HEALTH EFFECTS INSTITUTE

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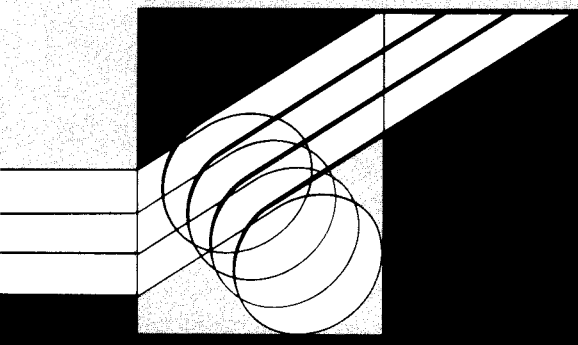
RESEARCH REPORT NO. 15



Susceptibility to Virus Infection with Exposure to Nitrogen Dioxide

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Includes the Report of the Institute's Health Review Committee

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

Funded equally by the U.S. Environmental Protection Agency (EPA) and 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. 84-3
Designed and printed by Prime Communications, Inc.

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LIST OF ABBREVIATIONS

ca	cold-adapted
cmu	cumulative methacholine units
ELISA	enzyme-linked immunosorbent assay
$FEF_{25-75\%}$	forced expiratory flow rate between 25 and 75% FVC
FEV_1	forced expiratory volume in one second (litres)
FVC	forced vital capacity (liters)
GSD	geometric standard deviation
HA	hemagglutinin
HAI	hemagglutination-inhibition
MDCK	Madin Darbin Canine Kidney
MEM	minimum essential medium
MMD	mass median diameter
NA	neuraminidase
$TCID_{50}$	50 percent tissue culture infectious dose

PREFACE

THE HEALTH EFFECTS INSTITUTE AND ITS RESEARCH PROCESS

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

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

INDEPENDENCE IN GOVERNANCE

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

TWO-SECTOR FINANCIAL SUPPORT

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

RESEARCH PLANNING AND PROJECT EVALUATION

HEI is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is developed by the Health Research Committee, a 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 the mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, which also reviews the procedures, independence, and quality of the selection process.

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

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

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

INTRODUCTION

In the summer of 1983, HEI issued a request for Applications (RFA 83-2) soliciting proposals on "Nitrogen Oxides and Susceptibility to Respiratory Infections." In the fall of 1983, Dr. Thomas J. Kulle of the University of Maryland, School of Medicine proposed a project entitled "Susceptibility to Virus Infections with Exposure to Nitrogen Dioxide." The HEI approved the three-year project and authorized expenditure of $590,258. The project began in January, 1984, and the final report was accepted by the Health Review Committee in July, 1987. The Health Review Committee's report, which follows the investigators' report, is intended to place the investigators' final report in perspective as an aid to the sponsors of HEI and to the public.

THE CLEAN AIR ACT

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

The determination of the appropriate standards for emissions of oxides of nitrogen depends in part on an assessment of the risks to health it presents. A controlled study of the effect of short-term exposure to nitrogen dioxide on human susceptibility to laboratory-induced respiratory viral infection can contribute knowledge useful in making the evaluations of 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. At that time, EPA deferred a decision on the need for separate short-term health standard pending receipt of results of additional research. Research of the type described above can contribute to the assessment of the appropriateness of the existing standards and the need for short-term standards.

BACKGROUND

Automotive emissions are one of the major sources of nitrogen dioxide (NO_2) in our environment. When inhaled, nitrogen dioxide travels through the respiratory airways to the alveoli in the deep lung, where it can cause a variety of harmful biological effects (reviewed in EPA 1981; WHO 1977).† In addition to these effects in the deep lung, it is known that a small fraction of the nitrogen dioxide is solubilized in the upper respiratory tract (Von Nieding et al., 1970),† where it may affect the incidence and course of respiratory infections and produce other effects.

Viruses are the major causative agents of respiratory infections in the general population. A large prospective study of Monto and Ulman (1974)† showed that 82 percent of the isolates from throat cultures taken within two days after the onset of symptoms were viral. Several types of virus cause respiratory disease; the most common among these are influenzas A and B, rhinoviruses, adenoviruses, parainfluenza type 1, and respiratory syncytial virus. The majority of respiratory infections occur in the upper respiratory tract and primarily involve the nose, throat, and trachea (Pennington 1988)†. Infants and young children are believed to be especially susceptible to respiratory infections because their immune system is not fully developed and because they are in frequent, close contact with others of the same age. Thus, the incidence of upper respiratory illnesses in infants, for which viruses are believed to be the major etiological factor, is 8 to 12 per year (Glezen et at., 1971; Loda et at., 1972; Belshe et al., 1983)†.

The possible interactions between nitrogen dioxide exposure and virus infection have been explored in several epidemiological studies (reviewed in EPA 1981; Samet 1987)†. Such studies have relied on comparisons of respiratory illness between residents in areas with high levels of nitrogen dioxide and those in low level areas of nitrogen dioxide, as well as comparisons between residents in homes with gas stoves and those in homes with electric stoves. (Gas stoves are the major source of indoor nitrogen dioxide.) In addition, these studies, which have focused on adults as well as children, generally have relied on self-reporting and recall of symptoms in response to questionnaires. Although effects on respiratory illness and symptoms, as well as on lung function, have been reported in several studies, the results of the epidemiological studies, taken as a whole, are contradictory and inconclusive and do not allow one to draw the conclusion that there is a causal relationship between exposure to ambient levels of nitrogen dioxide and respiratory health.

The major problems of epidemiological studies have been that few of them have measured nitrogen dioxide levels, and most have relied on subjective reporting and recall of symptoms; the microorganisms that cause these infections also have not been characterized in most of these studies. The confounding effects of other pollutants, including environmental tobacco smoke, also have not been adequately addressed.

The influence of nitrogen dioxide on susceptibility to respiratory infections has been demonstrated in several species of animals. In a series of early studies from the laboratory of Dr. Ehrlich, (Henry et al., 1970; Ehrlich 1973; Fenters et al., 1971)†, it was shown that exposure to influenza virus in conjunction with 5- to 10-ppm nitrogen dioxide increased the mortality rate of animals compared to virus-exposed animals that did not receive nitrogen dioxide. Since these early studies, several other investigators have shown that exposure to nitrogen dioxide has an effect on host defense mechanisms, which renders the host more susceptible to respiratory infections (Pennington 1988; Green 1984; Gardner 1982)†.

Although the available evidence that nitrogen dioxide enhances susceptibility to respiratory infections from animal studies is strong, it does not directly help in resolving the question of human risk from exposure to the levels of nitrogen dioxide most commonly found in ambient environments.

† See References, page 25.

First, most studies have found positive effects at 2 to 3 ppm or greater nitrogen dioxide; current ambient levels of nitrogen dioxide rarely exceed 0.3 ppm. Second, most animal studies have examined mortality as the end point of infection. However, respiratory infections in humans are seldom lethal. If nitrogen dioxide had an effect on respiratory infections, it would be expected to affect the incidence of infections or exacerbate the severity of respiratory symptoms. Finally, most animal studies have employed bacterial challenge in conjunction with nitrogen dioxide; however, most human respiratory infections are of viral origin.

In recent years, some investigators have performed animal studies that use viral pathogens and various indicators of morbidity as the end point. Such studies have provided useful information on the mechanism of action of nitrogen dioxide in altering the host susceptibility to infections. However, the lowest level of nitrogen dioxide at which effects have been demonstrated in such studies is 2 to 3 ppm nitrogen dioxide. In addition, none of the animal-pathogen models closely mimics the etiological and epidemiological factors that are known to affect respiratory infections in the human population.

In conclusion, the results of animal studies indicate there are adverse effects observed with exposures above 2 to 3 ppm of nitrogen dioxide. However, in order to determine the relevance of these results to ambient levels of human exposure, more sensitive methods of analysis and more common human respiratory pathogens need to be used. Chamber exposures overcome a major criticism of epidemiological studies because they allow controlled air-borne concentrations of nitrogen dioxide. Increased susceptibility to respiratory infections has not been demonstrated with short-term exposures below 2 ppm nitrogen dioxide in animals. Ambient levels of nitrogen dioxide are almost always below 0.3 ppm; therefore, we do not know whether ambient levels would have an adverse health effect in the human population. Because the epidemiological literature suggests that nitrogen dioxide exposure is likely to have an effect on either the incidence or severity of respiratory infections, human chamber studies that involve experimental viral infections constitute an important area of research that is currently absent.

Dr. Kulle and his co-workers have studied the effects of nitrogen dioxide exposure on the incidence and severity of influenza virus infections in healthy nonsmoking adult humans. They used a cautious approach in this preliminary study for ethical reasons. As a result, they chose an attenuated (less virulent) virus strain that was able to replicate at temperatures found in the nose at about 33°C but not at the temperature found in the trachea (about 39°C). Volunteers were exposed to varying concentrations of nitrogen dioxide or clean air for short periods, two days before and one day following virus administration. The extent of virus infection was determined by measuring the amounts of virus specific antibody (IgA or IgG) present in nose and in the blood, the viral titer, and duration of virus shedding.

INVESTIGATORS' REPORT

by Thomas J. Kulle, Ph.D.
Mary Lou Clements, M.D., M.P.H.

Susceptibility to Virus Infection with Exposure to Nitrogen Dioxide

ABSTRACT

The interaction between nitrogen dioxide (NO_2) exposure and human susceptibility to respiratory virus infection was investigated in a placebo-controlled, randomized, blinded trial that was conducted in an environmentally controlled research chamber over a three-year period. Healthy, nonsmoking volunteers, 18 to 35 years old, who were seronegative to influenza A/Korea/82 (H3N2) virus, were randomly assigned either to breathe filtered clean air (clean air group) or nitrogen dioxide (exposure group) for two hours a day for three consecutive days. The nitrogen dioxide concentrations were 2 ppm (Year 1), 3 ppm (Year 2), and 1 or 2 ppm (Year 3). Live, attenuated cold-adapted (*ca*) influenza A/Korea/82 reassortant virus was administered intranasally to all subjects after the second day of exposure. Only one of the 152 volunteers had any symptoms, and that subject had only a low-grade fever. No adverse changes in pulmonary function or nonspecific airway reactivity to methacholine were observed after 2 or 3 ppm nitrogen dioxide exposure, virus infection, or both. Infection was defined by virus recovery, a four-fold or greater increase in serum or nasal wash influenza-specific antibody titers, or both. The infection rates of the groups exposed to nitrogen dioxide and those breathing clean air were: 12/21 (2 ppm nitrogen dioxide) versus 15/23 (clean air) in Year 1; 17/22 (3 ppm nitrogen dioxide) versus 15/21 (clean air) in Year 2; and 20/22 (2 ppm nitrogen dioxide) and 20/22 (1 ppm nitrogen dioxide) versus 15/21 (clean air) in Year 3. Although the differences were not statistically significant, the groups exposed to 1 or 2 ppm nitrogen dioxide in the last year became infected more often (91 percent) than those breathing clean air (71 percent). The frequencies of infection in two of the four groups exposed to nitrogen dioxide were higher than the 56 to 73 percent infection rate observed in previous studies in healthy human volunteers with the same dose of *ca*-influenza A (H3N2) virus. Our findings suggest, but do not prove, that nitrogen dioxide alone may play a role in increasing the susceptibility of adults to respiratory virus infections.

INTRODUCTION

Serious toxic effects, including bronchospasm and pulmonary edema, as a result of high-level exposure to nitrogen dioxide (NO_2) have been well documented (World Health Organization, 1977; National Research Council, 1977; Gardner et al., 1979; U.S. Environmental Protection Agency, 1981; Morrow, 1984). Nitrogen dioxide, a by-product of burning fuels, has been found in industrial emissions, in automobile exhaust, and in homes using gas for heating and cooking. This common air pollutant is alleged to be toxic at the low levels found in ambient urban environments (Speizer et al., 1980; Hoek et al., 1984a,b; Remijn et al., 1985; Lenner, 1987; Orehek et al., 1976; Dawson and Schenker, 1979; Linn et al., 1985). However, the biologic importance of exposure to lower ambient nitrogen dioxide concentrations, specifically the impact of nitrogen dioxide on pulmonary function and its role in respiratory infections, has been a subject of controversy that has been unresolved by numerous experimental and epidemiologic studies in human populations.

Results of studies in animals generally have supported the hypothesis that nitrogen dioxide concentrations as low as 1.5 ppm adversely affect pulmonary function, increase susceptibility to respiratory virus infection, or both (Jakab, 1980; Ehrlich and Henry, 1968; Fenters et al., 1973, 1979; Lefkowitz et al., 1986). Several pathophysiologic mechanisms may contribute to altered susceptibility to virus infections, such as a decrease in the production of interferon, altered mucous secretion, changes in ciliary activity, and decreased activity of pulmonary alveolar macrophages. These studies have evaluated the effects in the lower airways, and have not examined viral or bacterial inocula delivered intranasally.

Several epidemiologic studies on the health effects of nitrogen dioxide exposure have yielded inconclusive results. In a four-year longitudinal study, Melia and co-workers (Melia et al., 1977, 1978, 1979; Goldstein, 1979; Florey et al., 1979) found that higher rates of respiratory illness occurred in children from households with gas stoves and higher ambient nitrogen dioxide levels than in households with lower levels of nitrogen dioxide. In contrast, Keller et al. (1979a,b) found no significant differences in the incidence of respiratory illness of children in homes with gas stoves compared to those in homes with electric stoves. Other studies (Speizer et al., 1980; Hoek et al., 1984a; Remijn et al., 1985; Mostardi et al., 1981) identified weak associations between nitrogen dioxide and respiratory illness but also recognized several potentially confounding variables that were not controlled for in earlier epidemiologic studies, including imprecision in the measurement of nitrogen dioxide, parental cigarette smoking, and exposure to sulfer dioxide and other pollutants. Thus, the role of low levels of nitrogen dioxide in increasing susceptibility to respiratory virus infections has not been clearly established.

This study addressed the hypothesis that inhalation of nitrogen dioxide will increase influenza infectivity in healthy nonsmoking adults. We evaluated the effect of brief (two-hour) exposures to 1 to 3 ppm nitrogen dioxide on infectivity. In a previous study in our chamber facility, we found no significant decrements in pulmonary function with two-hour exposure to 0.5 ppm nitrogen dioxide (Kerr et al., 1979), a more representative atmospheric level associated with automobile

exhausts (Lenner, 1987; Dawson and Schenker, 1979). We therefore chose to begin the investigation of the effects of nitrogen dioxide on infectivity at a higher dose range. We selected a live, attenuated, cold-adapted (*ca*) influenza A reassortant virus as the respiratory virus agent because *ca* viruses replicate briefly in the upper respiratory tract, especially in the nose, the portal of entry for nitrogen dioxide. Moreover, since these well-characterized viruses are safe and non-transmissible, they can be employed in open (outpatient) studies (Clements et al., 1983, 1984a, 1986; La Montagne et al., 1983; Murphy et al., 1984).

AIMS

Our study was designed to answer the following questions:

1) Does nitrogen dioxide exposure alter human susceptibility to respiratory virus infection?

2) Is there a nitrogen dioxide dose-dependent response associated with susceptibility to infection?

3) Is bronchial reactivity increased or pulmonary function decreased by nitrogen dioxide exposure, virus infection, or a combination of nitrogen dioxide exposure and virus infection?

An originally proposed fourth aim was deleted. This aim was to answer, "Does the timing of exposure affect the susceptibility to virus infection?" Instead of addressing this aim, the third year study addressed further the question of a dose-response relationship between the level of nitrogen dioxide exposure and viral infectivity.

METHODS

The methods section is presented with a general overview of the study design, followed by a detailed description of the study protocol and specific research methods.

STUDY DESIGN

This study was a placebo-controlled, randomized, masked trial performed over a period of three years. In each year, groups of subjects at rest were exposed for three days to nitrogen dioxide (exposure group) or filtered clean air (clean air group) in a controlled human environmental chamber. Each day subjects completed a symptom questionnaire, and at the end of the second exposure day, each subject was inoculated with an attenuated *ca* influenza vaccine virus. On each of the four days after inoculation, the volunteers were examined for symptoms and had nasal washes collected to detect virus replication. Serum and nasal wash specimens were collected before and three-to-four weeks after virus administration to measure systemic and local antibody responses to the vaccine virus.

The three-year exposure protocol is shown in Table 1. In Years 1 and 2, pulmonary function and bronchial reactivity were measured for the first four study days and nine and 30 days post-inoculation. In order to concentrate on the question of viral infectivity, no pulmonary function or airway reactivity measurements were made during the third study year. This modification, and the elimination of the baseline clean air day (Day 1) for both the clean air group and the exposure

Table 1. Three-Year Exposure Protocol

	Day 1 (Baseline**)	Day 2	Day 3	Day 4
Year 1				
Clean Air Group (n = 23)	Air	Air	Air*	Air
Exposure Group (2 ppm, n = 21)	Air	NO_2	NO_2	NO_2
Year 2				
Clean Air Group (n = 21)	Air	Air	Air*	Air
Exposure Group (3 ppm, n = 22)	Air	NO_2	NO_2*	NO_2
Year 3				
Clean Air Group (n = 21)	Air	Air*	Air	
Exposure Group (2 ppm, n = 22)	NO_2	NO_2*	NO_2	
Exposure Group (1 ppm, n = 22)	NO_2	NO_2*	NO_2	

*The virus was administered intranasally (0.25 ml per nostril) to each volunteer within 30 minutes after leaving the environmental chamber.
**A clean air day (Day 1) was included for both groups in Years 1 and 2 to obtain baseline pulmonary function measurements.

group, allowed a shortening of the environmental chamber requirements for each subject. As a result of this modification, it was possible to study 22 additional subjects in the third year.

The nitrogen dioxide concentration to which the subjects were exposed was 1 ppm in Year 3, 2 ppm in Years 1 and 3, and 3 ppm in Year 2.

SUBJECTS

Healthy, nonsmoking volunteers were recruited from the Baltimore metropolitan area and included college students and employees at the University of Maryland. The volunteers, 18 to 35 years old, were accepted if they fulfilled the following criteria:

1) seronegative to influenza A virus, defined by a serum hemagglutination-inhibition (HAI) antibody titer of 1:8 or less to influenza A/Korea/82 and A/Philippines/82 (H3N2) viruses;

2) nonallergic to bovine or egg allergens or to erythromycin, amphotericin B, or neomycin;

3) in excellent health by medical history and physical examination, with no upper respiratory infection;

4) normal baseline blood studies (CBC, BUN, glucose), negative Hepatitis B surface antigen, and negative pregnancy test;

5) normal pulmonary function, defined by a forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) > 80 percent of predicted.

Volunteers were informed of the study purposes, experimental protocol and procedures, and the potential risk from participation in the study. The study protocol was approved by the institutional review boards at the University of Maryland School of Medicine and the Johns Hopkins University School of Public Health. Each subject signed a statement of informed consent prior to participation.

DAILY SUBJECT PROTOCOL

The daily subject protocol for Years 1 and 2 is shown in Table 2. All influenza A-seronegative volunteers who agreed to participate in the study were randomly assigned to two groups, a clean air group that breathed only clean air and an exposure group that inhaled nitrogen dioxide. During environmental chamber confinement in the first year of the study (1984), the exposure group breathed clean air for two hours on Day 1 and 2 ppm nitrogen dioxide for two hours each on Days 2, 3, and 4; the clean air group breathed clean air on all four days (Table 1). Each subject was exposed for the two-hour period at the same time each day, to minimize diurnal variation in lung function. In the second year of the study (1985), all procedures were carried out in an identical manner, with the exception that the exposure group received 3 ppm nitrogen dioxide for two hours each on Days 2, 3, and 4. The studies were carried out in the spring or early summer of each year, when influenza was no longer circulating in the area.

The study protocol, as modified for Year 3 (1986), is shown in Table 3. All influenza A-seronegative volunteers who agreed to participate in the study were randomly assigned to one of three groups, a clean air group that breathed only clean air, or one of two groups that inhaled either 1 ppm or 2 ppm nitrogen dioxide (exposure groups). During environmental chamber confinement, the exposure groups breathed 1 ppm

Table 2. Daily Subject Protocol for Years 1 and 2

	Pre-Screening	Screening	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 12	Day 32	Day 33
Physical Exam		X										
Screening Blood (Antibodies)	X											
30 ml Blood for Antibodies		X										X
Urinalysis Pregnancy test (Females)		X										
Environmental Chamber Confinement			X	X	X	X						
Veal Infusion Nasal Washing					X	X	X	X	X			
Ringers Lactate Nasal Washing		X	X								X	X
Virus Inoculation					X							
Spirometry		X	X	X	X	X				X		X
Nonspecific Airways Reactivity		X	X	X	X	X				X		X

Table 3. Daily Subject Protocol for Year 3

	Pre-Screening	Screening	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 21	Day 22
50 ml Blood for Antibodies, Consent Form, History, Urinalysis	X									X
Physical Exam, Pulse & Temperature Chart Training		X								
Environmental Chamber			X	X	X					
Nasal Washing	X	X		X	X	X	X	X	X	X
Virus Inoculation				X						
Spirometry	X									

or 2 ppm nitrogen dioxide for two hours a day on Days 1, 2, and 3, and the clean air group breathed clean air for two hours a day on all three days. The chamber exposures were carried out in May 1986, when influenza was no longer circulating in the area.

EXPOSURE FACILITY

The environmentally controlled chamber at the University of Maryland was designed so that input air passed through high efficiency particulate absolute and activated carbon filters. The clean room, wherein the clean air group subjects stayed during chamber confinement, has a volumetric ventilation rate of 238 m^3/min in a 4.0 x 4.3 x 2.4 m room, which provided 5.8 air changes per minute. A ventilation rate of 8.5 m^3/min in the 2.1 x 4.3 x 2.4 m exposure room, which housed the exposure group subjects, enabled a complete air change every 2.6 minutes.

GENERATION AND MONITORING OF NITROGEN DIOXIDE

Nitrogen dioxide from 1 to 5 percent nitrogen dioxide cylinders, balance in nitrogen, was accurately metered into the chamber exposure room with the use of a pressure regulator, needle valve, and rotameter. The nitrogen dioxide concentration was continuously monitored by a Model 1600 NOx Chemiluminescent Monitor (Columbia Scientific Instruments, Austin, Texas) and a TGM-555 Toxic Gas Colorimetric Analyzer (CEA Instruments, Emerson, NJ), based upon the Griess- Saltzman procedure (Saltzman, 1954) as modified by Lyshkow (Lyshkow, 1965). Monitors were dynamically calibrated daily with a Model 230 Dynacalibrator (VICI Metronics, Santa Clara, CA), employing NBS-traceable permeation tubes.

VIRUSES

The live, attenuated, *ca* influenza A/Korea/82 (H3N2) reassortant virus employed in this study derived its six internal RNA segments from the attenuated *ca* influenza A/Ann Arbor/6/60 (H2N2) donor virus, while the two remaining genes [i.e., those that code for viral surface antigens, hemagglutinin (HA) and neuraminidase (NA) glycoproteins] were derived from the wild-type influenza A/Korea/1/82 (H3N2) virus. The passage history of the influenza A/Korea/1/82 (H3N2) virus has been described previously (Snyder et al., in preparation). The influenza A/Korea/82 wild-type virus is antigenically similar to the epidemic influenza A (H3N2) virus, the influenza A/Philippines/82 virus. The *ca* influenza A/Korea vaccine virus was cloned, as described previously (Spring et al., 1977). Virus suspensions administered to volunteers were prepared in the allantoic cavity of avian leukosis virus-free SPF eggs. All virus suspensions administered to the volunteers satisfied the safety requirements of the Bureau of Biologics, Food and Drug Administration, as described in the code of federal regulations. A $10^{6.5}$ tissue culture infectious dose ($TCID_{50}$) of the *ca* influenza A/Korea/82 (H3N2) virus (Lot E-104, CR-59, clone 19-1) was administered intranasally to seronegative volunteers. The same suspension of the *ca* virus was used in the studies conducted in all three years to ensure comparability of the virus inoculum.

VIRUS INOCULATION AND VOLUNTEER EXAMINATION

The virus was administered intranasally (0.25 ml per nostril) to the volunteers within 30 minutes after they left the environmental chamber on the second exposure day: study Day 3 (Years 1 and 2) and study Day 2 (Year 3). The volunteers were examined before virus inoculation, and daily for four days thereafter, to determine whether or not illness occurred. Each volunteer recorded his or her own temperature and pulse rate at home four times a day for four days. Nasal-wash specimens for virus isolation were obtained before inoculation and for four successive days after inoculation.

Influenza-like illness was defined by one of the following criteria:

1) fever — oral temperature $\geq$ 37.8°C;

2) systemic illness — the occurrence of myalgia, or chills

and sweats, or both;

3) upper respiratory illness — the development of rhinorrhea, pharyngitis, or both, for two consecutive days; or

4) lower respiratory illness — the presence of a persistent cough for two days.

VIRUS ISOLATION

Ten milliliters of veal infusion broth were used for collection of nasal wash specimens before virus administration, and daily for four days thereafter. We inoculated 0.1 ml of wash fluid into each of four Madin Darbin Canine Kidney cells (MDCK) mono-layered cultures. The MDCK cells were grown as monolayers on 24-well plates and were maintained with a serum-free liquid overlay of Eagle's minimal essential medium, gentamicin, amphotericin, and trypsin. The quantity of virus present in the nasal wash specimens was determined on frozen aliquots by titration of decimal dilutions of wash fluid (four wells per dilution) on MDCK cells. These methods have been previously described (Murphy et al., 1985).

ANALYSIS OF SERA AND NASAL WASHES FOR ANTIBODY

Sera and nasal wash specimens were collected before, and three-to-four weeks after, administration of the virus to detect systemic and local respiratory tract antibody responses to the vaccine virus. Nasal wash specimens were collected by rinsing each nostril with 10 ml of Ringer's lactate solution; the procedure was repeated up to three times with 20-minute spacings, and the individual washes were pooled. The pooled washes were concentrated to contain approximately 10 mg of IgA/100 ml by dialysis against Aquacide 1A.

To detect serum IgA and IgG antibodies and nasal wash IgA antibodies to *ca* influenza A/Korea/82 (H3N2) virus, serum and nasal wash specimens were tested by enzyme-linked immunosorbent assay (ELISA) with the use of specific rabbit anti-human immunoglobulin. Briefly, U-bottomed polystyrene microtiter plates (Immunlon 1) were used with a ladder of reagents from the solid phase up, consisting of HA and NA glycoproteins, human serum or nasal wash, rabbit anti- human IgA or IgG, and goat anti-rabbit IgG serum conjugated with alkaline phosphatase in substrate. The influenza A/Korea/82 HA and NA glycoproteins together (HA-NA) were employed as the antigen in the ELISA. The ELISA titer was expressed as the highest dilution in which the optical density of the antigen-containing well was at least twice the optical density of the respective control well lacking antigen. Nasal wash IgA concentration was determined with the use of an ELISA capture assay with goat anti-human IgA as capture antibody, followed by a ladder of nasal wash, rabbit anti-human IgA, goat anti-rabbit IgG conjugated with alkaline phosphatase, and p-nitrophenyl phosphate as substrate. The details of this method have been described elsewhere (Snyder et al., in preparation).

A virus possessing the A/Korea/82 HA and one having the A/Philippines/82 HA were used as antigens in the HAI test (Dowdle et al., 1979). If an antibody response was detected only by one assay, the test was repeated to confirm the result.

DATA ANALYSIS

Influenza Infection

Infection was defined by evidence of virus recovery, a significant rise between pre-vaccination and post-vaccination nasal wash or serum antibody titers, or both. Specifically, influenza A *ca* virus infection was defined by any of the following criteria: isolation of *ca* influenza A virus, a four- fold or greater rise of serum HAI antibody, serum IgG HA-NA ELISA antibody titers, or serum or nasal wash IgA HA-NA ELISA antibody titers. For statistical calculations, virus titers were transformed to $\log_{10}$ and antibody titers to $\log_2$. Comparisons between group means were made by two-tailed Student's t-test, Mann Whitney U test, or ANOVA.

The effect of nitrogen dioxide exposure on the susceptibility to infection was determined by comparing the rates of infection between the nitrogen dioxide exposure groups and the clean air groups. Infection rates and percentages of volunteers with antibody responses for different groups were compared by Chi square, Fisher's exact test, or Mantel-Haentzel test, as appropriate.

Duration of virus shedding (replication) was based on the day of collection of the last culture-positive nasal wash specimen, e.g., if virus was isolated from nasal wash specimens of a volunteer on Days 3 and 4 after inoculation, the total duration of virus shedding was four days, or if only isolated on Day 3, the duration of virus shedding was three days. Data from all infected volunteers were used for calculating the duration of virus shedding and the magnitude of virus shed, since virus replication would have had to occur prior to the production of an antibody response. That the virus was not isolated from each infected person can be attributed to the likelihood that the timing of the sample collection did not correspond to the time of the virus replication. Although the MDCK tissue culture system is the most sensitive tissue culture line available for influenza virus isolation, it is not sensitive enough to detect levels of virus replication less than 0.50 $\log_{10}$ $TCID_{50}$ per ml. For calculation of peak mean titer of virus shed, a titer of 0.50 $\log_{10}$ was assigned to each infected volunteer from whom virus was not isolated.

RESULTS

ENVIRONMENTAL CHAMBER CONCENTRATIONS

The nitrogen dioxide concentrations during the study were rigidly controlled in the exposure room, in which the nitrogen dioxide exposure group subjects resided. The mean nitrogen dioxide level was 2.00 $\pm$ 0.01 ppm (mean $\pm$ standard deviation) for Year 1, 3.01 $\pm$ 0.01 ppm for Year 2, and 1.01 $\pm$ 0.00 ppm or 2.01 $\pm$ 0.01 ppm for the two exposure groups in Year 3. No nitrogen dioxide was detected in the clean room (clean

air group) by the CSI-1600 NO_x monitor, which has a minimum detectability of 0.01 ppm nitrogen dioxide. Chamber room temperatures were maintained at 22.2 ± 0.5°C and relative humidity at 60 ± 3 percent.

VOLUNTEER SELECTION

In the first year, 360 individuals were screened serologically; 70 were both seronegative (seroeligible) and eligible by the other criteria, and 44 agreed to participate in the study. Altogether, 44 volunteers completed the study; 21 were exposed to 2 ppm nitrogen dioxide and 23 breathed clean air.

In the second year, 211 individuals were screened serologically; 52 were seronegative and eligible, and 43 agreed to participate in the study. In total, 43 volunteers completed the study, with 22 exposed to 3 ppm nitrogen dioxide and 21 in the clean air group.

In the third year of the study, 246 people were screened serologically; 70 were both seronegative and eligible by other criteria, and 65 agreed to participate and completed the study. The subjects were randomly assigned to one of three groups: one breathed clean air (n = 21); the second, 2 ppm nitrogen dioxide (n = 22); and the third, 1 ppm nitrogen dioxide (n = 22). As shown in Table 4, the preinoculation antibody titers for each group were similar; this attested to the success of the randomization.

CLINICAL RESPONSE TO *ca* VACCINE VIRUS

As expected, the *ca* vaccine virus was well-tolerated by the adult volunteers. Only one person developed any symptoms of an influenza-like illness: a temperature elevation of 37.9°C (100.2°F) on Day 2 after virus inoculation. This did not restrict the volunteer's activity. The minimally ill volunteer, who was in the 3 ppm nitrogen dioxide (Year 2) exposure group, developed an antibody response to the vaccine virus, but the virus was not cultured from the nasal wash specimens of this volunteer. No changes in the appearance of the nasal mucosa of the volunteers in any nitrogen dioxide- exposed group were observed.

VIROLOGIC RESPONSE TO *ca* VACCINE VIRUS

The data in Table 5 summarize the virologic responses of the 87 volunteers in all three years to inoculation with $10^{6.5}$ $TCID_{50}$ of *ca* influenza A/Korea/82 reassortant virus. Volunteers are grouped by year and by nitrogen dioxide or clean air exposure.

In the first year, the virus was recovered from nasal wash specimens in 6 of 21 (29 percent) volunteers exposed to 2 ppm nitrogen dioxide, and in only 1 of 23 (4 percent) of those breathing clean air. The difference in the proportions of virus shedding approached statistical significance (p = 0.07, Fisher's exact test). The amount of *ca* virus isolated from the volunteers was very small. The duration of virus shedding was longer in infected subjects exposed to 2 ppm nitrogen dioxide compared to infected subjects who breathed clean air. As noted above, an infection was defined as either virus isolation or an antibody response. The magnitude of virus shed also was greater in the 2 ppm nitrogen dioxide group compared to the clean air group (p = 0.06, two-tailed Mann Whitney U test).

In the second year, the virus was recovered from only 3 of 22 (14 percent) in the 3 ppm nitrogen dioxide group, compared to 1 of 21 (5 percent) in the clean air group. There were no statistically significant differences between infected volunteers in the nitrogen dioxide group and the clean air group with respect to the frequency or duration of virus shedding or the magnitude of virus shed.

In the third year, low titers of virus were recovered from the nasal washes of 8 of 65 volunteers. There were no statistical differences between the nitrogen dioxide-exposed and control groups in the frequency or duration of virus shedding or in the magnitude of virus shed.

ANTIBODY RESPONSES TO *ca* VACCINE VIRUS

Data in Tables 4 and 5 summarize the local (nasal wash) and serum antibody responses of volunteers in the nitrogen dioxide and clean air groups. The clean air groups from the studies conducted in all three years were similar in their antibody responses ("any antibody response" = 65, 71, and 71 percent respectively, for Years 1 through 3). There were more frequent (but not significantly different) serum antibody responses among those volunteers exposed to 3 ppm nitrogen dioxide (73 percent —Year 2) than in those exposed to 2 ppm nitrogen dioxide (57 percent —Year 1).

For the third-year study, the rate of "any antibody response" to virus inoculation was higher in each of the nitrogen dioxide exposure groups (91 percent for both 1 and 2 ppm) than for the clean air group (71 percent), but the differences were not statistically significant.

COMPARISON OF RESPONSES OF NITROGEN DIOXIDE-EXPOSED AND CLEAN AIR GROUPS IN YEARS 1 THROUGH 3

The clean air groups for Years 1 through 3 were comparable based on their rates of infection, overall antibody response, mean duration of virus shedding, and mean peak titer of virus shed (Table 5). There was a significant ($p < 0.03$) increase in the frequency of antibody responses in the 2 ppm nitrogen dioxide group in the third year, as compared with those in the 2 ppm nitrogen dioxide group in the first year. Statistically significant differences were found between the responses of the 2 ppm groups for total antibody (57 percent versus 91 percent, $p < 0.03$), serum HAI A/Korea (5 percent versus 36 percent, $p < 0.02$) and A/Philippines (0 percent versus 41 percent, $p < 0.0009$) antibodies, serum ELISA IgG anti-HA-NA (29 percent versus 77 percent, $p < 0.004$), and nasal wash ELISA IgA anti-HA-NA (29 percent versus 68 percent, $p < 0.03$) antibody.

Table 4 shows the serum and nasal wash antibody titers of the three clean air groups and the four nitrogen dioxide exposure groups before and after virus administration. Most of the pre-inoculation antibody levels measured by serum HAI, serum ELISA IgG HA-NA, or nasal wash ELISA IgA HA-NA assays were remarkably similar.

There did appear to be a general increase in pre-inoculation ELISA IgA (HA-NA) antibody level in the serum of volunteers in the third year (mean $\log_2$ titer = 8.0-8.1) compared to those in the studies in the first two years (mean $\log_2$ titer = 5.5-6.2). There were no consistent differences in antibody responses between subjects in the first year versus the second year or in the second year versus the third year.

As shown in Table 5, there was no apparent dose-dependent relationship between the level of nitrogen dioxide exposure and the rate of infection with *ca* influenza A/Korea

Table 4. Summary of Mean Antibody Responses to *ca* Influenza A/Korea Virus Inoculation in Volunteers Exposed to NO_2 or Clean Air

NO_2 Exposure	No. of Volunteers	Mean (log 2) Antibody Titer ± SD							
		Serum HAI A/Korea		Serum IgG ELISA		Serum IgA ELISA		Nasal Wash IgA ELISA	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
Year 1									
2 ppm	21	1.3 ± 0.5	1.8 ± 0.8	10.2 ± 2.3	10.9 ± 2.2	5.5 ± 1.6	6.4 ± 1.5	5.3 ± 1.7	6.7 ± 1.2
None	23	1.5 ± 0.7	2.3 ± 0.8	10.9 ± 1.4	11.8 ± 1.4	5.8 ± 1.9	7.6 ± 1.6	6.3 ± 2.2	7.5 ± 1.7
Year 2									
3 ppm	22	1.2 ± 0.4	2.2 ± 1.2	9.1 ± 1.9	10.6 ± 1.7	5.5 ± 2.2	7.7 ± 2.0	6.1 ± 2.1	6.8 ± 1.9
None	21	1.3 ± 0.5	2.1 ± 1.0	9.9 ± 1.5	11.0 ± 1.6	6.2 ± 1.9	7.4 ± 1.8	5.1 ± 2.6	6.5 ± 2.4
Year 3									
2 ppm	22	1.4 ± 0.8	3.1 ± 1.8	11.6 ± 1.5	13.6 ± 3.3	8.1 ± 2.7	10.8 ± 2.7	5.7 ± 1.8	8.7 ± 2.4
1 ppm	22	1.1 ± 0.3	3.3 ± 1.7	10.9 ± 2.4	12.8 ± 2.5	8.1 ± 2.5	11.4 ± 2.8	5.2 ± 1.8	8.1 ± 2.3
None	21	1.5 ± 0.9	2.9 ± 1.6	11.6 ± 1.6	13.4 ± 1.3	8.0 ± 2.4	10.2 ± 2.8	6.2 ± 2.5	8.3 ± 2.1

Table 5. Summary of Responses of NO_2- and Air-Exposed Seronegative Adults to *ca* Influenza A/Korea/1/82 (H3N2) Reassortant Virus[1]

NO_2 Exposure	No. of Volunteers	Percent Infected	Percent Shedding	Mean Duration[2] (Days ± S.D.)	Mean Peak Titer[2] ($\log_{10}$ $TCID_{50}$/ml ± S.D.)	Percent With						
						Serum HAI A/Korea	Serum HAI A/Philippines	Serum IgG ELISA	Serum IgA ELISA	Nasal wash IgA ELISA	Any Antibody Response	Illness[3]
Year 1												
2 ppm	21	57[5]	29	1.6 ± 1.8	0.9 ± 0.70	5[5]	0[5]	29[5]	38	29[5]	57[5]	0
None	23	65	4	0.3 ± 1.0	0.6 ± 0.20	17	17	43	61	41[4]	65	0
Year 2												
3 ppm	22	77	14	0.5 ± 1.2	0.6 ± 0.10	23	41	59	64	23	73	5
None	21	71	5	0.2 ± 0.8	0.5 ± 0.07	24	14	43	57	38	71	0
Year 3												
2 ppm	22	91[5]	14	0.4 ± 1.1	0.7 ± 0.50	36[5]	41[5]	77[5]	64	68[5]	91[5]	0
1 ppm	22	91	5	0.1 ± 0.4	0.5 ± 0.05	64	32	59	64	77	91	0
None	21	71	19	0.7 ± 1.3	0.8 ± 0.60	38	19	67	57	57	71	0

[1] Virus isolation, antibody response, or both, signified infecion.

[2] Data from infected volunteers were used for calculations. The lowest detectable quantity of viral shedding was $\log_{10}$ 0.75 $TCID_{50}$/ml.

[3] Volunteers were considered ill if they developed any of the following syndromes: fever (≥ 37.8°C); systemic illness —the occurrence of myalgia, chills and sweats; upper respiratory tract illness —rhinitis, pharyngitis, or both, observed on two consecutive days; and lower respiratory tract illness —a persistent cough lasting for at least two days.

[4] Nasal wash specimens from one volunteer were not available for testing.

[5] Differences in percentages between indicated responses of NO_2-exposed groups in Years 1 and 3 were statistically significant ($p < 0.03$).

virus. The infection rates were highest in the volunteers exposed to nitrogen dioxide concentrations of 1 ppm (91 percent) and 2 ppm (91 percent) in the third year. Compared to the group breathing clean air, infection rates were only slightly higher in the group exposed to 3 ppm nitrogen dioxide (77 percent, Year 2) and were slightly lower in the group exposed to 2 ppm nitrogen dioxide (57 percent, Year 1).

DISCUSSION

Our study was designed to evaluate the role of nitrogen dioxide in altering susceptibility to virus infection in healthy adult volunteers. The scientific basis for this inquiry has been addressed by previous investigators, beginning with several large, population-based, observational studies in the 1960s that examined epidemiologic aspects of respiratory illness in children (Ware et al., 1984; Weiss et al., 1980; Colley and Reid, 1970; Holland et al., 1969; Lunn et al., 1967; Leeder et al., 1974). These investigations, among others, led to an appreciation of the importance of air pollutants as causes of adverse health effects. In 1970, Shy et al. reported a relationship between respiratory symptoms and ambient nitrogen dioxide concentrations in children living near Chattanooga, Tennessee. These findings subsequently were supported by the work of Melia and co-workers (Melia et al., 1978, 1979; Goldstein et al., 1979; Florey et al., 1979), who found a similar association in a five-year longitudinal study of 5,758 English and Scottish children. In a survey of 9,280 children in six U.S. cities, Speizer et al. (1980) also found significant associations between home cooking stoves, illness histories, and impaired lung function. Because these epidemiologic studies were undertaken in such large populations, only estimates, rather than actual measures of individual exposures to nitrogen dioxide, were used. Over the next decade, Mostardi et al. (1981) and Hoek et al. (1984a) identified weak associations between nitrogen dioxide or gas stoves and respiratory illness.

Not all of the results of subsequent studies, however, concurred with these findings, and some yielded sharply contradictory data. Keller et al. (1979b) surveyed 441 families in Columbus, Ohio and New York and reported no evidence to suggest that cooking with gas was associated with increases in respiratory disease or decreases in pulmonary function. Similarly, Schenker et al. (1983) surveyed 4,071 children in western Pennsylvania and suggested that socioeconomic status, rather than the use of gas cooking stoves, influenced the prevalence of respiratory symptoms, illness, or both. Robertson et al. (1984), in a long- term study of occupational exposures, also found no relationship between nitrogen dioxide exposure and respiratory illness in 560 adult British coal miners. Like the previous studies, all of these investigations involved large populations and used estimates of individual exposures to nitrogen dioxide rather than actual individual measurements. Melia et al. (1978), and Schenker et al. (1983), did attempt to control for such potentially confounding variables as underlying pulmonary status, parental cigarette smoking, and exposures to other pollutants. However, methodologic problems in their studies included the insensitivity of the nitrogen dioxide measurements, the lack of uniformity in determining the respiratory illnesses, the effects of underlying immunologic variability in the populations studied, and the different respiratory pathogens to which the study populations were exposed.

Goldstein et al. (1973), Ehrlich and Henry (1968), and Fenters et al. (1979) conducted quantitative studies in animals, and their results tended to support an association between nitrogen dioxide exposures and infection with bacteria or viruses. On the basis of the previous studies, it has not been possible to exclude or confirm with certainty a causal relationship between ambient nitrogen dioxide exposures and respiratory virus infections.

Green (1984) discussed an experimental animal model system that employed a bacterial aerosol to quantify six major components of antimicrobial defense mechanisms of the bronchopulmonary tree, utilizing nonpathogenic organisms. The defense functions appear remarkably similar across animal species, with available human data suggesting that findings obtained using the model may be extrapolated to humans. Reported animal studies employ bacterial or viral aerosols of respirable sizing; thus, these aerosols do not deposit primarily in the nasal cavity. Therefore, appropriate animal models evaluating susceptibility to infection with nitrogen dioxide and a nasal inoculum do not exist.

We conducted a placebo-controlled, randomized, double-masked experimental study to assess the role of brief exposures of 1 through 3 ppm nitrogen dioxide in increasing the susceptibility of healthy adults to respiratory infection with influenza A virus. Our study was designed to minimize some of the problems that have been recognized in other studies. To control for the duration and concentration of nitrogen dioxide exposures, we conducted our study in an environmentally controlled chamber. In an effort to eliminate factors other than the nitrogen dioxide or the virus that might influence the outcome of the study, we selected healthy, non-smoking volunteers who, by serology, were susceptible to infection with the influenza virus and who had normal pulmonary function. Finally, we administered a live attenuated (avirulent) *ca* influenza A reassortant virus intranasally, as a proxy for a virulent respiratory virus. We used this type of virus because it has been well characterized in terms of safety, the dose required to induce infection in the upper respiratory tract, and its ability to stimulate antibodies in the nose and blood of susceptible adults (Clements et al., 1983, 1984a; Clements and Murphy, 1986; LaMontagne et al., 1983; Murphy et al., 1984).

In our study, volunteers exposed to 1 ppm or 2 ppm nitrogen dioxide in the third year became infected with the attenuated *ca* influenza A virus more frequently than did those who breathed clean air. By contrast, there were no differences in the infection rates between the groups exposed to nitrogen dioxide and the clean air groups in the first two years of the study. The differences in infection rates between the groups exposed to nitrogen dioxide and the clean air for each study were not statistically significant. However, the rate of infection (91 percent) achieved by the $10^{6.5}$ $TCID_{50}$ dose of *ca* virus in two of four nitrogen dioxide-exposed groups was much higher than the rates that have been achieved by the same dose of *ca* influenza A (H3N2) viruses in seronegative adults in several previous studies. In general, this dose of *ca* influenza A virus infects between 56 percent and 73 percent of HI-seronegative adults (Clements et al., 1983, 1984b). A similar level of infectivity (65 percent to 71 percent) was observed in our clean air subjects. Thus, these differences in frequency of infection, although not statistically significant, may have biologic significance and may indicate some effect of nitrogen dioxide on susceptibility to virus infection.

There was a difference in the susceptibility of adults exposed to 2 ppm nitrogen dioxide between the studies conducted in the first and third years. The reason for this different response to 2 ppm nitrogen dioxide and virus inoculation is not clear. It is possible that the variation in results may be due to the small number of volunteers studied each year. Another possible explanation is that the immunologic status of the volunteers changed somewhat between 1984 (Year 1) and 1986 (Year 3). During the winter of 1984-85, an epidemic of influenza A/Philippines/82 (H3N2) virus, which was antigenically related to influenza A/Korea/82 virus, occurred for the first time in Baltimore. No other epidemics of influenza A viruses occurred during 1985-86. Although we selected volunteers with a low or absent level of serum HAI antibodies, we could not control for all forms of immunity. There is some evidence, based on the pre-inoculation IgA antibody levels in serum (Table 4), that the volunteers in the third year may have been exposed previously to an influenza A/Korea-like virus. Thus, these individuals, when inoculated with the *ca* virus, may have been more likely to mount an antibody response. It is, therefore, conceivable that susceptibility to influenza virus infection in persons exposed to the same dose of nitrogen dioxide may vary depending on if and when they have been previously infected with a related influenza virus. A trial with larger numbers of seronegative adults would need to be carried out within the same year, to determine whether nitrogen dioxide enhances the susceptibility of influenza A infection in persons who have been exposed previously to ("primed with") a similar influenza A virus. If the susceptibility to influenza virus infection in volunteers exposed to nitrogen dioxide varies following naturally acquired influenza infections, this could explain, in part, why the findings of several epidemiological studies carried out in different years have yielded conflicting results.

We did not demonstrate any significant adverse changes in pulmonary function with nitrogen dioxide exposure either prior to or after virus inoculation (see Appendix). The lack of changes in pulmonary responses with exposure to nitrogen dioxide in this study is consistent with most other reported studies (Fischer et al., 1985; Hazucha et al., 1983; Kleinman et al., 1983; Linn et al., 1985a,b; Mohsenin, 1986; Kerr et al., 1979). Decrements in pulmonary function in normal subjects have not been observed to result from two-hour exposures to 1 ppm nitrogen dioxide with intermittent exercise (Hackney et al., 1978), or with levels as high as 4 ppm for one-and-one-quarter hours with 15 minutes of heavy exercise (Linn et al., 1985b). Studies with virulent viruses administered by aerosol to persons who exercise, or to patients with pulmonary conditions such as asthma, may be required to determine any interactive effect of virus and nitrogen dioxide on pulmonary function.

The studies of airway reactivity in Year 2 yielded an interesting, albeit unexpected, finding. In the clean air group, repeated challenge with 438 cumulative methacholine units (cmu) was associated with a statistically significant diminished response on the second, third, and fourth study days. This effect was not present on the twelfth and thirty-third study days. The most likely explanation for this result is that cholinergic adaptation was occurring, as has been described by McDonnell et al. (1986) and Beckett (1987). A similar effect was not observed in Year 1, probably because a lower maximum dose was used (379 vs. 438 cmu). The exposure group in Year 2 (3 ppm nitrogen dioxide) did not show a diminished response to daily sequential methacholine challenge.

Several possible explanations exist for this interesting result. First, competing effects could be occurring. The nitrogen dioxide exposure could be subtly increasing reactivity, but the effect is negated by the cholinergic adaptation. Second, the nitrogen dioxide exposure could be increasing the access of the methacholine to the cholinergic receptor, thus producing an increased effective dose which negates adaptation. Finally, the nitrogen dioxide could modify the cholinergic receptor, and thus alter its response characteristics. These speculations obviously require further investigation. Studies of the effect of oxidants on the cholinergic receptor should be done with repeat methacholine challenge at 4-to-12-hour intervals, when the adaptation is more pronounced (Beckett, 1987). Nevertheless, this finding is intriguing, particularly since asthma is associated with cholinergic hyperresponsiveness, and nitrogen dioxide has been shown by some investigators to increase nonspecific reactivity in people with asthma (Bauer et al., 1986a).

It is possible that in those studies where associations between nitrogen dioxide exposures and alterations in pulmonary responses have been found, underlying pulmonary conditions such as asthma (Orehek et al., 1976; Bylin et al., 1985), exposure to allergens, other air pollutants (Orehek et al., 1976; Bylin et al., 1985), smoking (Orehek et al., 1976; Bylin

et al., 1985), and longer exposures and higher levels of nitrogen dioxide (vonNieding et al., 1979; Bylin et al., 1985) may have influenced the outcomes. Although our findings showed no pulmonary effect of 1 through 3 ppm nitrogen dioxide in healthy nonsmokers, the effect of the nitrogen dioxide in those with underlying diseases or with exposure to other air pollutants may be more profound.

The lack of pulmonary function changes 7 to 14 days after intranasal administration of live attenuated *ca* influenza A virus vaccines was demonstrated previously in adults with chronic obstructive airways disease (Gorse et al., 1986; MRC Advisory Group, 1984). It is not surprising that upper respiratory infection after intranasal administration of live attenuated influenza A viruses, such as the *ca* reassortant viruses, does not cause alterations in pulmonary function. Experimental challenge with intranasally administered influenza A wild-type viruses does not appear to cause significant abnormalities, whereas naturally acquired influenza infection does (Clements and Murphy, 1986; Hoskins et al., 1979; Feldman et al., 1985; Johnson et al., 1985; Murphy et al., 1982). Except for a few studies of live influenza virus candidate vaccines that were not attenuated satisfactorily (Hobbins et al., 1982; Rosenzweig et al., 1975; Nicholson et al., 1976), previous studies of other (non-*ca*) live attenuated influenza virus vaccines have consistently demonstrated no significant changes in numerous pulmonary function tests in healthy adults (Zeck et al., 1976; Prevost et al., 1975; Kava and Laitinen, 1985, MRC Advisory Group, 1980), in patients with chronic obstructive airway disease (Goldstein et al., 1979; Hoek et al., 1984a; Bauer et al., 1986b, 1987; Bartsch et al., 1977; Fell et al., 1977) and in patients with asthma (Mohsenin, 1986; Winson et al., 1977).

Overall, the results of our study to assess the effect of nitrogen dioxide on increased susceptibility to upper respiratory virus infection with *ca* influenza A virus were inconclusive. However, there was some evidence in the third year of our study to suggest that exposure to 1 to 2 ppm nitrogen dioxide may enhance the susceptibility to *ca* virus infection in individuals who have been previously infected with similar influenza viruses. Without further confirmatory studies, it would be premature to extrapolate from these results to the general population, where the impact of nitrogen dioxide might be expected to differ with the prevalence of underlying diseases, smoking, exposure to other pollutants, age, and other factors that we did not explore. Additional studies with larger numbers of volunteers may be required to achieve conclusive results. These results, and other previous studies, suggest a natural or experimental challenge with a virulent virus is more sensitive than an attenuated *ca* virus to detect an effect of nitrogen dioxide on susceptibility to infection, virus replication, pulmonary function, or airway reactivity. However, studies involving natural challenge would be very difficult to conduct, and challenge studies with wild-type virus would be difficult to justify on ethical grounds.

REFERENCES

American Thoracic Society Statement, 1979. Snowbird workshop on standarization of spirometry. Am Rev Resp Dis; 119:831-838.

Bartsch P, Dierckx JP, Frans A, Gillard C, Jovanovic D, Stanescu D, 1977. Live influenza vaccine in patients with chronic bronchopulmonary diseases. A multicenter study with two consecutive vaccinal strains. Dev Biol Stand; 39:113-121.

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

Bauer MA, Utell MJ, Smeglin AM, Speers DM, Gibb RF, Morrow PE, 1986b. Effects of low-level nitrogen dioxide on lung function in exercising subjects with chronic obstructive pulmonary disease (COPD). Am Rev Resp Dis; 133: A215 (Abstract).

Bauer MA, Utell MJ, Speers DM, Gibb FR, Morrow PE, 1987. Effects of 0.30 ppm nitrogen dioxide on lung function and breathing patterns in subjects with chronic obstructive lung disease (COPD). Am Rev Resp Dis; 135(4): A58 (Abstract).

Beckett WS. 1987. Tolerance to methacholine by repeated inhalation challenge. Am Rev Resp Dis; 135:A473 (Abstract).

Bylin G, Lindvall T, Rehn T, Sundin B, 1985. Effects of short-term exposure to ambient nitrogen dioxide concentrations on human bronchial reactivity and lung function. Eur J Resp Dis; 66:205-217.

Chai H, Farr FS, Froehlich LA, Mathison DA, McLean JA, Rosenthal RR, Sheffer AL, Spector SL, Townley RG, 1975. Standardization of bronchial inhalation challenge procedures. J Allergy Clin Immunol; 56:323.

Clements ML, O'Donnell S, Levine MM, Chanock RM, Murphy BR, 1983. Dose response of a/Alaska/6/77 (H3N2) cold-adapted reassortant vaccine virus in adult volunteers: Role of local antibody in resistance to infection with vaccine virus. Infect Immun; 40(3):1044-1051.

Clements ML, Betts RF, Maassab HF, Murphy BR, 1984a. Dose response of influenza A/Washington/897/80 (H3N2) cold-adapted reassortant virus in adult volunteers. J Infect Dis; 149:814-815.

Clements ML, Betts RF, Murphy BR, 1984b. Advantages of live attenuated cold- adapted influenza A virus over inactivated vaccine for A/Washington/80 (H3N2) wild-type virus infection. Lancet; 1:705-708.

Clements ML, Murphy BR. 1986. Development and persistence of local systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccines. J Clin Microbiol; 23:66-72.

Colley JRT, Reid DD, 1970. Urban and social origins of childhood bronchitis in England and Wales. Br Med J; 2:213-217.

Dawson SV, Schenker MB, 1979. Health effects of inhalation of ambient concentrations of nitrogen dioxide. Am Rev Resp Dis; 120:281-292.

Dowdle WR, Kendal AP, Noble GR, 1979. Lennette EH and Schmidt NJ (eds.) Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections, 5th Ed. Washington, Amer. Pub Health Assoc., p. 585.

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

Feldman S, Wright PF, Webster RG, Roberson PK, Mahoney J, Thompson J, Doolittle M, Lott L, Johnson P, Christoph RC, 1985. Use of influenza A virus vaccines in seronegative children: Live cold-adapted versus inactivated whole virus. J Infec Dis; 152:1212-1218.

Fell PJ, O'Donnell HF, Watson NP, Simmons RL, Hasell SK, 1977. Longer term effects of live influenza vaccine in patients with chronic pulmonary disease. Lancet; i:1282-1284.

Fenters JD, Findlay JC, Port CD, Ehrlich R, Coffin DL, 1973. Chronic exposure to nitrogen dioxide. Immunologic, physiologic, and pathologic effects in virus-challenged squirrel monkeys. Arch Environ Health; 27:85-89.

Fenters JD, Ehrlich R, Findlay J, Spangler J, Tolkacz V, 1979. Serologic response in squirrel monkeys exposed to nitrogen dioxide and influenza virus. Amer Rev Resp Dis; 104:448-451.

Fischer P, Remijn B, Brunekreef B, Van der Lende R, Schouten J, Quanjer P, 1985. Indoor air pollution and its effect on pulmonary function of adult non-smoking women: II. Associations between nitrogen dioxide and pulmonary function. Intern J Epidem; 14(2):221-226.

Florey CduV, Melia RJW, Chinn S, Goldstein BD, Brooks AGF, John HH, Craighead IB, Webster X, 1979. The relation between respiratory illness in primary schoolchildren and the use of gas for cooking. III. Nitrogen dioxide, respiratory illness and lung infection. Inter J. Epidem; 8(4):347-353.

Gardner DE, Miller FJ, Blommer EJ, Coffin DL, 1979. Influence of exposure mode on the toxicity of nitrogen dioxide. Environ Health Persp; 30:23-29.

Goldstein EM, Eagle MC, Hoeprich PD, 1973. Effect of nitrogen dioxide on pulmonary bacterial defense mechanism. Arch Environ Health; 26:202-04.

Goldstein BD, Melia RJW, Chinn S, Florey CduV, Clark D, John HH, 1979. The relation between respiratory illness in primary schoolchildren and the use of gas for cooking. II. Factors affecting nitrogen dioxide levels in the home. Intern J Epidem; 8(4):339-345.

Gorse GJ, Belshe RB, Munn NJ, 1986. Safety of and serum antibody response to cold-recombinant influenza A and inactivated trivalent influenza virus vaccines in older adults with chronic diseases. J Clin Microbiol; 24:336-342.

Green GM, 1984. Similarities of host defense mechanisms against pulmonary infectious diseases in animals and man. In: Miller FJ and Menzel DB (Eds). Fundamentals of extrapolation modeling in inhaled toxicants: Ozone and nitrogen dioxide; Washington; Hemisphere Publishing Corp.; 291-298.

Hackney JD, Thiede FC, Linn WS, Pederson EE, Spier CE, Law DC, Fisher DA, 1978. Experimental studies on human health effects of air pollutants. IV. Short-term physiological and clinical effects. Arch Environ Health; 33(4):171-181.

Hazucha MJ, Ginsberg JF, McDonnell WF, Haak ED Jr., Pimmel RL, Salaam SA, House DE, Bromberg PA, 1983. Effects of 0.1 ppm nitrogen dioxide on airways of normal and asthmatic subjects. J Appl Physiol; 54:730-739.

Hobbins TE, Hughes TP, Rennels MB, Murphy BR, Levine MM, 1982. Bronchial reactivity in experimental infection with influenza virus. J Infect Dis; 146(4):468-470.

Hoek G, Brunefreef B, Meijer R, Scholten A, Boleij J, 1984a. Indoor nitrogen dioxide pollution and respiratory symptoms of schoolchildren. Int Arch Occup Environ Health; 55:79-86.

Hoek G, Meijer R, Scholten A, Noij D, Lebret E, 1984b. The relationship between indoor nitrogen dioxide concentration levels and personal exposure: a pilot study. Int Arch Occup Environ Health; 55:73-78.

Holland WW, Halil T, Bennett AE, Elliott A, 1969. Factors influencing the onset of chronic respiratory illness disease. Br Med J; 2:205-208.

Hoskins TW, Davies RR, Smith AJ, Miller CL, Allchin A, 1979. Assessment of inactivated influenza A vaccine after three outbreaks of influenza A at Christ's Hospital. Lancet; 1(8106):33-35.

Jakab GJ, 1980. Nitrogen dioxide-induced susceptibility to acute respiratory illness: A perspective. Bull NY Acad Med; 56(9):847-865.

Johnson PR, Feldman S, Thompson JM, Wright PF, 1985. Comparison of long-term systemic and secretory antibody responses in seronegative children given live, attenuated or inactivated influenza A vaccine. J Med Virol; 17:325-335.

Kava T, Laitinen LA, 1985. Effects of killed and live attenuated influenza vaccine on symptoms and specific airway conductance in asthmatics and healthy subjects. Allergy; 40:42-47.

Keller MD, Lanese RR, Mitchell RI, Cote RW, 1979a. Respiratory illness in households using gas and electricity for cooking. I. Survey of incidence. Environ Res; 19:495-503.

Keller MD, Lanese RR, Mitchell RI, Cote RW, 1979b. Respiratory illness in households using gas and electricity for cooking. II. Symptoms and objective findings. Environ Res; 19:504-515.

Kerr HD, Kulle TJ, McIlhany ML, Swidersky P, 1979. Effects of nitrogen dioxide on pulmonary function in human subjects: An environmental chamber study. Environ Res; 19:392-404.

Kleinman MT, Bailey RM, Linn WS, Anderson KR, Whynot JD, Shamoo DA, Hackney JD, 1983. Effects of 0.2 ppm nitrogen dioxide on pulmonary function and response to bronchoprovocation in asthmatics. J Toxicol Environ Health; 12:815-826.

LaMontagne JR, Wright PF, Clements ML, Maasab HF, Murphy BF 1983. Prospects for live, attenuated influenza vaccines using reassortants derived from the A/Ann Arbor/6/60 (H2N2) cold-adapted (ca) donor virus. In: Laver WG (ed). The origin of pandemic influenza viruses. New York: Elsevier Science Publishing, Inc.; 243-247.

Leeder SR, Woolcock AJ, Blackburn CRB, 1974. Prevalence and natural history of lung disease in New South Wales schoolchildren. Int J Epidemiol; 3:15-23.

Lefkowitz SS, McGrath JJ, Lefkowitz DL, 1986. Effects of nitrogen dioxide on immune responses. J Toxicol Environ Health; 17:241-248.

Lenner M, 1987. Nitrogen dioxide in exhaust emissions from motor vehicles. Atmos Environ; 21(1):37-43.

Linn WS, Shamoo DA, Spier CE, Valencia LM, Anzar UT, Venet TG, Avol EL, Hackney JD, 1985a. Controlled exposure of volunteers with chronic obstructive pulmonary disease to nitrogen dioxide. Arch Environ Health; 40(6):313-317.

Linn WS, Soloman JC, Trim SC, Spier CE, Shamoo DA, Venet TG, Avol EL, Hackney JD, 1985b. Effects of exposure to 4 ppm nitrogen dioxide in healthy and asthmatic volunteers. Arch Environ Health; 40(4):234-239.

Lunn JE, Knowelden J, Handyside AJ, 1967. Patterns of respiratory illness in Sheffield infant schoolchildren. Br J Prev Sco Mid; 21:7-16.

Lyshkow NA, 1965. A rapid and sensitive colorimetric reagent for nitrogen dioxide in air. J Air Pollut Contr Assoc; 15:481.

MRC Advisory Group on Pulmonary Function Tests in Relation to Live Influenza Virus Vaccines, 1980. A study of live influenza virus vaccine in patients with chronic bronchitis. Br J Dis Chest; 74:121-127.

MRC Advisory Group on Pulmonary Function Tests in Relation to Live Influenza Virus Vaccines, 1984. Trials of live attenuated influenza virus vaccine in patients with chronic obstructive airways disease. Br J Dis Chest; 78:236-247.

McDonnell WF, Abdul-Salaam S, House DE, Hazucha MJ, 1986. Airway reactivity assessment by single dose and dose-response challenge to methacholine. Am Rev Resp Dis; 133:A375 (Abstract).

Melia RJW, Florey CduV, Altman DG, Swan AV, 1977. Association between gas cooking and respiratory disease in children. Br Med J; 2:149-152.

Melia RJW, Darley SC, Palmes ED, Goldstein BD, 1978. Differences in nitrogen dioxide levels in kitchens with gas or electric cookers. Atmos Environ; 12:1379-1381.

Melia RJW, Florey CduV, Chinn S, 1979. The relation between respiratory illness in primary school children and the use of gas for cooking. I. Results from a national survey. Inter J Epidem; 8(4):333-338.

Mohsenin V, 1986. Effect of nitrogen dioxide exposure on bronchial reactivity in normal and asthmatic subjects. Am Rev Resp Dis; 133: A215 (Abstract).

Morrow PE, 1984. Toxicological data on NOx: An overview. J Toxicol Environ Health; 13(2-3):205-227.

Mostardi RA, Woebkenberg NR, Ely DL, Conlon M, Atwood G, 1981. The University of Akron study on air pollution and human health effects. II. Effects on acute respiratory illness. Arch Environ Health; 36:250-255.

Murphy BR, Nelson DL, Wright PF, Tierney EL, Phelan MA, Chanock RM, 1982. Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. Infect Immun; 36:1102-1108.

Murphy BR, Clements ML, Madore HP, Steinberg J, O'Donnell S, Betts R, Demico D, Reichman RC, Dolin R, Maassab HF, 1984. Dose response of influenza A/California/10/78 (H1N1) cold-adapted reassortant influenza virus in adult volunteers. J Infect Dis; 149:816.

Murphy BR, Clements ML, Tierney EL, Black RE, Steinberg J, Chanock RM, 1985. Dose response of influenza A/Washington/897/80 (H3N2) avian-human reassortant virus in adult volunteers. J Infect Dis; 152(1):225-229.

National Research Council (NRC), 1977. Nitrogen oxides: Medical and biological effects of environmental pollutants. National Academy of Sciences. Washington, DC.

Nicholson KG, Tyrrell DAJ, Freestone DS, 1976. WRL 105 Strain (H3N2) live attenuated influenza vaccine: Acceptability, reactivity, and antibody response in normal, bronchitic, and geriatric volunteers. Lancet; i:1309-1311.

Orehek J, Massari JP, Gayrard P, Grimarud C, Charpin J, 1976. Effect of short- term, low-level nitrogen dioxide exposure on bronchial sensitivity of asthmatic patients. J Clin Invest; 57:301-307.

Prevost JM, Vereerstraeten-Schmerber J, Lamy F, De Koster JP, 1975. Live attenuated influenza virus vaccines in patients with chronic bronchopulmonary diseases. Scand J Resp Dis; 56:58-69.

Remijn B, Fischer P, Brunekreef B, Lebret E, Boleij JS, Noij D, 1985. Indoor air pollution and its effect on pulmonary function of adult non-smoking women: I. Exposure estimates for nitrogen dioxide and passive smoking. Intern J Epid; 14(2):215-220.

Robertson A, Dodgson J, Collings P, Seaton A, 1984. Exposure to oxides of nitrogen: Respiratory symptoms and lung function in British coal miners. Br J Indust Med; 41:214-219.

Rosenzweig DY, Dwyer DJ, Ferstenfield JE, Rytel NW, 1975. Changes in small airway function after live attenuated influenza vaccination. Am Rev Resp Dis; 111:399-403.

Saltzman BE, 1954. Colorimetric microdetermination of nitrogen dioxide in the atmosphere. Anal Chem; 26:1949.

Schenker MG, Samet JM, Speizer FE, 1983. Risk factors for childhood respiratory disease: The effect of host factors and home environment exposures. Am Rev Resp Dis; 128:1038-1043.

Shy CM, Creason JP, Pearlman ME, McClair KE, Benson FB, Young MM, 1970. The Chattanooga school children study: Effect of community exposure to nitrogen dioxide. J Air Poll Control Assoc; 20:582-588.

Snyder MH, Betts RF, DeBorde D, Tierney EL, Clements ML, Herrington D, Sears SD, Dolin R, Maassab HF, Murphy BR, in preparation. Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines.

Speizer FE, Ferris B Jr., Bishop YMM, Spengler JD, 1980. Respiratory disease rates and pulmonary function in children associated with nitrogen dioxide exposure. Am Rev Resp Dis; 121:3-10.

Spring SB, Maassab HF, Kendal AP, Murphy BR, Chanock RM, 1977. Cold-adapted variants of influenza A virus. I. Comparison of the genetic properties of ts mutants and five cold-adapted variants of influenza A virus. Virology; 77:337-343.

U.S. Environmental Protection Agency, 1981. Office of Air Quality Planning and Standards. Review of the National Ambient Air Quality Standards for Nitrogen Oxides. Assessment of Scientific and Technical Information. Research Triangle Park, North Carolina.

VonNieding G, Wagner HM, Krekeler H, Lollgen H, Fries W, Beuthan A, 1979. Controlled studies of human exposure to single and combined action of nitrogen dioxide, O_3, and SO_2. Int Arch Occup Environ Health; 43:195-210.

Ware JH, Docker DW 3d, Spiro A, Speizer FE, Ferris BG Jr., 1984. Passive smoking, gas cooking, and respiratory health of children living in six cities. Am Rev Resp Dis; 129(3):366-374.

Weiss ST, Tager IB, Speizer FE, Rosner B, 1980. Persistent wheeze: Its relation to respiratory illness, cigarette smoking and level of pulmonary function in a population sample of children. Am Rev Resp Dis; 122:697-707.

Winson IG, Smit JM, Potter CW, Howard P, 1977. Studies with live attenuated influenza virus in chronic bronchitis. Thorax; 32:726-728.

World Health Organization, 1977. Environmental health criteria No 4. Nitrogen oxides. Geneva.

Zeck R, Solliday N, Kehoe T, Berline B, 1976. Respiratory effects of live influenza virus vaccine: Healthy older subjects and patients with chronic respiratory disease. Am Rev Resp Dis; 114:1061-1067.

PULMONARY FUNCTION, NONSPECIFIC AIRWAY REACTIVITY, AND SYMPTOM MEASUREMENTS

The following measurements were obtained in Years 1 and 2 only, as discussed above in the study design section. During each day of chamber confinement, spirometric measurements were made before entering (t = 0 hours baseline) and exiting (t = 2 hours' exposure) the chamber on Days 1 through 4 and once on Days 12 and 33 (nine and 30 days post-inoculation). The spirometric measurements were made in accordance with the criteria set forth by the American Thoracic Society (American Thoracic Society Statement, 1979), using a 10-L Stead-Wells Spirometer interfaced with an Eagle Microprocessor (Warren E. Collins, Braintree MA). A minimum of three acceptable forced vital capacity (FVC) maneuvers were preformed. The two maneuvers with the highest sum of FVC and forced expiratory volume in one second (FEV_1) were used to determine the mean FVC, FEV_1, and $FEF_{25-75\%}$ (forced expiratory flow rate between 25 and 75% FVC).

Nonspecific airway reactivity was determined before exiting the chamber (t = 2 hours) on Days 1 through 4 as well as on Days 12 and 33 (nine and 30 days post- inoculation). Delayed effects on airway reactivity were sought, since airway reactivity has been shown to be increased up to four weeks after respiratory virus infection (Hobbins et al., 1982). A methacholine challenge was performed according to the method of Chai et al. (1975), with the following modifications. The methacholine aerosol (MMD = 1.4 um, GSD = 2.46) was administered in successively increasing doses, ranging from 3.1 to 50 mg/ml via a Bird micronebulizer No. 158. The subject inhaled from functional residual capacity to total lung capacity at an inspiratory flow rate of 30 L/min. Nebulization of the methacholine was triggered at 120 ml and continued for 0.6 second. Each subject received five inhalations of each successive concentration until a 25 percent reduction occurred in FEV_1 or the maximum dose of methacholine (50 mg/ml) was given. For each challenge, a dose-response curve was derived by a least-squares curve fitting. Since many normal subjects do not have a 25 percent decrease in FEV_1 with the maximum dose of 50 mg/ml, the percent decrease in FEV_1 for an equivalent maximum dose received on each day was recorded for statistical analysis.

Symptoms were collected before entering (t = 0 hours baseline) and before exiting (t = 2 hours' exposure) the environmental chamber on Days 1, 2, 3, and 4. The symptom questionnaire contained five questions regarding the presence and severity of nose or throat irritation, eye irritation, chest discomfort or tightness, cough, and headache. The subjects classified their response to each symptom as none, mild (present, but not annoying), moderate (annoying), or severe (debilitating).

ANALYSIS OF PULMONARY FUNCTION, NONSPECIFIC AIRWAY REACTIVITY, AND SYMPTOM MEASUREMENTS

A repeated-measures analysis of variance (ANOVA) was used to assess the effect of nitrogen dioxide exposure and viral infection on each pulmonary function. Two sets of data analyses were employed. One used as the dependent variable the ratio of the function response at t = 2 hours to the function response at t = 0 hours (t2/t0), comparing the nitrogen dioxide exposure effect for each function across Days 1 through 4; the other used as the dependent variable the function response at t = 2 hours only, comparing the nitrogen dioxide exposure effect (Days 2 through 4) and infectivity effect (Days 4, 12, and 33) for each function. A p value of 0.05 was considered statistically significant.

Four statistical comparisons were employed in the pulmonary function (FVC, FEV_1, and $FEF_{25-75\%}$) analysis, as follows:

1) Each function was compared between days within group (nitrogen dioxide exposure and clean air), using Tukey's test;

2) Each function was compared between groups (nitrogen dioxide exposure versus clean air) by day, using the t-test for two independent means;

3) Each function was compared between days within subsets of infected subjects (nitrogen dioxide exposure and clean air), using Tukey's test;

4) Each function was compared between groups (nitrogen dioxide exposure versus clean air) by day for the subsets of infected subjects, using the t-test for two independent means.

The same four sets of statistical comparisons were made to assess the effect of nitrogen dioxide exposure and infectivity on nonspecific airway reactivity.

Statistical comparisons 1 and 2 above were employed in determining symptom response to nitrogen dioxide. One dependent variable in the data analysis was the difference in symptom response at t = 2 hours to that at t = 0 hours (t2- t0).

PULMONARY RESPONSE (YEARS 1 AND 2)

Twenty-one subjects each year were analyzed for pulmonary changes in the clean air and nitrogen dioxide-exposure groups for Years 1 and 2. In the first year, two subjects were excluded from the pulmonary analysis because of either marked hyperreactivity to the methacholine aerosol or an upper respiratory illness on Day 33. One subject was excluded from the nitrogen dioxide-exposure group in Year 2 because of marked hyperreactivity to the methacholine aerosol.

Table 6 shows the symptom data for the clean air and 3 ppm nitrogen dioxide exposure groups in Year 2. No significant symptoms were reported.

The pulmonary function data (FVC and FEV_1 for both 2 and 3 ppm nitrogen dioxide) are shown in Tables 7 and 8. There are no significant adverse changes related to either nitrogen dioxide exposure or infectivity in pulmonary function (FVC, FEV_1, $FEF_{25-75\%}$). In the clean air group of the 2 ppm nitrogen dioxide study (Table 7), FVC and FEV_1 and $FEF_{25-75\%}$ (t2/t0 ratio) improved significantly on Day 4 compared to Day 1; and in the 3 ppm nitrogen dioxide-exposure group (Table 8), $FEF_{25-75\%}$ (t2/t0 ratio) improved significantly on Day 4 compared to Day 1. Each of these improvements was less than 3 percent.

In Year 1, the subjects in the clean air group received an average of 379 ± 101 cmu (cumulative methacholine units) and the subjects in the nitrogen dioxide group received 379 ± 107 cmu (2 ppm nitrogen dioxide, Table 9). Subjects in Year 2 all received 438 cmu (3 ppm nitrogen dioxide, Table 9). In the second year, the clean air group showed a significant decrease in methacholine responsiveness on the second, third, and fourth study day compared to the first study day. The decreased responsiveness was not present on the twelfth and thirty-third study days. By contrast, the nitrogen dioxide exposure group did not show a decrease in methacholine responsiveness on the second, third, and fourth study days. No significant changes in methacholine reactivity were observed in the first year of study.

Table 6. Percent of Individuals Reporting Symptoms with Exposure to 3 ppm NO_2

	Day 1	Day 2	Day 3	Day 4
Cough				
Clean Air Group	14% (3/21)	10% (2/21)	0% (0/21)	0% (0/21)
Exposure Group	5% (1/21)	5% (1/21)	0% (0/21)	0% (0/21)
Nose/Throat Irritation				
Clean Air Group	19% (4/21)	0% (0/21)	5% (1/21)	0% (0/21)
Exposure Group	5% (1/21)	10% (2/21)	10% (2/21)	10% (2/21)
Eye Irritation				
Clean Air Group	0% (0/21)	0% (0/21)	10% (2/21)	0% (0/21)
Exposure Group	5% (1/21)	5% (1/21)	5% (1/21)	0% (0/21)
Chest Irritation or Tightness				
Clean Air Group	10% (2/21)	5% (1/21)	0% (0/21)	0% (0/21)
Exposure Group	5% (1/21)	0% (0/21)	0% (0/21)	0% (0/21)
Headache				
Clean Air Group	14% (3/21)	0% (0/21)	0% (0/21)	10% (2/21)
Exposure Group	10% (2/21)	10% (2/21)	10% (2/21)	0% (0/21)

Note: The NO_2 exposure days are Days 2, 3, and 4 of the Exposure Group.

TABLE 7: Pulmonary Function Data at 2 ppm

		Forced Vital Capacity						Forced Expiratory Volume In One Second					
Clean Air Group		Day 1	Day 2	Day 3	Day 4	Day 12	Day 33	Day 1	Day 2	Day 3	Day 4	Day 12	Day 33
All (n = 21)	t = 0 hr (L)	4.66	4.64	4.56	4.55	—	—	3.90	3.87	3.85	3.84	—	—
	t = 2 hr (L)	4.51	4.48	4.48	4.49	4.53	4.51	3.73	3.75	3.75	3.77	3.78	3.76
	t_2/t_0 ratio	0.966	0.966	0.980	0.988*	—	—	0.956	0.967	0.971	0.980*	—	—
Infect (n = 14)	t = 2 hr (L)	4.56	4.56	4.54	4.53	4.58	4.54	3.83	3.87	3.88	3.87	3.88	3.85
NO_2 Exposure Group													
All (n = 21)	t = 0 hr (L)	4.50	4.46	4.47	4.40	—	—	3.85	3.86	3.88	3.86	—	—
	t = 2 hr (L)	4.35	4.33	4.34	4.32	4.32	4.35	3.73	3.70	3.74	3.75	3.72	3.75
	t_2/t_0 ratio	0.968	0.972	0.973	0.981	—	—	0.968	0.958	0.965	0.970	—	—
Infect (n = 12)	t = 2 hr (L)	4.33	4.28	4.32	4.30	4.30	4.34	3.79	3.79	3.84	3.81	3.82	3.84

* $p < 0.05$, Day 4 vs Day 1.

Table 8. Pulmonary Function Data at 3 ppm

		Forced Vital Capacity						Forced Expiratory Volume In One Second					
Clean Air Group		**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 12**	**Day 33**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 12**	**Day 33**
All (n = 21)	t = 0 hr (L)	4.89	4.84	4.85	4.83	—	—	4.14	4.12	4.13	4.12	—	—
	t = 2 hr (L)	4.78	4.76	4.74	4.72	4.79	4.73	4.04	4.03	4.03	4.00	4.07	4.00
	t_2/t_0 ratio	0.977	0.983	0.977	0.976	—	—	0.976	0.978	0.976	0.970	—	—
Infect (n = 15)	t = 2 hr (L)	4.83	4.80	4.77	4.75	4.84	4.78	4.15	4.14	4.12	4.09	4.17	4.09
NO_2 Exposure Group													
All (n = 21)	t = 0 hr (L)	4.64	4.64	4.65	4.66	—	—	3.91	3.93	3.96	4.00	—	—
	t = 2 hr (L)	4.57	4.56	4.59	4.52	4.58	4.54	3.85	3.84	3.92	3.91	3.91	3.89
	t_2/t_0 ratio	0.983	0.982	0.985	0.971	—	—	0.985	0.976	0.987	0.979	—	—
Infect (n = 16)	t = 2 hr (L)	4.48	4.50	4.50	4.42	4.50	4.45	3.83	3.83	3.87	3.86	3.87	3.86

Table 9. Bronchial Provocation with Methacholine — Percent Decrease FEV_1

		2 ppm						3 ppm						
Clean Air Group		**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 12**	**Day 33**		**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 12**	**Day 33**
All (n = 21)	t = 2 hr	11.0	10.7	10.8	10.4	12.1	13.4	(n = 21)	10.1	7.0#	7.5*	7.5*	10.5	10.5
Infect (n = 14)	t = 2 hr	9.5	8.9	10.5	9.6	10.5	12.9	(n = 15)	10.7	7.1	7.7	7.6	9.7	11.4
NO_2 Exposure Group														
All (n = 21)	t = 2 hr	13.7	12.7	12.3	12.5	16.6	14.0	(n = 21)	9.3	9.9	9.2	7.8	9.6	9.6
Infect (n = 12)	t = 2 hr	13.3	12.3	11.4	10.7	14.2	13.4	(n = 16)	9.0	9.0	7.5	6.6	8.6	9.4

* $p < 0.05$, Days 3 or 4 vs Day 1.
# $p < 0.01$, Day 2 vs Day 1.

HEALTH REVIEW COMMITTEE'S REPORT

GOALS AND OBJECTIVES

During the past 25 years, there have been numerous studies in laboratory animals, as well as epidemiological investigations, which suggested that exposure to oxidant air pollutants enhanced susceptibility to infection by inhaled microorganisms. Attempts to demonstrate this interaction in controlled clinical studies of viral infections in humans have not been reported. In this study, the investigators tested the hypothesis that human exposure to nitrogen dioxide increases susceptibility to laboratory-induced respiratory infection with cold-adapted influenza A virus.

The originally proposed objectives of this study were:

1) to determine the effects in human volunteers of nitrogen dioxide exposure on susceptibility to virus infection as determined by either virus recovery or significant rises in nasal wash or serum antibody, or both;

2) to evaluate the importance of timing of nitrogen dioxide exposure relative to virus inoculation;

3) to characterize the dose response relationships between nitrogen dioxide concentrations and susceptibility to infection; and

4) to detect changes in symptoms and pulmonary function or nonspecific airways reactivity secondary to either nitrogen dioxide exposure or virus infection, or both. All of these objectives were addressed in the research and described in the draft final report, with the exception of the studies of exposure timing, (#2 above) which was omitted after consultation with, and approval of, the HEI staff and the Health Research Committee.

SUMMARY OF INVESTIGATORS' CONCLUSIONS

Exposure of healthy, non-smoking volunteers to 1, 2, or 3 ppm of nitrogen dioxide for two hours a day for three consecutive days:

1. Did not significantly alter the rates of infection of subjects who were intranasally administered influenza A virus as compared to those control subjects who were air- exposed.

2. Did not produce adverse changes in pulmonary function or non-specific airway reactivity to methacholine after either 2 or 3 ppm nitrogen dioxide exposure or virus infection, or both.

3. Resulted in higher frequencies of infection in two of the four nitrogen dioxide-exposed groups than the historical range of infection rates in non-exposed volunteers. This suggested to the authors a possible tendency of nitrogen dioxide to increase susceptibility to respiratory virus infection.

Overall, the investigators concluded that the results of this study to assess the effect of nitrogen dioxide on subjects' susceptibility to upper respiratory virus infection with cold-adapted influenza A virus were inconclusive.

TECHNICAL EVALUATION

ASSESSMENT OF METHODS AND STUDY DESIGN

The study was a randomized, blinded study performed over a period of three years. In each year, groups of subjects were exposed for two hours per day for three successive days either to 1, 2, or 3 ppm of nitrogen dioxide or to filtered clean air in a controlled environmental chamber. At the end of the second exposure day, each subject was inoculated intranasally with attenuated cold-adapted influenza A/Korea/82 (H3N2) reassortant virus. For each of four days after inoculation, the volunteers were examined for symptoms and had nasal washes collected to detect virus replication. Serum and nasal wash specimens also were collected three to four weeks after the virus administration to measure systemic and local antibody responses to the virus. In the first two years of the study, pulmonary function and bronchial reactivity to methacholine were measured for the first four study days and at nine and 30 days post-inoculation. Volunteers were selected from groups of several hundred people who were recruited from the Baltimore metropolitan area. Criteria for selection included: 1) seronegative to influenza A virus; 2) nonallergic to bovine or egg allergens or selected antibiotics; 3) in excellent health with no upper respiratory infection; 4) normal baseline blood chemistry and normal pulmonary function; and 5) between the ages of 18 and 35 years. Each test group was made up of 21 to 23 subjects randomly distributed into control or nitrogen dioxide- exposure chambers. Only the chamber technicians were aware of individual group assignments, and interpretation of all data was done on coded specimens or volunteers' assigned numbers in order to avoid bias as much as possible.

The methods used for detecting infection with virus are appropriate, and the methods for assessing pulmonary function are standard methods and suitable for the purpose intended. Analysis of the exposure-chamber air concentrations showed very tight control, so the stated exposure level has very little variation. The choice of the highly attenuated, low-virulent virus used in the study may have contributed to the generally negative findings about the effects of nitrogen dioxide on virus infectivity or effects of nitrogen dioxide and viral infection on pulmonary function. The authors acknowledged that a more virulent virus might have led to different conclusions, but in the interest of safety to the volunteers (and their families or close associates who might be subject to

secondary infection) they felt the choice of the well-characterized, low virulent, cold-adapted influenza A virus was appropriate.

It is possible that the inclusion of only infected subjects when determining the duration of virus shedding may potentially lead to serious bias; the use of life-table analysis would seem more appropriate. Also, it is not clear that the investigators were justified in pooling the control group data for each of the three years, during which a high degree of variability in percent shedding between the groups was found.

The sample size of the experimental and control groups was probably too small to enable detection of small differences in infectivity between control and nitrogen dioxide-exposed subjects. The investigators acknowledged that criticism as a possible explanation for an apparent lack of significant effects, but in a separate communication they stated that they felt that their analyses permitted at least 70 percent power for detecting a difference between control and nitrogen dioxide-exposed groups as small as 0.8 standard deviations. Furthermore, they indicated that the sample sizes also were limited by practical considerations of cost and overall availability of qualified subjects who would be able to complete the study in a timely manner.

The investigators' choice of young, healthy, college-attending adults for subjects may have resulted in their using the most resistant subpopulation for viral infections. This could be a further factor in the generally negative results of the study.

INTERPRETATION OF RESULTS

The investigators have reached the appropriate conclusion that, under the conditions employed, exposures to 1, 2, or 3 ppm of nitrogen dioxide did not cause significant changes in susceptibility either to virus infection or to changes in pulmonary function or airway sensitivity, with or without superimposed cold-adapted influenza A virus exposure. The investigators tended to interpret certain insignificant trends in the data as indicative of possible increase in viral infectivity in nitrogen dioxide-exposed subjects; however, such a conclusion cannot be drawn on the basis of the present data. Furthermore, the authors speculate as to the possible reasons for lack of pulmonary function effects with nitrogen dioxide-exposures or nitrogen dioxide plus virus exposure. The authors' interpretations are speculative, and no data were obtained to support the suggested possibility that a subtle effect of nitrogen dioxide on the cholinergic system might explain observed differences in airway responsiveness to methacholine.

ATTAINMENT OF STUDY OBJECTIVES

The investigators completed the studies that had been proposed in their original specific aims, with the exception of the temporal relationship between nitrogen dioxide exposure and infectivity, which was omitted in accordance with an HEI agreement. Within the context of the method and experimental design employed, the study objectives were attained.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

This study does not resolve the question as to whether low-level exposure to nitrogen dioxide increases human susceptibility to respiratory viral infection. The highly attenuated virus used in this research may not accurately reflect the situation that would be attained with more virulent infectious viruses administered to human volunteers. Further clinical research to test this hypothesis directly could only be conducted after a careful review of ethical considerations and an assurance of adequate protection of subjects' safety and the safety of those who might be exposed secondarily. However, this work begins to fill a gap in existing nitrogen dioxide studies; previously there had been no attempt to model nitrogen dioxide and viral behavior in humans.

The nitrogen dioxide exposure periods used in this study were very limited (two hours on each of three successive days). It is not known if different exposure regimens would alter susceptibility to infection with the virus used.

CONCLUSIONS

The findings of this controlled clinical research project are inconclusive rather than unequivocally negative, so it would be wise not to draw any conclusions regarding the validity of the hypothesis that nitrogen dioxide exposure (1 to 3 ppm nitrogen dioxide for two hours a day, on three successive days) alters the frequency or severity of influenza infection in humans.

The results of this study in human volunteers suggests that safe, controlled clinical research on susceptibility to viral infections after nitrogen dioxide exposure may have serious limitations with respect to testing the original hypothesis of increased sensitivity in humans suggested by previous epidemiologic and animal studies. If the viral infectivity model used in this study is to have further application in this regard, it appears that a more virulent virus challenge, a different sequence or timing of nitrogen dioxide exposure, and a much larger study to test its validity would be required.

REFERENCES

Belshe RB, Van Voris LP, Mufson MA, 1983. Impact of viral respiratory diseases on infants and young children in a rural and urban area of southern West Virginia. Am J Epidemiol; 117:467.

Ehrlich R, Fenters JP, 1973. Influence of NO_2 on experimental influenza in squirrel monkeys. In: Proceedings of the 3rd International Clean Air Congress 8-12 Oct 1973: pp A11- A13. Dusseldorf, Federal Republic of Germany. Sponsored by The International Union of Air Pollution Prevention Association, Dusseldorf VDI-Verlag Gmbh.

EPA, 1981. Review of the National Ambient Air Quality Standards for Nitrogen Oxides: Assessment of Scientific and Technical Information. OAQPS. No. EPA450-5-82-002.

Fenters JP, Ehrlich R, Findeeg J, Spengler J, Tolkaiz V, 1971. Serologic response in squirrel monkeys exposed to NO_2 and influenza virus. Amer Rev Resp Dis; 104:448-451.

Gardner DE, 1982. Use of experimental airborne infections for monitoring altered host defenses. Environ Health Perspect; 43:99-107.

Glezen WP, Loda FA, Clyde WA, Senior RJ, Sheaffer CI, Conley WG, Denny FW, 1971. Epidemiologic patterns of acute lower respiratory disease of children in a pediatric group practice. J Pediatr; 78:397-406.

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

Henry MC, Findlay J, Spengler 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.

Loda FA, Glezen WP, Clyde WA, 1972. Respiratory disease in group day care. Pediatrics; 49:428.

Monto AS, Ullman BM, 1974. Acute respiratory illness in an American community. The Tecumseh study. JAMA; 227:164-169.

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

Samet JM, Marbury MC, Spengler JD, 1987. Health effects and sources of indoor air pollution. Unpublished results.

Von Nieding G, Wagner HM, Krekelrer H, Schmidt U, Muysers K, 1970. Adsorption of NO_2 in low concentrations in the respiratory tract and its acute effects on lung function and circulation. Presented at 2nd International Clean Air Congress of the International Union of Air Pollution Prevention Associations, Dec. 6-11, Washington D.C.

WHO, 1977. Environmental Health Criteria for Oxides of Nitrogen. World Health Organization, Geneva.

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RESEARCH REPORT No. 15: January, 1988