses between 2.5 and 5 ppm were tested, and the report does not formally analyze the data for trends to affirm dose-response relations. A conclusion that 5 ppm is the "threshold" implies that nitrogen dioxide has no detectable effects at 3.9 ppm.

INTERPRETATION OF RESULTS

A major concern in interpreting the results is the small number of animals on which the conclusions are based. This concern is further amplified because the animals were caged in groups. Although statistical significance was observed in a number of the small-sample experiments, one must reserve judgment with regard to their biological sig-

nificance. Hence, additional evidence will be necessary to establish relevant dose-response relationships or mechanistic explanations.

There are two noteworthy features of this research design. First, the animals were exposed to nitrogen dioxide both before and after the inoculation with virus (see Study Design section in this report). This protocol may be a more realistic model for human nitrogen dioxide exposure than previous studies in which pollutant exposure occurred either before or after administration of the infectious agent.

A second feature, the secondary challenge with the virus 30 days after the initial combined nitrogen dioxide and virus exposure, has provided useful information. The investigators interpret the results of increased sensitivity to viral reinfection as suggesting a deficit in the immune memory function induced by the initial combined exposure. This has important implications, in that reinfection of the respiratory tract is common in early childhood (Henderson et al. 1979), the age at which the influence of nitrogen dioxide on the frequency of respiratory illness is postulated to be greatest (Shy et al. 1970). It is possible that the virus itself may be immunosuppressive, and that exposure to the virus, rather than to nitrogen dioxide, was responsible for the subsequent increase in susceptibility; however, this hypothesis would still involve an interaction between nitrogen dioxide and virus at the time of initial exposure, which would have enhanced the initial infection, thus rendering the animal less immunocompetent on subsequent infection. As indicated by the authors, this observation offers an area for additional investigation.

Justification for the use of the murine animal model to explore the effect of exposure to nitrogen dioxide in humans is presented (see Discussion section of Investigators' Report). The authors point out anatomic and functional similarities between humans and laboratory animals, and they suggest that the qualitative differences among species may not seriously complicate interpretation of the health implications of the observed results. However, the laboratory model used here does not fully represent the actual conditions of human exposure; therefore, quantitative extrapolation to humans should be approached cautiously.

Also, the levels of nitrogen dioxide at which any effects were observed in this study are much higher than the levels to which people are generally exposed. Although the authors state that the concentration at which increased sensitivity to viral infection was noted in mice (5 ppm) is comparable to some reported indoor air concentrations of nitrogen dioxide, it should be emphasized that the concentrations of nitrogen dioxide studied here exceed the outdoor air quality standard for average nitrogen dioxide concentrations by approximately two orders of magnitude, and that they exceed the usual peak concentrations in the outdoor environment by at least one order of magnitude (U.S. Environmental Protection Agency 1985).

REMAINING UNCERTAINTIES AND FUTURE RESEARCH

While the results of this study are limited, they do suggest additional areas of research. A more precise determination of the actual concentrations required to enhance viral infectivity or reinfectivity could be achieved with larger sample sizes and better-controlled experiments. In addition, animal studies that contribute to knowledge of the mechanisms by which increased susceptibility occurs may lead to our understanding of the effect of nitrogen dioxide on human respiratory infections.

The suggestion that exposure to nitrogen dioxide adversely affects specific antiviral functions of the pulmonary macrophage and natural killer cells deserves confirmation and more extensive investigation. The observation that splenic lymphocytes, obtained from nitrogen-dioxide-exposed animals 30 days after primary infection, fail to proliferate in response to subsequent antigens needs more in-depth study to determine whether or not this deficit is related to the increased viral sensitivity noted in the reinfection experiments. Documentation of a deficit of local pulmonary production of gamma interferon during exposure to murine cytomegalovirus could help to clarify the relation.

CONCLUSIONS

This study has provided evidence that mice exposed to nitrogen dioxide, before and after inoculation with a murine virus, are rendered more sensitive to initial infection by the virus and more sensitive to reinfection by the virus 30 days later. The evidence is consistent with the hypothesis, put forward on the basis of epidemiological data, that exposure to nitrogen dioxide increases the susceptibility to viral infection. However, because of the small sample sizes and other problems noted above, the results are of limited value in terms of quantitative, dose-response relations.

Perhaps more importantly, this research provides the basis for formulation of hypotheses concerning the mechanism of increased sensitivity to virus that may, with further research, provide more sensitive tools and better understanding of the relations between nitrogen dioxide exposure and human sensitivities to viral infection. Considerable additional research is needed to confirm the proposed

viral mechanisms, and to work out the application of such mechanisms to the assessment and evaluation of air quality so that protection of human health can be assured.

REFERENCES

Acton JD, Myrvik QN. 1972. Nitrogen dioxide effects on alveolar macrophages. Arch Environ Health 24:48–52.

Amoruso MA, Witz G, Goldstein BD. 1981. Decreased superoxide anion radical production by rat alveolar macrophages following inhalation of ozone or nitrogen dioxide. Life Sci 28:2215–2221.

Brain JD, Knudson DE, Sorokin SP, Davis MA. 1976. Pulmonary distribution of particles given by intratracheal instillation or by aerosol inhalation. Environ Res 11:13–33.

Ehrlich R. 1980. Interaction between environmental pollutants and respiratory infections. Environ Health Perspect 35:89–100.

Ehrlich R, Fenters JP. 1973. Influence of NO_2 on experimental influenza in squirrel monkeys. In: Proceedings of the Third International Clean Air Congress, pp. A11-A13. Sponsored by the International Union of Air Pollution Prevention Association. VDI-Verlag, Dusseldorf, Federal Republic of Germany.

Ehrlich R, Silverstein E, Maigetter R, Fenters JD. 1975. Immunologic response in vaccinated mice during long-term exposure to nitrogen dioxide. Environ Res 10:217–223.

Fenters JD, Ehrlich R, Findlay J, Spangler J, Tolkacz V. 1971. Serologic response in squirrel monkeys exposed to NO_2 and influenza virus. Am Rev Respir Dis 104:448–451.

Gardner DE. 1982. Use of experimental airborne infections for monitering altered host defenses. Environ Health Perspect 43:99–107.

Gardner DE. 1984. Oxidant-induced enhanced sensitivity to infection in animal models and their extrapolations to man. J Toxicol Environ Health 13:423–439.

Gardner DE, Miller FJ, Blommer EJ, Coffin DL. 1979. Influence of exposure mode on the toxicity of NO_2. Environ Health Perspect 30:23–29.

Graham JA, Gardner DE, Blommer EJ, House DE, Menache MG, Miller FJ. 1987. Influence of exposure patterns of nitrogen dioxide and modifications by ozone on susceptibility to bacterial infectious disease in mice. J Toxicol Environ Health 21:113–125.

Green GM. 1984. Similarities of host defense mechanisms against pulmonary infectious diseases in animals and man. J Toxicol Environ Health 13:471–478.

Green GM, Jakab GJ, Low RB, Davis GS. 1977. Defense mechanisms of the respiratory membrane. Am Rev Respir Dis 115:479–514.

Henderson FW, Collier AM, Clyde WA Jr, Denny FW. 1979. Respiratory syncytial virus infections, reinfections, and immunity: A prospective, longitudinal study in young children. N Engl J Med 300:530–537.

Henry MC, Findlay J, Spangler J, Ehrlich R. 1970. Chronic toxicity of NO_2 in squirrel monkeys: III. Effect on resistance to bacterial and viral infection. Arch Environ Health 20: 566–570.

Jakab GJ. 1980. NO_2-induced susceptibility to acute respiratory illness: A perspective. Bull NY Acad Med 56: 847–855.

Katz GV, Laskin S. 1975. Pulmonary macrophage response to irritant gases. In: Air Pollution and the Lung (Aharanson EF, Ben-David A, Klingberg MA, eds.). Wiley & Sons, New York, NY.

Kulle TJ, Clements ML. 1988. Susceptibility to Virus Infection with Exposure to Nitrogen Dioxide. Research Report No. 15. Health Effects Institute, Cambridge, MA.

Lefkowitz SS, McGrath JJ, Lefkowitz DL, Spicer JB. 1984. Interferon production following NO_2 exposure. Int J Immunopharmacol 6(4):275–278.

McGrath JJ, Oyervides J. 1985. Effects of nitrogen dioxide on resistance to *Klebsiella pneumoniae* in mice. J Am Coll Toxicol 4:227–231.

Mochitate K, Takahashi Y, Ohsumi T, Miura T. 1986. Activation and increment of alveolar macrophages induced by nitrogen dioxide. J Toxicol Environ Health 17:229–239.

Morrow PE. 1984. Toxicological data on NO_x: An overview. J Toxicol Environ Health 13:205–227.

Pennington JE. 1988. Effects of automotive emissions on susceptibility to respiratory infections. In: Air Pollution, the Automobile, and Public Health: Research Opportunities for Quantifying Risk (Watson AY, Bates RR, and Kennedy D, eds.). National Academy of Sciences Press, Washington, DC.

Samet JM, Marbury MC, Spengler JD. 1987. Health effects and sources of indoor air pollution. Am Rev Respir Dis 136:1486–1508.

Schlesinger RB. 1987. Intermittent inhalation of nitrogen dioxide: Effects on rabbit alveolar macrophages. J Toxicol Environ Health 21:127–139.

Shy CM, Creason JP, Pearlman ME, McClain KE, Benson FB, Young MM. 1970. The Chattanooga schoolchildren study: Effects of community exposure to nitrogen dioxide. II. Incidence of acute respiratory illness. J Air Pollut Control Assoc 20:582–588.

U.S. Environmental Protection Agency. 1982. Air Quality Criteria for Nitrogen Oxides. EPA-600/8–82–026. Environmental Criteria and Assessment Office, Research Triangle Park, NC.

U.S. Environmental Protection Agency. 1985. Health research plan for the study of nitrogen dioxide. Staff paper. Office of Assistant Administrator of Research and Development, Washington, DC.

Valand SB, Acton JD, Myrvik QN. 1970. Nitrogen dioxide inhibition of viral-induced resistance in alveolar monocytes. Arch Environ Health 20:303–309.

Vollmuth TA, Driscoll KE, Schlesinger RB. 1986. Changes in early alveolar particle clearance due to single and repeated nitrogen dioxide exposures in the rabbit. J Toxicol Environ Health 19:255–266.

LIST OF HEI PUBLICATIONS

Special Reports

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
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research (Supplement)	January 1988

Research Reports

Report No.	Title	Principal Investigator	Publication Date
1	Estimation of Risk of Glucose 6-Phosphate Dehydrogenase-Deficient Red Cells to Ozone and Nitrogen Dioxide	M. Amoruso	August 1985
2	Disposition and Metabolism of Free and Particle-Associated Nitropyrenes After Inhalation	J. Bond	February 1986
3	Transport of Macromolecules and Particles at Target Sites for Deposition of Air Pollutants	T. Crocker	February 1986
4	The Metabolic Activation and DNA Adducts of Dinitropyrenes	F.A. Beland	August 1986
5	An Investigation into the Effect of a Ceramic Particle Trap on the Chemical Mutagens in Diesel Exhaust	S.T. Bagley	January 1987
6	Effect of Nitrogen Dioxide, Ozone, and Peroxyacetyl Nitrate on Metabolic and Pulmonary Function	D.M. Drechsler-Parks	April 1987
7	DNA Adducts of Nitropyrene Detected by Specific Antibodies	J.D. Groopman	April 1987
8	Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung	J.L. Mauderly	May 1987
9	Biochemical and Metabolic Response to Nitrogen Dioxide-Induced Endothelial Injury	J.M. Patel	June 1987
10	Predictive Models for Deposition of Inhaled Diesel Exhaust Particles in Humans and Laboratory Species	C.P. Yu	July 1987
11	Effects of Ozone and Nitrogen Dioxide on Human Lung Proteinase Inhibitors	D.A. Johnson	August 1987
12	Neurotoxicity of Prenatal Carbon Monoxide Exposure	L.D. Fechter	September 1987
13	Effects of Nitrogen Dioxide on Alveolar Epithelial Barrier Properties	E.D. Crandall	October 1987
14	The Effects of Ozone and Nitrogen Dioxide on Lung Function in Healthy and Asthmatic Adolescents	J.Q. Koenig	January 1988
15	Susceptibility to Virus Infection with Exposure to Nitrogen Dioxide	T.J. Kulle	January 1988
16	Metabolism and Biological Effects of Nitropyrene and Related Compounds	C.M. King	February 1988

Copies of these reports can be obtained by writing to the Health Effects Institute, 215 First Street, Cambridge, MA 02142.

Research Reports

Report No.	Title	Principal Investigator	Publication Date
17	Studies on the Metabolism and Biological Effects of Nitropyrene and Related Nitro-polycyclic Aromatic Compounds in Diploid Human Fibroblasts	V.M. Maher	March 1988
18	Respiratory Infections in Coal Miners Exposed to Nitrogen Oxides	M. Jacobsen	July 1988
19	Factors Affecting Possible Carcinogenecity 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
21	Maximal Aerobic Capacity at Several Ambient Concentrations of Carbon Monoxide at Several Altitudes	S.M. Horvath	December 1988
22	Detection of Paracrine Factors in Oxidant Lung Injury	A.K. Tanswell	February 1989
23	Responses of Susceptible Subpopulations to Nitrogen Dioxide	P.E. Morrow	February 1989

Copies of these reports can be obtained by writing to the Health Effects Institute, 215 First Street, Cambridge, MA 02142.

THE HEALTH EFFECTS INSTITUTE: AN OVERVIEW

HEI

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 Charles Powers, Partner, Resources for Responsible Management.

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-seven 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.

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, independent 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 Report, 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 Report, is then 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.

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 215 First Street, Cambridge, Massachusetts 02142 (617) 491-2926

Research Report Number 24 March 1989