

**Modulation of Pulmonary Defense  
Mechanisms Against Viral and Bacterial  
Infections by Acute Exposures to Nitrogen Dioxide**

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**Includes the Report of the Institute's Health Review Committee**

**Research Report Number 20**

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## ABBREVIATIONS

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ANOVA	analysis of variance
BHI	brain-heart-infusion broth
CP-HBSS	citrate-phosphate-buffered Hank's balanced salt solution
EDTA	ethylenediaminetetraacetic acid
EID <sub>50</sub>	50 percent median egg infectious dose
HBSS	Hank's balanced salt solution
HEPA filter	high-efficiency particulate air filter
HEPES	N-2-hydroxyethyl-piperazine-N-ethane sulfonic acid
N <sub>2</sub>	nitrogen
NO <sub>2</sub>	nitrogen dioxide
<sup>32</sup> P	radiolabeled phosphorus
PBS	phosphate-buffered saline
PMNs	polymorphonuclear leukocytes
ppm	parts per million
TSA	trypticase soy agar
TSB	trypticase soy broth

# Modulation of Pulmonary Defense Mechanisms Against Viral and Bacterial Infections by Acute Exposures to Nitrogen Dioxide

George J. Jakab

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### ABSTRACT

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The scientific literature suggests that ambient levels of nitrogen dioxide increase susceptibility to respiratory infections. However, this association has not been conclusively demonstrated. The epidemiologic data regarding this relationship are inconclusive because these studies have used parameters of "acute respiratory illness" that are not necessarily related to infectious episodes. Previous animal studies have used either mortality after bacterial infection with virulent bacteria or decreased rate of intrapulmonary killing of bacteria with low virulence. Studies using appropriate bacterial and viral challenge organisms, with morbidity as an endpoint, provide a better basis for extrapolation to humans. The investigations in animals suggest a relationship between nitrogen dioxide and increased susceptibility to respiratory infection, but studies in which functional parameters of host resistance to such infections have been used are few.

The aim of this work was to determine the threshold level of acute nitrogen dioxide exposure that would induce increased susceptibility to, and increased severity of, viral and bacterial infections. Physiologic parameters of host resistance to respiratory infections were used as endpoints. A composite picture was developed of dose-response relationships between nitrogen dioxide and the impairment of a spectrum of defense parameters in the murine respiratory tract against viral and bacterial challenges.

The salient findings of this study are as follows: (1) the intrapulmonary killing of *Staphylococcus aureus* was impaired at 5 ppm of nitrogen dioxide; (2) this effect was found at 2.5 ppm or less when nitrogen dioxide exposure was superimposed on lungs predisposed to lowered resistance through immunosuppression with corticosteroids; (3) the adverse effect of nitrogen dioxide occurred at lower concentrations when exposure followed bacterial challenge; and (4) during the course of murine Sendai virus infection, exposure to nitrogen dioxide for four hours per day did not alter the infection in the lungs, but rather it enhanced lung pathology.

The implications of these findings are that the antibacterial defenses of the lungs are susceptible to the inhibiting effects of short acute exposures of lower concentrations of nitrogen dioxide when the lungs are predisposed by bacteria present or, even more so, by immunosuppression. The alveolar macrophage phagocytic system is the defense component of the lungs that is most susceptible to the adverse effects of nitrogen dioxide. The finding that nitrogen dioxide increases virus-associated lung damage suggests that the increased severity of the disease process results from the proliferation of the virus to high titers, rather than from alterations of the infective process.

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### INTRODUCTION

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Nitrogen dioxide (NO<sub>2</sub>) causes lung injury at high concentrations (Lowry and Schuman 1956; National Research Council 1977), but its effects at levels encountered in outdoor and indoor air have been difficult to characterize. Most studies of the relationship between exposure to NO<sub>2</sub> and health have focused on respiratory symptoms and illnesses and on changes in pulmonary function. Experimental investigations support the choice of these outcome measures because NO<sub>2</sub> may damage the lung directly, through its oxidant properties, or indirectly, by altering the defense mechanisms of the lung, and thereby increasing its susceptibility to respiratory infections.

The current U.S. National Ambient Air Quality Standard for NO<sub>2</sub> is 0.05 parts per million (ppm) for the annual arithmetic mean of 24-hour values (U.S. Environmental Protection Agency 1981). Average 24-hour NO<sub>2</sub> concentrations in urban atmospheres vary widely, but in some cities they commonly reach the range of 0.05 to 0.2 ppm. Some urban communities report short-term (spike) elevated NO<sub>2</sub> concentrations as high as 0.5 ppm (California Air Resources Board 1983). Recognition, in the late 1970s, that indoor NO<sub>2</sub> sources also contribute to personal exposure, and that indoor concentrations often exceed outdoor concentrations in many homes (Shy et al. 1978), gave impetus to the study of the sources and effects of indoor NO<sub>2</sub>. Combustion of gas during cooking releases NO<sub>2</sub>. On the average, normal use of an unvented gas cooking range adds 0.025 ppm of NO<sub>2</sub> to the background concentration in the home (Spengler et al. 1983). The increase is greater during cold weather when the air exchange is usually

reduced. Peak levels in the kitchen may reach 0.2 to 0.4 ppm during cooking with a gas range (Spengler and Sexton 1983). Therefore, personal exposures to NO<sub>2</sub> are higher for people living in homes with gas stoves and ovens than for people living in homes with electric stoves. In addition, exposure from gas cooking stoves is widespread; 50 percent of homes in the United States have gas cooking appliances (U.S. Department of Commerce 1983).

Data on the health effects of NO<sub>2</sub> concentrations likely to be encountered by the general population are derived from experimental and epidemiologic studies. Some controlled human exposure studies suggest that levels comparable to those measured in homes may increase airway reactivity in some persons with asthma, but the results of other studies are inconclusive (Orehek et al. 1976; Hazucha et al. 1983; Kleinman et al. 1983; Morrow 1984; Bauer et al. 1986).

The majority of epidemiologic studies have been cross-sectional surveys of school children. The investigators generally assessed symptom status, pulmonary function, and retrospective illness histories obtained by parent-completed questionnaires. However, consistent evidence of excess respiratory symptoms in children exposed to gas stoves has not been demonstrated.

## NITROGEN DIOXIDE AND RESISTANCE TO RESPIRATORY INFECTIONS

### Epidemiology

Studies designed specifically to detect an association between NO<sub>2</sub> exposure and increased susceptibility to respiratory infections are few. The many epidemiologic studies that have been reported (Shy et al. 1970a,b; Pearlman et al. 1971a,b; Cohen et al. 1972; Speizer and Ferris 1973a,b; Melia et al. 1977, 1978, 1979; Ferris 1978; Florey et al. 1979, 1982; Goldstein et al. 1979, 1981; Keller et al. 1979a,b; Speizer et al. 1980a,b; Love et al. 1982a,b; Schenker et al. 1983; Hoek et al. 1984a,b; Ware et al. 1984; Fischer et al. 1985; Remijin et al. 1985) focused on the association between exposure to NO<sub>2</sub> and a group of symptoms generally termed "acute respiratory illness." The respiratory parameters examined (for example, runny nose, sore throat, cough, phlegm, wheezing, nasal congestion, colds going into the chest) are not necessarily related to infectious episodes, but rather may be due to such other factors as the irritant effect of NO<sub>2</sub> or increased bronchial hyperreactivity.

It is unclear how these symptoms of acute respiratory illness became equated with acute respiratory infections; there is no direct epidemiologic evidence of the relationship between exposure to NO<sub>2</sub> and increased acute

respiratory infection. There have been no epidemiologic studies in which infectious parameters were rigorously examined (for example, isolation of the infectious agent during symptomatic episodes and seroconversion). The Health Effects Institute is currently attempting to remedy this by supporting a feasibility study by Dr. Jonathan M. Samet of the University of New Mexico, Albuquerque, NM.

### Experimental Studies

Recognition of the association between exposure to NO<sub>2</sub> and the development of acute respiratory illness has led to the use of animal models that have used microbiologic parameters to evaluate toxicity. Specifically, the effect of exposure to NO<sub>2</sub> on the outcome of host-parasite interactions in the lung has been explored (Goldstein et al. 1976; Ehrlich 1980; Gardner 1984; Goldstein 1984; Green 1984). The aerosol model of rodent infections provides an excellent means of measuring pollutant-induced physiologic abnormalities of antibacterial activity, because sufficient similarity exists between the defense mechanisms of rodents and humans to permit the use of the rodent as a surrogate (Goldstein 1984; Green 1984). Detection of NO<sub>2</sub>-induced abnormalities in individual components of the pulmonary antibacterial system is a sensitive means of assessing potential toxicity and of providing evidence to test the hypothesis that NO<sub>2</sub> may increase susceptibility to respiratory infections in humans.

The bulk of the studies have been performed with a rodent model that is often referred to as the "infectivity model," developed by Ehrlich (1963, 1966, 1980), Coffin and Bloomer (1967), Coffin and Gardner (1972), Gardner and Graham (1972), and coworkers (Purvis and Ehrlich 1963; Ehrlich and Henry 1968; Henry et al. 1969, 1970; Ehrlich et al. 1979; Illing et al. 1980; McGrath and Oyervides 1982, 1985; Gardner 1984). This model links interference with pulmonary antibacterial activity to disease and mortality. Rather than infecting rodents with minimally virulent organisms (such as staphylococci) that do not provoke injurious responses in the rodent lung (Goldstein et al. 1974; Pierce et al. 1977), in the infectivity model rodents are challenged by aerosol inhalation with highly virulent *Streptococcus pyogenes* (group C) or *Klebsiella pneumoniae* before or after NO<sub>2</sub> exposure. When these pathogens are used the physiologic alterations in lung antibacterial defenses induced by NO<sub>2</sub> exposure result in bacterial proliferation and excessive mortality (base-line mortality in the infectivity model is approximately 40 percent with the *Klebsiella* species and 20 percent with *S. pyogenes*). Studies with this model have shown that when a species of *Klebsiella* is used as the infecting organism, a two-hour exposure to 3.5 ppm of NO<sub>2</sub> results in increased mortality

(Ehrlich 1966). When *S. pyogenes* was used, increased mortality was observed after a single three-hour exposure to 2 ppm of NO<sub>2</sub> (Ehrlich et al. 1977).

An important conclusion that can be supported from the use of the infectivity model is that NO<sub>2</sub> exposure has reduced the host defenses. Furthermore, these studies point to the importance of the infecting organism in the assessment of the health effects of NO<sub>2</sub> exposure, because the toxic effect occurred at a lower concentration when *S. pyogenes* was used as the infecting organism.

Considerable caution must be used in applying the conclusion from the infectivity model to humans (Weil 1972; Colucci et al. 1973; Krasovskii 1976; Bouhuys et al. 1978; Jakab 1980; Book 1982; Gardner 1984; Goldstein 1984; Green 1984; Garattini 1986). As Green (1984) states:

In man, the association is with increased symptoms of cough, phlegm, and colds going into the chest. These are nonspecific symptomatic changes that could be attributable to irritant as well as infectious etiologies. If infective episodes were involved in humans, as could be the case in chronic bronchitis, they would have the character of exacerbating purulent bronchitis instead of pneumonias and would be related to endogenous flora rather than to newly acquired pathogens. Such infections are nonlethal by replication of bacteria and invasion of tissues and bloodstream. The underlying health effect, except where severe chronic disease is present, is characterized as a minor morbidity rather than a mortality incident.

The aerosol model for rodent infections that (1) measures minor changes in symptomatology and physiology rather than major lethal events, (2) does not depend on the virulence of pathogenic microbial species, (3) involves infection as an outcome and not a cause of the physiologic derangement, and (4) concentrates on physiologic rather than pathologic measures of host defense (Colucci et al. 1973) was developed by Laurenzi and coworkers (1964), Green and Goldstein (1966), and their colleagues. This system of analysis utilizes quantitative bacteriologic monitoring of lung tissues in animals exposed to NO<sub>2</sub>. This methodology provides an in vivo evaluation of microbicidal function of the lung; thus, the model uses physiologic parameters of host resistance against bacterial challenge as an endpoint (Green and Goldstein 1966). In one of the few studies in which this model was applied to evaluate the toxicity of NO<sub>2</sub>, mice were challenged with aerosolized *S. aureus* and then exposed to various concentrations of NO<sub>2</sub> (0 to 14.8 ppm) for four hours (Goldstein et al. 1973). The lungs were then removed and the quantities of viable bacteria that remained in the lungs were determined. Animals exposed to NO<sub>2</sub> levels of 1.9 ppm cleared bacteria

as well as animals in the control groups did. However, pulmonary bactericidal capacity was progressively impaired with increasing concentrations in groups exposed to 3.8 ppm NO<sub>2</sub> or greater.

The above finding points to the lack of a clear association between exposure to NO<sub>2</sub> and increased susceptibility to respiratory infections. The epidemiologic data are ambiguous: Infection cannot be affirmed as causative, whereas the experimental model used most often at ambient levels of NO<sub>2</sub> exposure measures death as an endpoint, rather than minor changes in morbidity that are applicable to the human situation.

The remainder of this introduction deals with respiratory defense mechanisms against viral and bacterial infections as a basis for understanding the experimental results and the possible interaction between NO<sub>2</sub> and respiratory infections in human populations.

## RESPIRATORY DEFENSE MECHANISMS AGAINST BACTERIAL INFECTIONS

Although bacteria enter the lungs daily by inhalation of small droplets or by aspiration from the upper respiratory tract, the distal airways and the alveoli are normally sterile. This is because the normal lung has the inordinate capacity to inactivate bacteria (Green et al. 1977). Bronchopulmonary defense mechanisms against bacterial infections depend primarily on the integrated activity of the phagocytic and immune systems (Green et al. 1977). In the normal lung, the alveolar macrophage serves as the surveillance phagocyte (Goldstein et al. 1977a). This resident phagocytic system can be augmented by the intraalveolar influx of polymorphonuclear leukocytes (PMNs) to provide the lungs with additional defense capabilities (Rehm et al. 1979, 1980). Specific immune mechanisms augment the biocidal defenses of the lung by enhancing phagocytic activity (Jakab 1976).

Experimental studies have shown that exposure to NO<sub>2</sub> impairs pulmonary antibacterial defenses (Ehrlich 1966; Ehrlich and Henry 1968; Buckley and Loosli 1969; Goldstein et al. 1973; Ehrlich et al. 1977), and studies have examined the effects of NO<sub>2</sub> on various lung-defense and cell-function parameters (Sherwin et al. 1968; Gardner et al. 1969; Valand et al. 1970; Acton and Myrvik 1972; Giordano and Morrow 1972; Freeman et al. 1974; Hattori and Takemura 1974; Aranyi et al. 1975; Hadley et al. 1977; Schiff 1977; Amoroso et al. 1981), but the effect of NO<sub>2</sub> on the integrated defenses has not been systematically approached. It is possible to study the individual components of the coordinated biocidal mechanisms of the lungs by using different challenge organisms to probe specific defense parameters. In this research, *S. aureus* was used

as a probe to study the functional integrity of the alveolar macrophage phagocytic system, and the gram-negative bacteria *Proteus mirabilis* and *K. pneumoniae* were used to examine the dual phagocytic systems of the lungs consisting of both the resident alveolar macrophages and newly recruited inflammatory PMNs into the alveolar spaces (Pierce et al. 1977; Rehm et al. 1979, 1980). Finally, *Pasteurella pneumotropica*, a gram-negative rodent respiratory bacterium (Brennan et al. 1965, 1969), was used to examine the effect of NO<sub>2</sub> on pulmonary antibacterial defenses against an organism endogenous to the host.

The rationale for the selection of gram-positive and gram-negative organisms to probe the individual defense mechanisms of the lungs is as follows. Inhalation challenges with the number of *S. aureus* used in these studies do not result in an inflammatory response of the lungs (Pierce et al. 1977; Lipscomb et al. 1983); therefore, intrapulmonary killing of this organism is dependent on the alveolar macrophage phagocytic system (Goldstein et al. 1977a; Lipscomb et al. 1983; Onofrio et al. 1983). On the other hand, inhalation challenges with gram-negative organisms result in a brisk inflammatory response of the lungs by an influx of PMNs (Jay et al. 1976; Pierce et al. 1977; Rehm et al. 1980). That the PMNs play a role in resistance to gram-negative infections is indicated by the observation that intrapulmonary killing of gram-negative bacteria, but not of *S. aureus*, is suppressed in neutropenic animals (Rehm et al. 1979), and recruitment of PMNs into the lungs prior to bacterial challenges enhances pulmonary bactericidal activity (Rylander et al. 1975).

At the cellular level, the bactericidal armamentarium of the pulmonary phagocyte rapidly inactivates and degrades inhaled organisms within hours of their entrance into the alveolar region (Kim et al. 1976). The ability of the resident macrophage to seek out, ingest, and inactivate bacteria results from the integration of a number of complex events. Phagocytes are attracted to bacteria by chemotactic factors either elaborated from the bacteria or formed as a result of the interaction of the bacteria with lung tissue. Bacterial ingestion is triggered by the attachment of the bacteria to both specific immunologic receptors and nonspecific membrane receptors. Once ingested, the bacteria are internally isolated in phagosomes. Lysosomes, containing microbicidal and degradative enzymes (Goldstein 1983), fuse with the phagosomes to form the phagolysosome, in which intracellular processing of the bacteria occurs. In addition to lysosomal killing mechanisms, alveolar macrophages are capable of inactivating organisms by oxygen-dependent mechanisms, such as those involving the superoxide radical anion, myeloperoxidase, and hydrogen peroxide; these microbicidal compounds are of primary importance in bacterial killing by phagocytes.

The bacterial challenges used herein with *S. aureus*, *P. mirabilis*, *K. pneumoniae*, and *P. pneumotropica* do not establish an infective process. Regardless of the challenge dose delivered by aerosol inhalation, the bacteria are rapidly eliminated; the lungs are virtually sterile approximately 24 to 48 hours after the bacterial challenge (Green and Kass 1964a,b; Laurenzi et al. 1964).

Infection is defined as "the multiplication of organisms" (Stedman 1972), and self-limiting infections in the healthy host represent the success of the host defense mechanisms in controlling the process. Because of the extraordinary capacity of lung defenses to maintain the sterility of the respiratory membrane, the best available model of a self-limiting bacterial infection is with the intracellular microorganism *Listeria monocytogenes*. After aerosol inhalation, this organism sets up a smoldering long-term infection in the lung that lasts approximately seven days (Leford et al. 1978, 1979; Jakab et al. 1981). Thereafter, with the appearance of the cell-mediated immune response (Truitt and Mackaness 1971), the bacteria are rapidly inactivated. The cell-mediated immune responses against *L. monocytogenes* infection involve specifically sensitized T-lymphocytes that secrete biologically active proteins, termed lymphokines. These lymphokines interact with alveolar macrophages to endow the latter with enhanced intracellular microbicidal activity (Harrington-Fowler et al. 1981; Miyata et al. 1982).

#### RESPIRATORY DEFENSE MECHANISMS AGAINST VIRAL INFECTIONS

In experimental models of Sendai virus and influenza virus infection, the severity and duration of the resulting illness depend on the infectious dose of the virus delivered to the respiratory tract (Jakab 1975). With infectious doses that cause moderate-to-severe pneumonitis, the virus proliferates rapidly in the lungs, reaching peak titers approximately three to five days after viral infection. Thereafter, pulmonary virus titers rapidly decline, with infectious virus no longer recoverable after the ninth day of infection (Appell et al. 1971; Jakab and Dick 1973).

During the acute stages of the infection, the ciliated epithelial cells of the conducting airways are the principal sites of viral replication. The ciliated epithelium degenerates and desquamates in the affected areas. Maximum histopathologic changes occur approximately a week after viral infection. At this time, the affected areas of the lung parenchyma are characterized by hyperemia and thickening of the alveolar walls, with interstitial infiltration with leukocytes and capillary thrombosis. The alveoli are congested and edematous, and contain leukocytic exudates. The virus-induced lesion begins to resolve by the ninth day of

the infection, as evidenced by the beginning of repair of the damaged areas of mucosa and the resolution of the consolidation in the lung parenchyma (van Nunen and van der Veen 1967; Robinson et al. 1968).

Host defenses against the virus infection include interferon, which nonspecifically prevents viral replication (Charlton and Blandford 1977; Zee et al. 1979; Wyde et al. 1982), and the specific antiviral immune response (Virelizier et al. 1979; Ada et al. 1981; Ennis 1982). Interferon concentrations in the lung are usually at their highest levels on approximately the fifth day of infection, and then decline as the virus disappears (Heath 1979; Hoshino et al. 1983). Specific antiviral immunoglobulins are detected in the lungs by the third day of infection, and are recovered from bronchial washings by day eight (Scott and Walker 1976; Charlton and Blandford 1977); serum antibodies usually appear by day eight (Heath 1979). In addition to the humoral immune response, cytotoxic T-lymphocytes sensitized to the viral antigen also appear in the lungs during the third day of infection. This response peaks at approximately day seven and then declines. Cytotoxic T-lymphocytes destroy virus-infected cells through a process involving the lysis of the target cell containing the viral antigen (Yap and Ada 1978; Wells et al. 1983).

## SUMMARY

Our knowledge of whether or not exposure to ambient levels of NO<sub>2</sub> increases susceptibility to respiratory infections is inconclusive. The epidemiologic data regarding this relationship leave room for doubt, as these studies have used parameters of acute respiratory illness that are not necessarily related to infectious episodes. Experimental studies in which functional parameters of host resistance to respiratory infections have been used are few. However, these few experimental studies indicate that acute NO<sub>2</sub> exposure at concentrations of 3.8 ppm or greater (Goldstein et al. 1973) impairs the intrapulmonary killing of bacteria in the lungs.

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## AIMS

The overall goal of this study was to determine the threshold concentration of NO<sub>2</sub> that, during an acute exposure, increases the susceptibility to, and the severity of, viral and bacterial respiratory infections. This aim was accomplished through studies of functional resistance mechanisms of the murine lung against bacterial and viral challenges; this provides a composite picture of the dose-response relationship between exposure to NO<sub>2</sub> and impairment of a spectrum of respiratory defense parameters. The specific objectives were to determine the concentration of NO<sub>2</sub> that corresponds with each of the following effects:

1. Decreases physical transport from the lungs;
2. Decreases intrapulmonary bacterial killing;
3. Decreases in vivo alveolar macrophage phagocytic activity;
4. Decreases auxiliary (PMN) phagocytic activity in the lungs;
5. Increases the severity of bacterial infection;
6. Increases the severity of viral infection;
7. Decreases intrapulmonary bacterial killing in the predisposed lung.

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## METHODS

### ANIMALS

White female Swiss mice weighing 20 to 23 g (Hilltop Laboratory Animals, Scotsdale, PA), and having no serologic evidence of Sendai virus infection, were used. Bacterial agglutination tests demonstrated serum antibodies against *P. pneumotropica*, but not against *S. aureus*, *P. mirabilis*, and *K. pneumoniae*. The animals were housed in filter-topped cages and provided with food and water ad libitum. Virus-infected animals and noninfected animals were kept in separate rooms. National Institutes of Health (NIH) Guidelines for care and use of laboratory animals were followed.

### BACTERIAL AGENTS

*S. aureus* (FDA strain 209P, phage type 42D), *P. mirabilis* (a laboratory strain), *K. pneumoniae* (a clinical isolate), *P. pneumotropica* (a fresh isolate from the nasopharynx of a mouse not from the supplier of the animals used herein), and *L. monocytogenes* (ATCC strain 7644) were used in our experiments.

Stock bacterial cultures were prepared by inoculating each of the organisms into 1,000 ml of brain-heart-infusion broth (BHI) and incubating the suspension for 24 hours at 37°C in a rotary-shaker water bath (Model G76; New Brunswick Scientific, New Brunswick, NJ). Thereafter, each bacterial preparation was concentrated 10-fold by centrifugation and suspended in 80 ml of BHI. After adding 20 ml of sterile glycerin to each suspension, the bacterial preparations were divided into 1-ml portions and frozen at -20°C. The purity of each culture was verified before use.

### VIRAL AGENTS

Stock viral cultures of parainfluenza 1 (Sendai) virus and mouse-adapted influenza A/PR8/34 virus were prepared by inoculating 0.1 ml of the virus into the allantoic cavity of 10-day-old embryonated chicken eggs (Truslow Farms, Chestertown, MD). The eggs were then incubated at 35°C for two days, chilled overnight at 4°C, and the allantoic

fluid from each egg was harvested and pooled. After centrifugation (500 × g; 10 minutes at 4°C), each virus preparation was divided into small portions and frozen at -70°C. Ten dozen eggs were used to prepare each virus stock. The infectious titer of the Sendai and influenza virus stock preparations was 10<sup>9</sup> 50 percent median egg infectious dose (EID<sub>50</sub>). The identities of the viruses were verified by serologic (hemagglutination-inhibition) tests against NIH reference reagents: V321-511-558 for Sendai virus and V301-511-552 for influenza A/PR8/34 virus.

### PREPARATION OF BACTERIA FOR INHALATION CHALLENGE

Of the stock bacterial preparations of *S. aureus*, *P. mirabilis*, *K. pneumoniae*, *P. pneumotropica*, and *L. monocytogenes*, 1 ml was inoculated into 200 ml of trypticase soy broth (TSB) and incubated at 37°C in a rotary-shaker water bath. After 18 hours the cultures were centrifuged (3,000 × g; 10 minutes), washed twice with 0.1 M phosphate-buffered saline (PBS; pH 7.6), and resuspended in 10 ml of TSB.

For the studies dealing with physical translocation of bacterial particles, the staphylococci were incubated in 150 ml of phosphorus-free culture medium containing 1 mCi of <sup>32</sup>P (Jakab and Green 1972). After 18 hours at 37°C in a shaking water bath, the labeled staphylococci were centrifuged, washed twice with PBS to remove all the unattached radiolabel, and resuspended in 8 ml of TSB.

### BACTERIAL CHALLENGE

A previously described (Ruppert et al. 1976) modification of the Henderson (1952) aerosol apparatus was used to challenge the animals with the bacteria by inhalation. The apparatus consists, in sequence, of a Collision atomizer activated by compressed air, a 6- by 60-cm length of Plexiglas tubing to mix the nebulized agent with diluting air, a large Plexiglas cylindrical chamber (28 by 80 cm) containing six cylindrical wire cages, a fiberglass prefilter, two absolute-type bacterial high-efficiency particulate air (HEPA) filters, and a vacuum pump. The atomizer was activated with 15 psi of compressed air and, at a rate of 6 liters per minute, produced a continuous cloud of small infectious droplets, 97 percent of which had an aerodynamic particle diameter of 3.5 μm or less (Jakab and Green 1972), as determined with an Andersen (1958) sampler.

The outlet of the nebulizer emptied into the open end of the small Plexiglas tubing, which acted as a mixing chamber in which the bacterial cloud from the nebulizer was mixed with a larger volume of air. The secondary air entered the mixing chamber around the nozzle of the atomizer. The vacuum pump, located at the downstream end of the apparatus, maintained the secondary air flow of approximately

20 liters per minute, as measured with a hot wire anemometer. Each end of the large cylindrical exposure chamber was removable, and was equipped at the center with a rectangular baffle plate suspended perpendicularly to the air stream. This baffle plate removed large droplets, and provided more uniform mixing and distribution of the bacteria in the exposure chamber.

The six cylindrical cages located in the exposure chamber were made of stainless-steel woven wire. Each holds 12 mice that are individually separated, in order to prevent the huddling of the animals, which would alter the number of bacterial particles the animals inhaled. To ensure more uniform distribution of the aerosol in the chamber, the cages rested on 2-cm-high offsets, which allowed a 2-cm airspace between the cages and the chamber wall. This space was then blocked by placing Plexiglas rings between the cages; the bacterial particles were channeled through the space holding the animals (Ruppert et al. 1976). The aerosol-generation apparatus, exposure chamber, and filter system were located in a HEPA-filtered reverse laminar flow hood (Baker Co., Sanford, ME). Animals were challenged for 30 minutes with each bacterial cloud, during which time 1 to 5 × 10<sup>5</sup> of each of the bacteria were deposited in the lungs.

### VIRAL INFECTION

Animals were infected with either parainfluenza 1 (Sendai) or influenza A/PR8/34 virus for 30 minutes in the same chamber, under identical conditions as were used for the bacterial challenges. For Sendai virus a 1:5 dilution of the stock virus was used, whereas a 1:50 dilution was used for infection with influenza A/PR8/34 virus. Each virus infection resulted in a moderate-to-severe pneumonitis, from which the animals readily recovered.

### BACTERICIDAL ASSAY

Pulmonary bactericidal activity was assessed by previously described methods (Ruppert et al. 1976). Briefly, animals were killed by luxation of the neck immediately (zero time) or four hours after cessation of bacterial challenge. The lungs were aseptically removed, trimmed of the trachea and major bronchi, and homogenized in 3 ml of iced TSB with an all-glass tissue homogenizer (Model K41; Tri-R Instruments, Rockville Center, NY). A 1.0-ml aliquot of the lung homogenate was diluted 10-fold in sterile PBS, and a 0.1-ml aliquot of the appropriate dilution was cultured quantitatively in quadruplicate on Petri-X dishes by standard microbiologic pour-plate methods. Trypticase soy agar (TSA) supplemented with 5 percent sodium chloride (NaCl) was used for *S. aureus*, TSA was used for *L. monocytogenes* and *K. pneumoniae*, and bismuth sulfite agar was used for *P. mirabilis*. *P. pneumotropica* was cultured on prepared TSA-5 percent sheep blood agar (Jakab and Dick 1973). The

Petri dishes were incubated for 48 hours at 37°C, and were then visually counted with a Quebec Colony Counter (Model 3325; American Optical, Buffalo, NY). Pulmonary bactericidal activity in each animal was calculated as the percentage of initial viable bacteria remaining after four hours by the following formula (Ruppert et al. 1976) (see Appendix A):

$$\text{Percent viable bacteria remaining} = \frac{\text{bacterial count (4 hr)}}{\text{mean bacterial count (0 hr)}} \times 100.$$

### RADIOASSAY PROCEDURE

Quantitative measurement of <sup>32</sup>P activity was performed on another 1.0-ml aliquot of the lung homogenate from animals challenged with radiolabeled *S. aureus* (Green and Goldstein 1966). The samples were prepared for liquid scintillation counting by digestion in 2.0 ml of 10x hyamine hydroxide (New England Nuclear, Boston, MA), overnight, at room temperature, in 20-ml screw-cap glass counting vials. After digestion, 5 ml of absolute ethyl alcohol and 10 ml of toluene-based liquid scintillation solution (Omni-fluor; New England Nuclear, Boston, MA) were added. The samples were assayed in a liquid scintillation spectrometer (Beckman Instruments Inc., Fullerton, CA). After correction for dilution, quench, and background, radioactivity was expressed as counts per minute per milliliter of lung homogenate assayed.

Physical transport of <sup>32</sup>P-labeled *S. aureus* from the lungs was determined by following the decline in radiotracer activity. The <sup>32</sup>P counts in the lungs of individual animals killed after four hours were expressed as a percentage of the mean radiotracer counts obtained from the animals killed immediately (Jakab and Green 1972).

### VIRUS TITRATION

At various times after Sendai virus infection, groups of animals were killed and their lungs removed aseptically. Each lung was homogenized in 2 ml of iced citrate phosphate-buffered Hank's balanced salt solution (CP-HBSS; pH 7.0). The lung homogenates were then incubated for five minutes at 37°C in a rotary-shaker water bath in order to elute the virus from the tissue. Thereafter, the pooled homogenates were centrifuged (500 × g, 5 minutes at 4°C), the supernatant aliquotted in 1.0-ml portions, and frozen at -70°C until assay.

Infectious pulmonary virus titers were determined by allantoic cavity inoculation of 10-day-old embryonated chicken eggs (Lennette and Schmidt 1974). A 0.1-ml inoculum of a 10-fold dilution of the pooled lung homogenate supernatant fluid (in CP-HBSS supplemented with 100 units of penicillin and 100 μg of streptomycin) was inoculated into each of four eggs. The eggs were then incubated at 35°C for two days, chilled at 4°C overnight, and then harvested

for allantoic fluid. Thereafter, 0.5 ml of each allantoic fluid was mixed with 0.5 ml of a 0.5 percent suspension of sheep erythrocytes, and the tubes were examined for hemagglutinin activity after incubation for one hour at room temperature. The EID<sub>50</sub> endpoint was calculated by the Karber method (Lennette and Schmidt 1974).

### COLLECTION OF PULMONARY CELLS

Total and differential cell counts of free pulmonary cells were made four hours after the inhalation challenge with bacteria (Astry et al. 1983). The animals were killed by brainstem compression and were bled by cardiac puncture. The lungs were surgically removed in toto. Pulmonary cells were collected by inserting a Pasteur pipette into the trachea and introducing and withdrawing 1.5 ml of lavage solution (0.85 percent NaCl, 0.1 percent glucose, 3 mM ethylenediaminetetraacetic acid [EDTA], and 20 mM N-2-hydroxyethyl-piperazine-N-ethane sulfonic acid [HEPES] buffer) three times; a total of 4.5 ml of lavage solution was used for each lung. After collection, the total number of cells from each animal was counted with the use of a hemacytometer. The cell suspensions were then centrifuged (400 × g; 10 minutes), the supernatants discarded, and the cells resuspended in Hank's balanced salt solution (HBSS) at 5 × 10<sup>5</sup> cells/ml. Duplicate sets of pulmonary cells were prepared by cytocentrifuging 0.2 ml of the cells so prepared (1,000 rpm for 10 minutes in a Cytospin II; Shandon Instruments, Sewickley, PA). Morphologic differentiation was performed on the cytocentrifuged preparations, which were stained with Diff-Quick (Dade Diagnostics, Agua, PR), by microscopically counting not less than 300 cells and scoring them as being either alveolar macrophages, PMNs, or lymphocytes.

### ALBUMIN DETERMINATIONS IN PULMONARY LAVAGE FLUID

The concentration of albumin was quantified in the first lavage fluid. After centrifugation to remove the lavaged cells, 20 μl of the supernatant was incubated with 100 μl of a commercially available 0.01 percent bromocresol green solution (Albumin Color Reagent; Sigma Chemical Co., St. Louis, MO). After 15 minutes at room temperature, the optical density was measured at 630 nm and the quantity of albumin (mg/ml) was estimated from a standard curve.

### LUNG WET:DRY WEIGHT RATIOS

Separate groups of mice were killed, and the lungs were excised, weighed, lyophilized overnight (Speedvac Concentrator; Savant Instruments Inc., Farmingdale, NY), and weighed again.

## NITROGEN DIOXIDE INHALATION EXPOSURE CHAMBERS

Two identical stainless-steel horizontal-flow environmental exposure chambers (Baker Company, Sanford, ME) were used in these studies (Appendix B). These chambers have been described and tested for reliable flow dynamics (Hemenway et al. 1982; Hemenway and MacAskill, 1982).

## NITROGEN DIOXIDE EXPOSURE

The NO<sub>2</sub> generation and quantification system used herein was newly developed in collaboration with our consultant, Dr. David R. Hemenway (Department of Civil and Mechanical Engineering, University of Vermont). Concurrent with this project, Dr. Hemenway was a coinvestigator of another project funded by the Health Effects Institute that dealt with NO<sub>2</sub> exposures (HEI Agreement No. 83-5). This newly developed NO<sub>2</sub> exposure system is detailed elsewhere (Hemenway and Jakab 1987).

Nitrogen dioxide was generated (Appendix C) by mixing the gas phase of liquid NO<sub>2</sub> (99.5 percent chemically pure grade; Matheson, East Rutherford, NJ) with nitrogen (N<sub>2</sub>) in a 50-liter Teflon gas-sampling bag (Pollution Measurement Corp., Chicago, IL). The mixing ratio of N<sub>2</sub>:NO<sub>2</sub> depended on the desired exposure concentrations. A stainless-steel and Teflon diaphragm pump (Model NO5; KNF Neuberger, Inc., Princeton, NJ), attached to a stainless-steel calibrated metering valve, delivered NO<sub>2</sub> to a small pre-mixing chamber for final dilution with HEPA-filtered room air before it entered one of the stainless-steel horizontal exposure chambers. Air flow to the exposure chambers was maintained at 8 cubic feet per minute, which provided a theoretical air turnover of 20 volume changes per hour.

Chamber concentrations of NO<sub>2</sub> were continuously monitored with a chemiluminescent NO<sub>2</sub> analyzer attached to a chart recorder (Columbia Scientific Instruments, Austin, TX), as described by Fontinin and associates (1970). The NO<sub>2</sub> monitor was calibrated by a primary calibration system with a permeation tube, as previously described (Hughes et al. 1977). A multipoint calibration was performed before each experiment.

Nitrogen dioxide concentrations were preset prior to the placement of the animals in the chamber. To place the animals in the chambers, the NO<sub>2</sub> flow into the diluent air was halted, the chamber flushed with the diluent air, the animals placed in the chamber, and the NO<sub>2</sub> flow started again. Once the NO<sub>2</sub> exposure levels were preset, evacuation of NO<sub>2</sub> from the chamber, transfer of the animals, and reestablishment of the preset concentration took approximately five minutes. Since the NO<sub>2</sub> concentrations were recorded with the chart recorder, the levels of NO<sub>2</sub> were calculated by measuring the area under the curve with an electronic graphics calculator (Numonics Corp., Lansdale,

PA). The exposure levels were reported as time-weighted averages for the exposure periods. Since real-time monitoring allows rapid adjustment for excursions from the target concentrations, the infrequent and slight drifts were immediately readjusted. The time-weighted exposure averages were within  $\pm 5$  percent of the target concentrations.

Two horizontal-flow exposure chambers were used in these studies. In one the mice were exposed to various levels of NO<sub>2</sub>; the other served as a control chamber to expose animals to an identical flow of HEPA-filtered room air without the NO<sub>2</sub>. The animals were housed in individual stainless-steel wire cages during the exposure periods.

## EXPERIMENTAL DESIGN

The experimental design depended primarily on the host-defense parameter being tested, or the specific question being asked about the effect of NO<sub>2</sub> exposure on resistance to respiratory infections.

### Intrapulmonary Antibacterial Defenses

Two basic protocols were used for the studies dealing with the effects of NO<sub>2</sub> exposure on the intrapulmonary killing of bacteria (*S. aureus*, *P. mirabilis*, *K. pneumoniae*, and *P. pneumotropica*). The first was to elucidate the effect of exposure to NO<sub>2</sub> after bacterial challenge. For these experiments, the animals were challenged with the bacteria and divided into three equal groups. One group was killed immediately after bacterial challenge (0 hr), whereas the second and third groups were exposed to NO<sub>2</sub> or ambient air, respectively, for four hours and then killed.

The second protocol was to assess the effect of preexposure to NO<sub>2</sub> on pulmonary bactericidal activity against *S. aureus* and *P. mirabilis*. For these studies, half the mice were exposed to ambient air for four hours. All the animals were then challenged with the bacterium. Half the NO<sub>2</sub>-preexposed group and half the ambient-air control group were killed immediately after bacterial challenge. The remaining half of each group were allowed to breathe ambient room air (in cages placed in the animal holding room) and then were killed at four hours.

Each group consisted of six to 10 animals. Depending on the protocol and the concentration of NO<sub>2</sub> used, each experiment was run at least two times and the results of the runs were pooled. The replicates of the experiments were needed for statistical evaluation.

### Resistance to Bacterial Respiratory Infection

To determine the effect of NO<sub>2</sub> exposure on resistance to long-term bacterial infection, mice were infected by aerosol inhalation with *L. monocytogenes* and then divided into two groups. In separate experiments, one group was exposed for four hours per day to either 10 or 20 ppm of NO<sub>2</sub>, while

the respective control groups were allowed to breathe ambient air for four hours per day in the second exposure chamber. Groups of mice were assayed at three, five, 10, and 14 days after bacterial infection.

### Resistance to Sendai Virus Infection

To examine the effect of NO<sub>2</sub> exposure on resistance to viral infections, mice were infected by aerosol inhalation with a sublethal dose of Sendai virus and then divided into two groups. In separate experiments, one group was exposed for four hours per day to either 5, 10, or 20 ppm of NO<sub>2</sub>, while the respective control groups were allowed to breathe ambient air for four hours per day in the second exposure chamber. Groups of virus-infected mice and noninfected mice were assayed at three, five, seven, and ten days after infection in order to determine pulmonary virus titers.

### Predisposed Host

#### *Influenza-Virus-Associated Bacterial Superinfections.*

Mice were infected by aerosol inhalation with a dose of influenza A/PR8/34, which caused a moderate pneumonitis. Eight days after infection, virus-infected mice and noninfected mice were challenged by aerosol inhalation with *S. aureus*. In separate experiments, half the virus-infected mice and noninfected mice were exposed for four hours to either 5 or 10 ppm of NO<sub>2</sub>, while the other half of the two groups were allowed to breathe ambient air for four hours in the second exposure chamber. Intrapulmonary bactericidal activity was assessed at four hours after bacterial challenge, as described above.

**Corticosteroid Treatment.** Mice were injected intramuscularly in the rear leg with 4 mg of sterile methylprednisolone acetate suspension (Depo-Medrol; Upjohn Co., Kalamazoo, MI) contained in a 0.1-ml volume. Control animals were injected intramuscularly with 0.1 ml of PBS. Four days later, all mice were challenged by aerosol inhalation with *S. aureus*, and the corticosteroid-treated animals and nontreated animals were separated into two groups. In separate experiments, half the treated mice and nontreated mice were exposed for four hours to either 1.0, 2.5, 5.0, or 10.0 ppm of NO<sub>2</sub>, while the other half of the two groups were allowed to breathe ambient air for four hours in the second exposure chamber. Intrapulmonary bactericidal activity was assessed at four hours after bacterial challenge, as described above.

### Summary of Experimental Design

1. Exposure after challenge: bacterial challenge → NO<sub>2</sub> for four hours → assay.
2. Exposure before challenge: NO<sub>2</sub> for four hours → bacterial challenge → four hours → assay.
3. Exposure before and after challenge: NO<sub>2</sub> for four hours → bacterial challenge → NO<sub>2</sub> for four hours.
4. Resistance to bacterial infection: infect with *L. monocytogenes* → expose to NO<sub>2</sub> for four hours per day → assay on days 0, 3, 5, 10, and 14.
5. Resistance to virus infection: infect with Sendai virus → expose to NO<sub>2</sub> for four hours per day → assay on days 0, 3, 5, 7, and 10.
6. Predisposed host 1: infect with influenza virus → eight days → bacterial challenge → NO<sub>2</sub> for four hours → assay.
7. Predisposed host 2: corticosteroid injection → four days → bacterial challenge → NO<sub>2</sub> for four hours → assay.

### STATISTICAL ANALYSIS

Statistical analysis was carried out using the differences between control groups and treated groups (excess survival of bacteria in the lungs) as the measurement analyzed. The experimental design was a randomized block design, with treatment combinations defined by concentration and presence or absence of exposure to NO<sub>2</sub> before or after bacterial challenge. The blocks were replications of the experiments, representing the different days on which the experiments were performed, and each concentration had between one and four replicates.

In order to determine if the between-replication variability was greater than the within-replication variability, a one-way analysis of variance was computed for the means of the differences of the replications, and the replication error term was compared with a comparable pooled variance term (calculated by dividing by the harmonic means of the group numbers [n]). If the replication error term was significantly greater than the pooled error term, a one-way analysis of variance, using the means of the differences, was the analysis adopted to determine significance. If the error terms were not significantly different, implying equal within- and between-replication variability, the analysis of variance was calculated by pooling all replications within a concentration. The post-hoc analysis used to determine which groups were significantly different was the Duncan's multiple range test.

Statistical design for the experiments with four exposure groups (the immunosuppressant and influenza virus experiments) was a randomized block design with four treatment combinations, defined by concentration of NO<sub>2</sub>. The blocks are replications of the experiments representing the different days on which the experiments were performed. A three-way analysis of variance was used to determine whether or not the between-replication variability was significantly different from the within-replication variability. If it was

significantly different, the F-ratios for testing treatment effects utilized appropriate error variances based on variations among replications and not the within-subgroup variation. Again, the Duncan's multiple range test was the post-hoc analysis used to determine significance.

## RESULTS

The data from the pooled runs at each NO<sub>2</sub> concentration are presented in the figures, followed by the appropriate reference to one of the appendices in which the individual value for each replicate is presented in tabular form.

### NITROGEN DIOXIDE GENERATION AND QUANTIFICATION

The NO<sub>2</sub> generation and quantification system is detailed in the Methods section, and in one of the papers that resulted from these studies (Hemenway and Jakab 1987).

Figure 1 shows a typical curve, indicating the excellent stability of the system over a four-hour exposure period. In general, the stability was better than  $\pm 2$  percent.

### PULMONARY ANTIBACTERIAL DEFENSES (NITROGEN DIOXIDE EXPOSURE AFTER BACTERIAL CHALLENGE)

#### *Staphylococcus aureus*

Pulmonary antibacterial defenses against *S. aureus* are primarily dependent on the alveolar macrophage phagocytic system (Goldstein et al. 1977a). Therefore, *S. aureus* was used as the indicator organism to probe the effect of NO<sub>2</sub> on the functional integrity of the alveolar macrophages. In these studies, groups of mice were challenged with *S. aureus* and then exposed for four hours to NO<sub>2</sub> target concentrations of 2.5, 4, 5, 10, and 15 ppm. The bactericidal activity data are presented in Figure 2 (Appendix D).

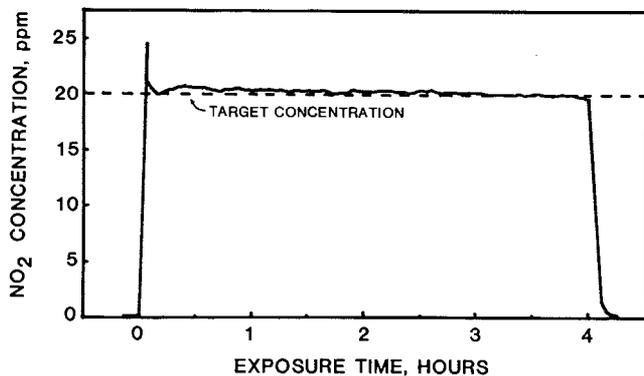


Figure 1. Inhalation chamber response using the Teflon bag generation system.

Pulmonary bactericidal activity against *S. aureus* decreased progressively with exposure to increasing concentrations of NO<sub>2</sub>. These differences were significant for the comparison of controls and mice exposed to 5 ppm of NO<sub>2</sub> or greater.

#### *Proteus mirabilis*

As stated above, pulmonary antibacterial defenses against *S. aureus* are primarily dependent on alveolar macrophages (Goldstein et al. 1977a), whereas lung defenses against gram-negative bacteria, such as our indicator organism, *P. mirabilis*, consist of both alveolar macrophages and PMNs (Jay et al. 1976; Rehm et al. 1980). The peripheral phagocyte is not a constituent of the cell populations of normal murine lungs, but it can migrate to the lung in response to inhaled gram-negative bacteria and thus provide auxiliary phagocytic defense capabilities to the lung (Pierce et al. 1977; Rehm et al. 1979). Failure of the PMNs to migrate to the lung is associated with a decrease of pulmonary defenses against gram-negative bacteria, but not against *S. aureus* (Rehm et al. 1979; Astry et al. 1983).

In these studies, groups of mice were challenged with *P. mirabilis* and exposed for four hours to target concentrations of 5, 10, 15, 17.5, 20, and 25 ppm of NO<sub>2</sub>. The bactericidal activity data are presented in Figure 3 (Appendix E). Nitrogen dioxide concentrations of 5 ppm had no effect on the intrapulmonary killing of *P. mirabilis*. In contrast, 10 ppm of NO<sub>2</sub> significantly enhanced the bactericidal activity of NO<sub>2</sub>-exposed lungs, and the trend toward this enhancement was still evident at 15 ppm. In-

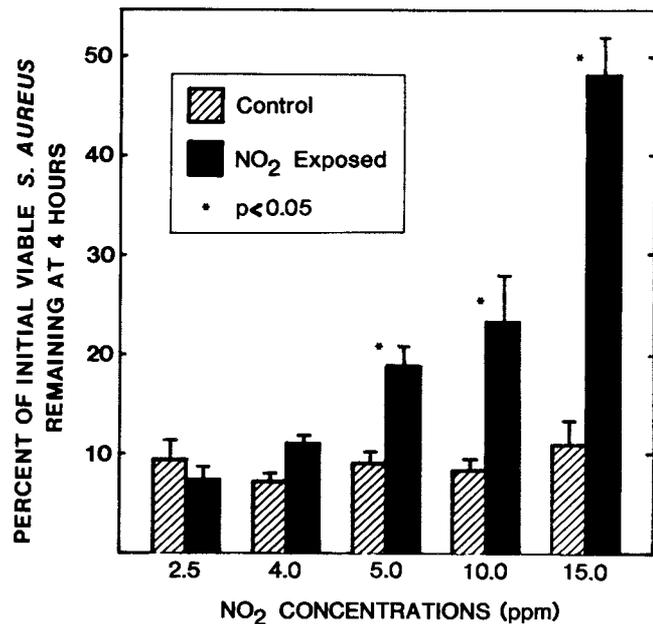


Figure 2. Comparison of intrapulmonary killing of *S. aureus* between mice exposed to either ambient air (control) or NO<sub>2</sub> for four hours after bacterial challenge. Each value represents the mean  $\pm$  SE of 14 to 22 determinations.

creasing the NO<sub>2</sub> exposure concentrations to 17.5 ppm resulted in equivalent rates of intrapulmonary bacterial killing. Finally, at exposure concentrations of 20 ppm, a significant suppression of bactericidal activity of the lung against this organism was observed. This impairment continued in a dose-dependent manner at 25 ppm; at 30 ppm mice began to die if exposed to NO<sub>2</sub> after *P. mirabilis* challenge.

The initial enhancement, followed by the suppression, of pulmonary bactericidal activity against *P. mirabilis* at increasing NO<sub>2</sub> exposure concentrations was unexpected. To gain insights into the possible mechanism of this effect (that is, the recruitment of additional phagocytes into the alveolar area), the lungs of control, NO<sub>2</sub>-exposed, *P. mirabilis*-challenged, and *P. mirabilis*-challenged-plus-NO<sub>2</sub>-exposed mice were lavaged in order to quantify total and differential cell counts on the retrieved cell populations.

Figure 4 (Appendix F) shows that  $6.3 \pm 0.4 \times 10^5$  cells were lavaged from the lungs of control animals, of which more than 98 percent were alveolar macrophages. Four-hour exposure to 10 ppm of NO<sub>2</sub> increased the total cell yield to  $8.1 \pm 1.2 \times 10^5$ , but did not significantly alter the differential cell population. When mice were exposed to our standard challenge of *P. mirabilis*, the lavageable cell yield increased to  $37.5 \pm 2.0 \times 10^5$  cells, compared with the control group receiving no treatment. Most of the cells (90.7  $\pm$  1.2 percent) from the group receiving the bacterial challenge were PMNs. Exposure of *P. mirabilis*-challenged animals to 10 ppm of NO<sub>2</sub> resulted in almost a twofold increase of the retrieved cell numbers, and an increase of the PMNs to  $95.2 \pm 0.3$  percent.

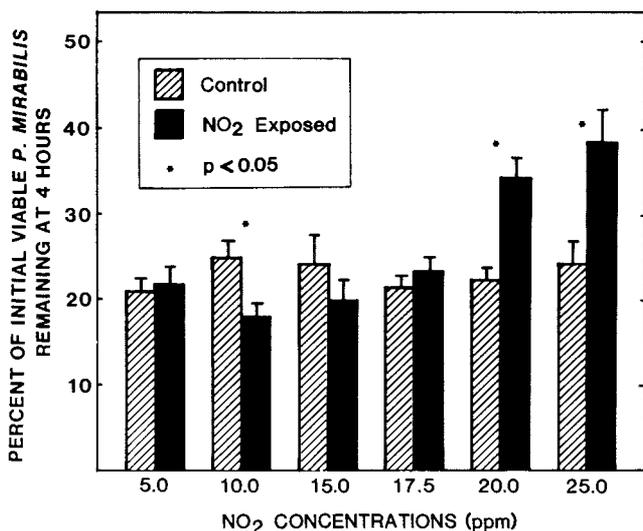


Figure 3. Comparison of intrapulmonary killing of *P. mirabilis* between mice exposed to either ambient air (control) or NO<sub>2</sub> for four hours after bacterial challenge. Each value represents the mean  $\pm$  SE of 20 to 24 determinations.

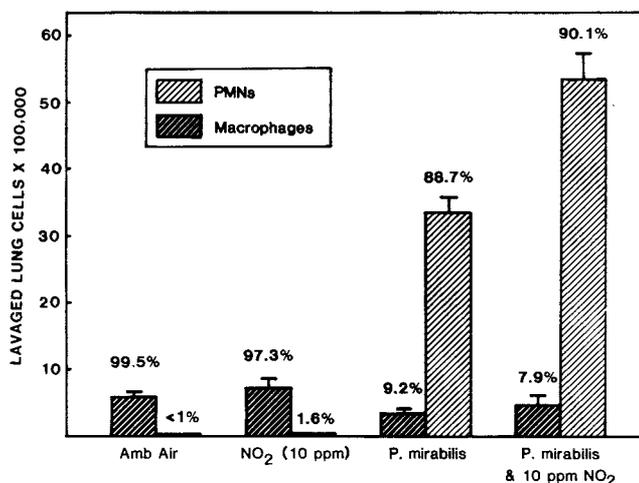


Figure 4. Comparison of total and differential counts of cells lavaged from murine lungs at four hours after treatment with either ambient air, 10 ppm NO<sub>2</sub>, *P. mirabilis*, or *P. mirabilis* plus 10 ppm NO<sub>2</sub>. The numbers on top of the bars represent the percentages of the alveolar macrophages and PMNs retrieved (lymphocytes were less than 2 percent throughout). Each value represents the mean  $\pm$  SE of eight determinations.

Figure 5 (Appendix F) presents the experiment with *P. mirabilis*-challenged mice that were exposed to 25 ppm of NO<sub>2</sub> for four hours before bronchoalveolar lavage. Exposure to NO<sub>2</sub> at this concentration reflected the results obtained with 10 ppm of NO<sub>2</sub> (Figure 4).

#### *Klebsiella pneumoniae*

To determine whether NO<sub>2</sub> acted as a coinflammatory agent with another gram-negative organism, which in this case was a respiratory pathogen, experiments were performed with *K. pneumoniae*. In these studies, groups of mice were challenged with *K. pneumoniae* and then exposed for four hours to 10 ppm of NO<sub>2</sub>, at which time pulmonary bactericidal activity and bronchoalveolar lavage cell counts were performed.

Exposure to 10 ppm of NO<sub>2</sub> significantly reduced the intrapulmonary killing of *K. pneumoniae*;  $45.6 \pm 3.0$  percent of the initial viable bacteria remained in the lungs of NO<sub>2</sub>-exposed mice, compared with  $28.1 \pm 1.7$  percent in the lungs of control animals that breathed ambient air (Appendix G).

The data for the lavage studies performed at four hours after bacterial challenge are presented in Figure 6 (Appendix H). From the lungs of control mice,  $3.8 \pm 0.3 \times 10^5$  cells were retrieved, of which more than 99 percent were alveolar macrophages. Four hours of exposure to 10 ppm NO<sub>2</sub> increased the total cell yield to  $4.8 \pm 0.3 \times 10^5$  and increased the PMN response to  $3.0 \pm 0.4$  percent. When mice were challenged with *K. pneumoniae*, the lavageable cell yield increased to  $13.5 \pm 1.3 \times 10^5$  and the PMN

response to  $67.5 \pm 3.0$  percent. Exposure of *K. pneumoniae*-challenged mice to 10 ppm of NO<sub>2</sub> resulted in  $20.1 \pm 2.2 \times 10^5$  total cells retrieved, of which  $67.1 \pm 2.5$  percent were PMNs.

### *Pasteurella pneumotropica*

As stated above, *S. aureus*, *P. mirabilis*, and *K. pneumoniae* were used as indicator organisms to probe the effect of NO<sub>2</sub> exposure on the functional integrity of the alveolar macrophage and auxiliary (PMN) phagocytic systems of the lungs. Bacterial challenge with *P. pneumotropica* was also used to probe the effect of NO<sub>2</sub> exposure on the defense mechanism of the lung against an organism endogenous to the respiratory tract of the experimental animal model. It should be remembered that these mice had serum antibody against *P. pneumotropica*, but no demonstrable antibody against the other organisms.

In these studies, groups of mice were challenged with *P. pneumotropica* and then exposed for four hours to 5, 10, 15, 20, 25, or 30 ppm of NO<sub>2</sub>. The bactericidal activity data are presented in Figure 7 (Appendix I). Nitrogen dioxide concentrations of 5, 10, 15, and 20 ppm had no statistically significant effect on bactericidal activity. Increasing the exposure levels of NO<sub>2</sub> to 25 and 30 ppm induced significant bactericidal dysfunction.

The lavage cell counts for *P. pneumotropica*-challenged animals exposed for four hours to 10 ppm of NO<sub>2</sub> are presented in Figure 8 (Appendix J). From the lungs of control mice,  $6.3 \pm 0.6 \times 10^5$  cells were retrieved, of which more than 99 percent were alveolar macrophages. After four

hours of exposure to 10 ppm of NO<sub>2</sub>, the total cell yield was  $7.0 \pm 4.7 \times 10^5$ , of which  $1.7 \pm 0.4$  percent were PMNs. When mice were challenged with *P. pneumotropica*, the lavageable cells increased to  $43.6 \pm 4.7 \times 10^5$  of which  $89.5 \pm 1.4$  percent were PMNs. Exposure of *P. pneumotropica*-challenged mice to 10 ppm of NO<sub>2</sub> for four hours resulted in a decrease of the total cells retrieved, to  $35.0 \pm 3.0 \times 10^5$ , of which  $92.5 \pm 1.1$  percent were PMNs.

### Summary

The data on pulmonary bactericidal activity and broncho-alveolar lavage in which mice were exposed to more than one concentration of NO<sub>2</sub> are summarized in Figures 9 and 10.

Previously, the bactericidal data for *S. aureus*, *P. mirabilis*, and *P. pneumotropica* (Figures 2, 3, and 7) were presented as the actual values of the percentage of viable bacteria remaining at four hours in control animals and in those exposed to various concentrations of NO<sub>2</sub>. Since the rate of intrapulmonary killing varies among the different bacteria (Jakab 1976), comparisons of the effect of NO<sub>2</sub> on the absolute bactericidal activity are difficult. Therefore, the differences in bactericidal values (excess bacterial survival or killing) between control and NO<sub>2</sub>-exposed groups were calculated. For example, if the actual value was 10 percent of the bacteria remaining at four hours in the control group and 15 percent in the NO<sub>2</sub>-exposed group, the difference (excess bacterial survival) would be 5 percent. Figure 9 presents the data as the differences, expressed as difference in percentage change, between control and NO<sub>2</sub>-exposed groups among the various bacteria.

The data reveal three different trends in the modulation of pulmonary antibacterial defenses by exposure to NO<sub>2</sub>. With *S. aureus*, a clear dose-response relationship is evident in that pulmonary bactericidal activity decreased progressively with increasing concentrations of NO<sub>2</sub>. With *P. mirabilis*, NO<sub>2</sub> exposure to 10 ppm significantly enhanced bactericidal activity (signified by a negative value), and at 15 ppm this enhancing trend was still evident. Increasing NO<sub>2</sub> exposures to 20 ppm and 25 ppm caused a significant suppression of pulmonary antibacterial defenses. Finally, with *P. pneumotropica*, yet another pattern emerged, in that significant suppression was observed only at NO<sub>2</sub> exposure levels of 25 and 30 ppm.

Figure 10 presents a summary comparison of the lavage cell determinations obtained from mice challenged with *S. aureus* (Appendix K), *P. mirabilis*, *P. pneumotropica*, and *K. pneumoniae* and exposed to NO<sub>2</sub>. Bacterial challenge with *P. mirabilis* recruited the most PMNs into the lungs, followed by *P. pneumotropica* and *K. pneumoniae*. Exposure to NO<sub>2</sub> significantly increased PMN recruitment into the lungs after *P. mirabilis* challenge, but had no signifi-

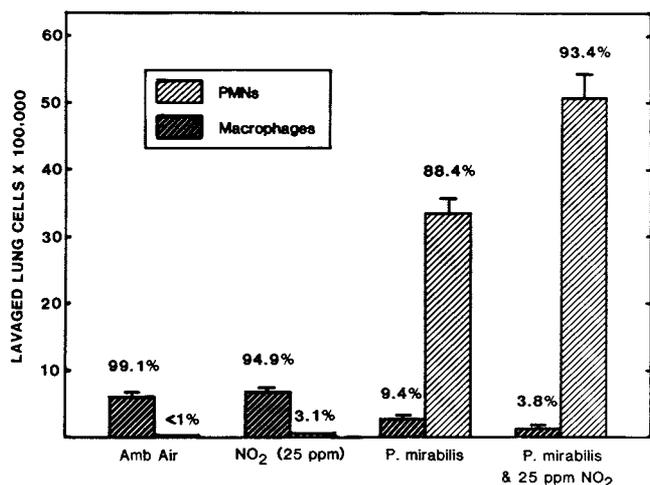


Figure 5. Comparison of total and differential counts of cells lavaged from murine lungs at four hours after treatment with either ambient air, 25 ppm NO<sub>2</sub>, *P. mirabilis*, or *P. mirabilis* plus 25 ppm NO<sub>2</sub>. The numbers on top of the bars represent the percentages of the alveolar macrophages and PMNs retrieved (lymphocytes were less than 2 percent throughout). Each value represents the mean  $\pm$  SE of eight determinations.

cant effect on pulmonary PMN recruitment with the other bacterial challenges.

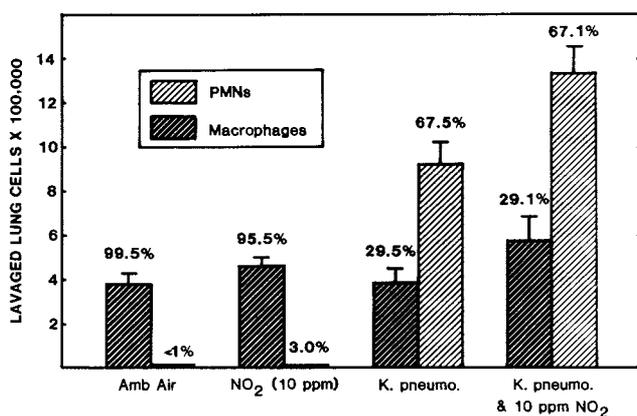
#### PULMONARY ANTIBACTERIAL DEFENSES (NITROGEN DIOXIDE EXPOSURE BEFORE BACTERIAL CHALLENGE)

The data for the experiments in which mice were exposed to various concentrations of  $\text{NO}_2$  for four hours and then challenged with either *S. aureus* or *P. mirabilis* are presented in Figure 11 (Appendices L and M). Two exposure levels were used in these studies. The first was the lowest concentration for which a bactericidal dysfunction against the organism was observed in the experiments described above using post-bacterial challenge and  $\text{NO}_2$  exposure. The second concentration was approximately one-and-a-half to two times higher.

Preexposure levels of 5 ppm of  $\text{NO}_2$  for four hours before bacterial challenge had no effect on the subsequent intrapulmonary killing of *S. aureus*, whereas 10 ppm significantly impaired bactericidal activity. Comparison of lung antibacterial defenses against *P. mirabilis* between exposure levels of 20 ppm and 30 ppm showed no effect at the lower concentration but a significant enhancement at the higher concentration.

#### PULMONARY ANTIBACTERIAL DEFENSES (NITROGEN DIOXIDE EXPOSURE BEFORE AND AFTER BACTERIAL CHALLENGE)

Previously it was demonstrated that 5 ppm was the lowest concentration of  $\text{NO}_2$  exposure that resulted in pulmonary

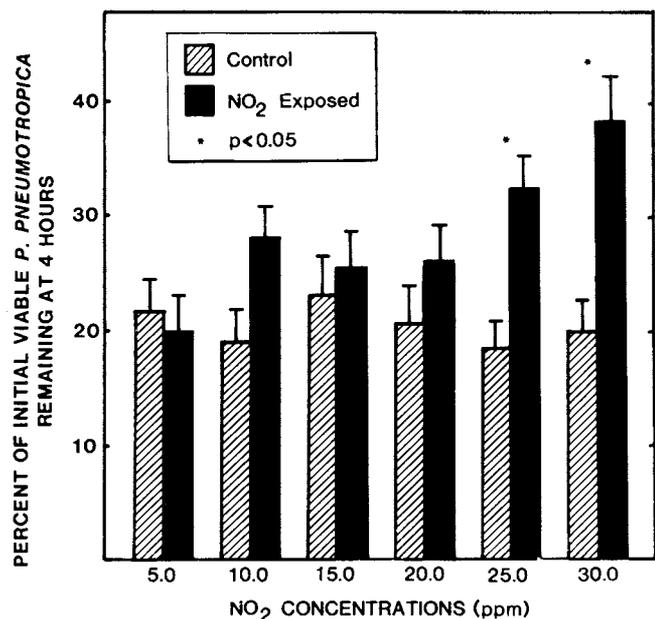


**Figure 6.** Comparison of total and differential counts of cells lavaged from murine lungs at four hours after treatment with either ambient air, 10 ppm  $\text{NO}_2$ , *K. pneumoniae*, or *K. pneumoniae* plus 10 ppm  $\text{NO}_2$ . The numbers on top of the bars represent the percentages of the alveolar macrophages and PMNs retrieved (lymphocytes were less than 2 percent throughout). Each value represents the mean  $\pm$  SE of eight determinations.

bactericidal dysfunction, and that this occurred when the challenge organism was *S. aureus* and  $\text{NO}_2$  exposure followed bacterial challenge (Figure 2). In order to determine whether or not this threshold dose could be reduced, mice were exposed to 2.5 ppm of  $\text{NO}_2$  for four hours before and after staphylococcal challenge. The four groups of mice were treated as follows: (1) ambient air, bacterial challenge, ambient air; (2) ambient air, bacterial challenge,  $\text{NO}_2$  exposure; (3)  $\text{NO}_2$  exposure, bacterial challenge, ambient air; and (4)  $\text{NO}_2$  exposure, bacterial challenge,  $\text{NO}_2$  exposure. The respective bactericidal values for the groups were  $11.8 \pm 1.1$  percent,  $10.8 \pm 0.9$  percent,  $9.7 \pm 0.9$  percent, and  $10.0 \pm 0.8$  percent (Appendix N), showing that exposure to 2.5 ppm of  $\text{NO}_2$  for four hours both before and after bacterial challenge did not have a detrimental effect on pulmonary defenses against *S. aureus*.

#### PULMONARY ANTIBACTERIAL DEFENSES IN THE PREDISPOSED HOST

The above series of studies clearly demonstrated that, in the normal host, perturbations of pulmonary antibacterial defenses were induced only at exposures to high levels of  $\text{NO}_2$ . In fact, the lowest concentration of  $\text{NO}_2$  that induced a detrimental effect against pulmonary challenges with *S. aureus* was 4 ppm (Figure 2). Hosts predisposed by a vari-



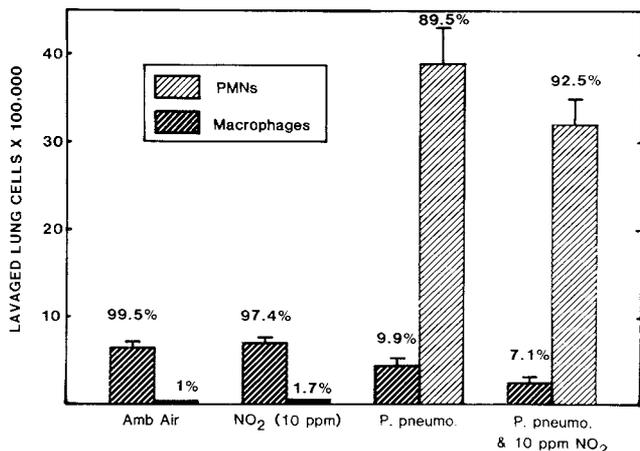
**Figure 7.** Comparison of intrapulmonary killing of *P. pneumotropica* between mice exposed to either ambient air (control) or  $\text{NO}_2$  for four hours after bacterial challenge. Each value represents the mean  $\pm$  SE of 20 to 30 determinations.

ety of factors such as virus infection and corticosteroid treatment are known to be more susceptible to infections of the lungs (reviewed by Huber et al. 1977).

**Influenza Virus**

Influenza virus infections are known to predispose the host to bacterial superinfections (Martin et al. 1959; Loosli 1967; Nichol and Cherry 1967; Jarstrand and Tunevall 1974). Experimental studies have shown that maximal suppression of pulmonary antibacterial defenses occurs approximately a week after viral infection (Green 1966; Jakab 1981a,b). In the studies reported here, groups of mice were infected by aerosol inhalation with influenza virus and then challenged eight days later with aerosolized *S. aureus*. Thereafter, mice were exposed for four hours to NO<sub>2</sub> at concentrations of 5 or 10 ppm. The groups consisted of control mice (noninfected and not exposed to NO<sub>2</sub>), virus-infected mice, and virus-infected plus NO<sub>2</sub>-exposed mice.

Figure 12 (Appendix O) presents the bactericidal data. Influenza virus infection on day eight significantly suppressed pulmonary antibacterial defenses: more than 40 percent of the initial viable staphylococci remained in the virus-infected lungs, compared with less than 11 percent in noninfected control animals. As demonstrated previously, NO<sub>2</sub> exposure at 5 and 10 ppm impaired the intrapulmonary killing of staphylococci in a dose-dependent manner. Nitrogen dioxide exposure superimposed on the virus infection had no effect on the intrapulmonary killing of *S. aureus* at 5 ppm, but at 10 ppm, NO<sub>2</sub> exposure significantly contributed to the virus-induced bactericidal defect.

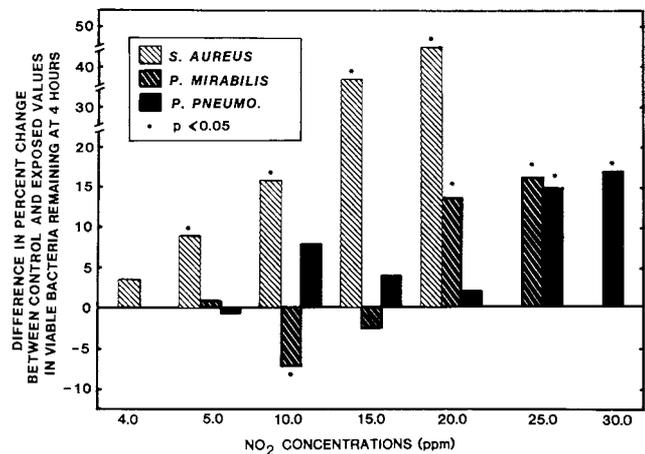


**Figure 8.** Comparison of total and differential counts of cells lavaged from murine lungs at four hours after treatment with either ambient air, 10 ppm NO<sub>2</sub>, *P. pneumotropica*, or *P. pneumotropica* plus 10 ppm NO<sub>2</sub>. The numbers on top of the bars represent the percentages of the alveolar macrophages and PMNs retrieved (lymphocytes were less than 2 percent throughout). Each value represents the mean ± SE of eight determinations.

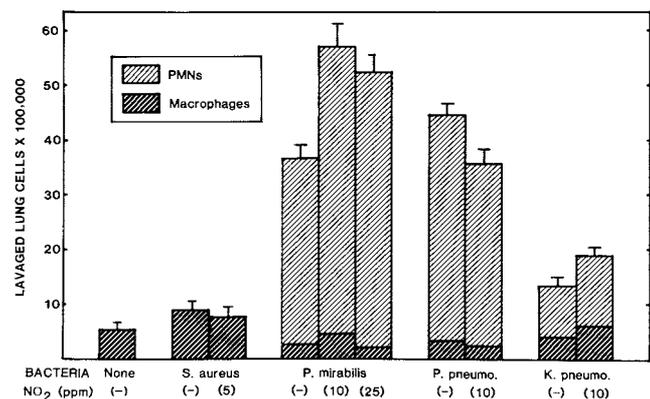
**Corticosteroid Treatment**

Corticosteroids are known to suppress pulmonary antibacterial defenses (Green and Kass 1964a; Pennington 1977; Blackwood and Pennington 1982; Nugent and Pesanti 1982). In these experiments, mice were treated by intramuscular injection with 0.1 ml of a depot corticosteroid, while control mice received an intramuscular injection of 0.1 ml of PBS. Four days later, corticosteroid-treated animals and control animals were challenged with *S. aureus* and exposed for four hours to NO<sub>2</sub> concentrations of either 1, 2.5, 5, or 10 ppm.

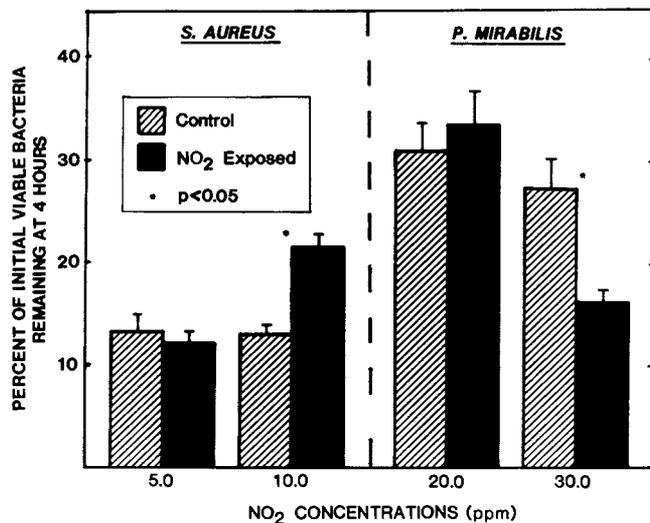
Figure 13 (Appendix P) presents the bactericidal data. Treatment with the corticosteroid significantly suppressed pulmonary antibacterial defenses, as more than 17 percent



**Figure 9.** Summary comparison of intrapulmonary killing of *S. aureus*, *P. mirabilis*, and *P. pneumotropica* between mice exposed either to ambient air or to various concentrations of NO<sub>2</sub> for four hours after bacterial challenge. The data are presented as the difference in percent change between control and NO<sub>2</sub>-exposed bactericidal values.



**Figure 10.** Summary comparison of total and differential counts of cells lavaged from murine lungs at four hours after challenge with either *S. aureus*, *P. mirabilis*, *P. pneumotropica*, or *K. pneumoniae* with or without treatment with NO<sub>2</sub>.

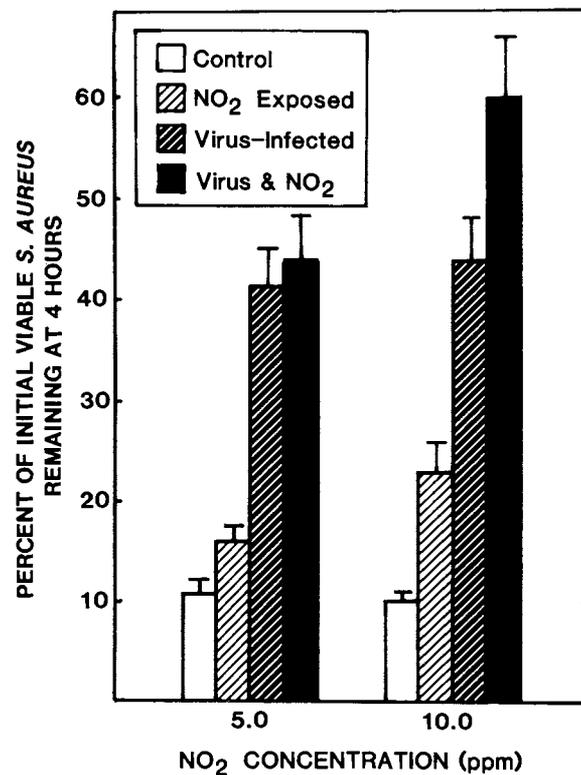


**Figure 11.** Comparison of intrapulmonary killing of *S. aureus* or *P. mirabilis* between mice exposed to either ambient air (control) or NO<sub>2</sub> for four hours before bacterial challenge. All animals were exposed to ambient air after bacterial challenge. Each value represents the mean  $\pm$  SE of 16 to 24 determinations.

of the initial viable staphylococci remained in the lungs of treated animals, while less than 10 percent remained in the lungs of the nontreated control mice. As demonstrated previously, NO<sub>2</sub> exposure at 5 and 10 ppm impaired the intrapulmonary killing of staphylococci in a dose-dependent manner, whereas exposures of 1 and 2.5 ppm had no significant effects. In contrast, the combination of corticosteroid treatment and NO<sub>2</sub> exposure significantly impaired the intrapulmonary killing of *S. aureus* at 2.5 ppm, and pulmonary bactericidal activity decreased progressively with exposure to increasing concentrations of NO<sub>2</sub>.

## PARTICLE CLEARANCE

In order to determine whether or not short-term NO<sub>2</sub> exposure altered the physical translocation of particles from the lungs, animals were challenged by aerosol inhalation with <sup>32</sup>P-labeled *S. aureus* and then exposed for four hours to NO<sub>2</sub> concentrations of either 10, 15, or 20 ppm. Physical clearance of the bacteria, as determined by decline in radiotracer activity in the lungs, is presented in Figure 14. Physical removal of radiolabeled staphylococci was not affected by exposure to NO<sub>2</sub>. Similar amounts of the isotope were recovered from the lungs of control and NO<sub>2</sub>-exposed mice for each of the exposure levels studied. However, as documented previously with unlabeled staphylococcal challenges (Figure 2), pulmonary bactericidal activity decreased progressively with exposures to increasing concentrations of NO<sub>2</sub>.



**Figure 12.** Comparison of intrapulmonary killing of *S. aureus* in mice infected with influenza A/PR8/34 virus eight days previously that were exposed to either ambient air or NO<sub>2</sub> for four hours per day throughout the virus infection. Each value represents the mean  $\pm$  SE of 20 determinations.

## BACTERIAL INFECTION

After bacterial challenge, the organisms used above (*S. aureus*, *P. mirabilis*, *P. pneumotropica*, and *K. pneumoniae*) are rapidly eliminated from the lungs. Because of this, an infective process is not established since the intrapulmonary defense mechanisms eliminate the viable bacteria successfully in a relatively short time. In contrast, aerosol exposure of mice to *L. monocytogenes* establishes a smoldering long-term infection; the viable bacterial population in the lungs does not change appreciably during the first week after exposure, but the organism is slowly eliminated after that point (Lefford et al. 1978, 1979; Jakab et al. 1981). To determine the effect of NO<sub>2</sub> exposure on pulmonary resistance to *L. monocytogenes*, mice were exposed to an infectious aerosol of the organism for 30 minutes and then exposed for four hours per day to NO<sub>2</sub> concentrations of either 10 or 20 ppm. During the remainder of the day the animals were housed in filter-topped stainless-steel cages. At three, five, 10, and 14 days after infection, control and

NO<sub>2</sub>-exposed mice were killed and the viable *L. monocytogenes* were counted. Figure 15 shows that exposure to NO<sub>2</sub> concentrations of 10 ppm and 20 ppm for four hours per day had no significant effect on the course of the *L. monocytogenes* infection.

**VIRAL INFECTION**

The murine respiratory virus parainfluenza 1 (Sendai) was used to determine whether NO<sub>2</sub> exposure increases the severity of a respiratory infection that is endogenous to the host (Parker et al. 1964; Parker and Reynolds 1968).

**Infectious Pulmonary Virus Titers**

Mice were infected by aerosol inhalation with a dose of the virus that induces a moderate pneumonitis from which all of the animals readily recovered. Within one hour of infection, the mice were exposed to NO<sub>2</sub> for four hours per day for 10 days. In three separate experiments, NO<sub>2</sub> concentrations of either 5, 10, or 20 ppm were used. At three, five, seven, and ten days after virus infection, groups of five mice were killed, their lung homogenates were pooled, and infectious pulmonary virus titers were determined by standard egg-inoculation techniques.

Figure 16 shows that exposure to NO<sub>2</sub> concentrations of 5 ppm for four hours per day had no effect on the proliferation and elimination of the infectious virus from the lungs. At the exposure level of 10 ppm, the proliferation of the virus was identical in control and NO<sub>2</sub>-exposed groups. Finally, at levels of 20 ppm, less infectious virus was recovered from the lungs of NO<sub>2</sub>-exposed animals than

from those allowed to breathe ambient air; however, this was overcome by day five. Starting on day four, NO<sub>2</sub>-exposed mice died in ever-increasing numbers. This was expected from preliminary range-finding experiments; therefore, additional mice were included to obtain mortality data in the 20-ppm NO<sub>2</sub>-exposure experiment. Gross examination of the lungs of moribund animals showed massive surface consolidation involving up to 80 percent of the lung surface area.

**Lung Wet: Dry Weight Ratios and Albumin in Lung Lavages**

Death from viral pneumonitis is associated with overwhelming lung damage that results in pulmonary insufficiency for adequate alveolar gas exchange. The above results of the virus titrations showed that NO<sub>2</sub> exposure at 5 and 10 ppm did not alter the *infective process* of the viral pneumonitis, whereas the results of the experiment with exposure to 20 ppm suggested that NO<sub>2</sub> exposure *potentiates lung pathology*, a contention supported by the observation that NO<sub>2</sub> exposure acts as a concomitant inflammatory agent with *P. mirabilis* (Figures 4 and 5).

In order to determine whether or not NO<sub>2</sub> exposure altered lung damage during Sendai virus pneumonitis, infected mice were exposed to either 10 or 20 ppm of NO<sub>2</sub> for four hours per day for eight days. At this time, at the height of the virus-induced pulmonary pathology (Heath 1979) wet: dry weight ratios and the albumin content of the lungs were determined as indices of acute lung damage. Figure 17 (Appendix Q) shows that 10 and 20 ppm of NO<sub>2</sub> did not increase the lung wet: dry weight ratios upon expo-

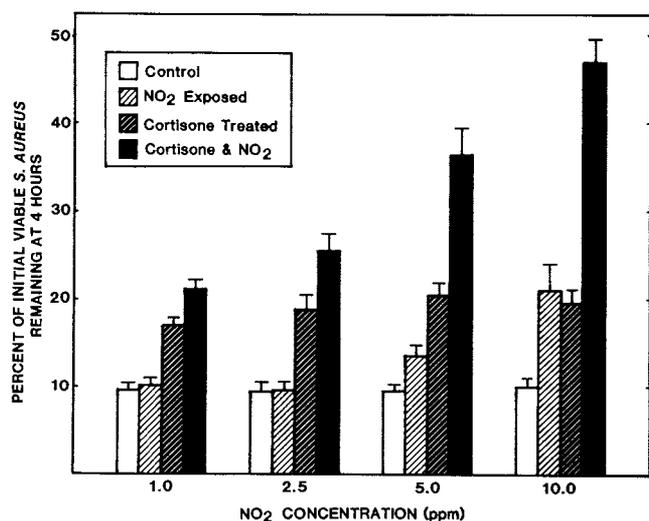


Figure 13. Comparison of intrapulmonary killing of *S. aureus* in mice treated with corticosteroids for four days. Exposure to NO<sub>2</sub> was for four hours after bacterial challenge. Each value represents the mean ± SE of 10 to 20 determinations.

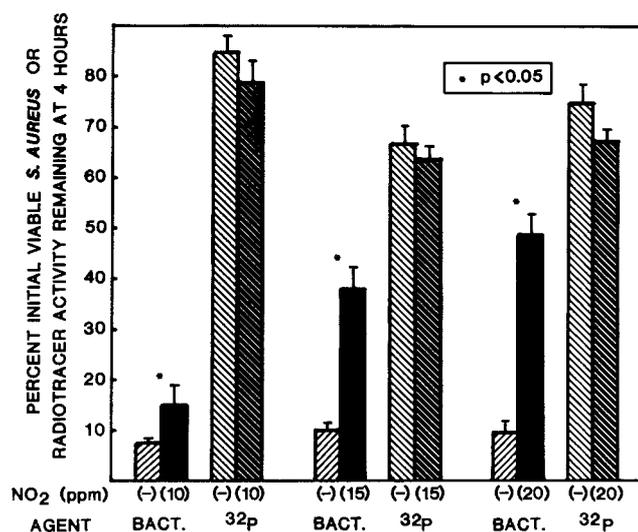


Figure 14. Comparison of intrapulmonary bacterial killing and radiotracer removal in murine lungs following inhalation challenge with <sup>32</sup>P-labeled *S. aureus*. Each value represents the mean ± SE of 20 to 26 determinations.

sure for four hours per day for eight days. At each NO<sub>2</sub> concentration, Sendai virus infection significantly increased the lung wet:dry weight ratios.

Lung lavage albumin concentrations are presented in Figure 18. In the two separate experiments the albumin concentrations, expressed as mg/ml of lavage fluid, were 0.193 ± 0.036 and 0.184 ± 0.073, respectively. Exposure to NO<sub>2</sub> concentrations of 10 and 20 ppm for four hours per day for eight days resulted in albumin concentrations of 0.195 ± 0.039 for 10 ppm and 0.202 ± 0.071 for 20 ppm. Sendai virus infection significantly elevated the lung albumin concentrations of the control animals to 0.834 ± 0.074 for experiment 1 and 0.908 ± 0.072 for experiment 2. Exposure of Sendai-virus-infected mice to 10 and 20 ppm of NO<sub>2</sub> significantly increased the lung albumin concentrations to 1.359 ± 0.126 and 1.869 ± 0.242, respectively.

**Lung Differential Cell Determinations**

In order to determine whether or not NO<sub>2</sub> exposure superimposed on Sendai virus infection results in an increased inflammatory response in the lungs (as previously

demonstrated with *P. mirabilis*), mice were infected with the virus and exposed to either 10 or 20 ppm of NO<sub>2</sub> for four hours per day for eight days. The total and differential cell counts retrieved by bronchoalveolar lavage are presented in Figures 19 and 20 (Appendix R).

From the lungs of noninfected animals, 3.4 ± 0.3 × 10<sup>5</sup> cells were retrieved, of which more than 98 percent were alveolar macrophages. Nitrogen dioxide exposure for four hours per day for eight days increased the cell yield to 4.2 ± 0.2 × 10<sup>5</sup> and 4.7 ± 0.3 × 10<sup>5</sup>, respectively, at NO<sub>2</sub> levels of 10 and 20 ppm, with the increase coming primarily from the influx of inflammatory PMNs. From Sendai-virus-infected animals, 10.3 ± 0.9 × 10<sup>5</sup> cells were lavaged, of which 86.6 percent were macrophages, 10.4 percent were lymphocytes, and 3.0 percent were PMNs. Exposure of Sendai-virus-infected animals to NO<sub>2</sub> increased both the cell yield and the inflammatory cell response in a dose-dependent manner (Figures 19 and 20), until at 20 ppm 17.8 ± 1.7 × 10<sup>5</sup> cells were retrieved from the lungs, of which

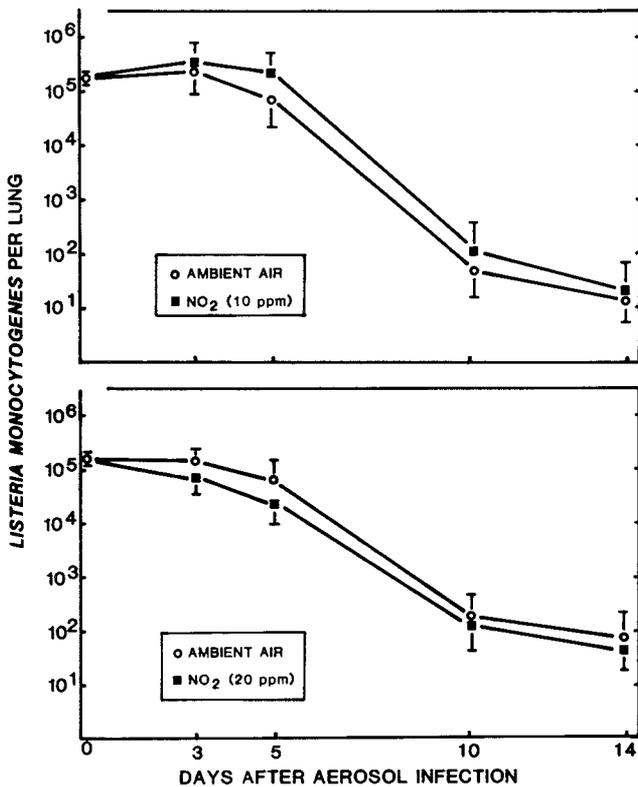


Figure 15. Comparison of the population of viable *L. monocytogenes* in the lungs of mice exposed for four hours per day to NO<sub>2</sub>. Each value represents the mean ± SE of six determinations.

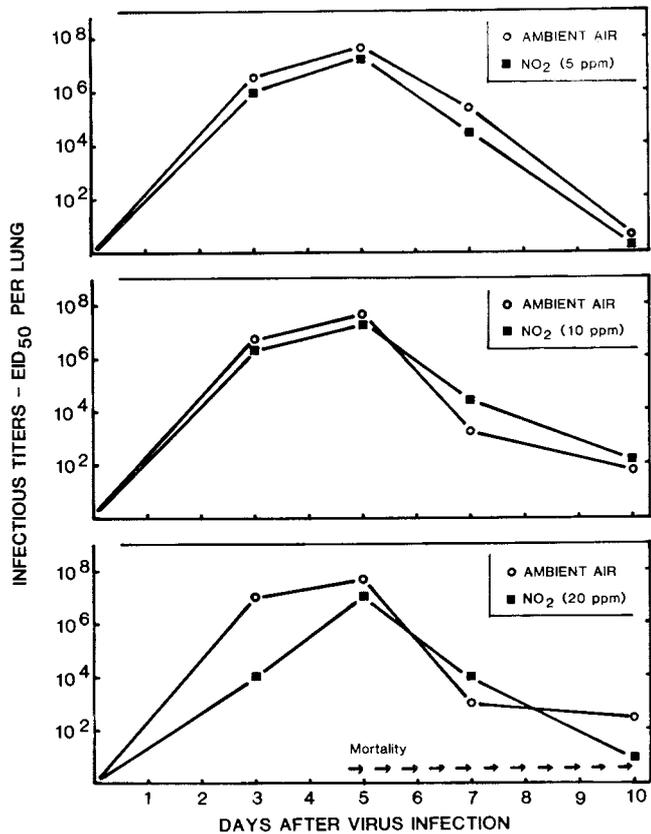
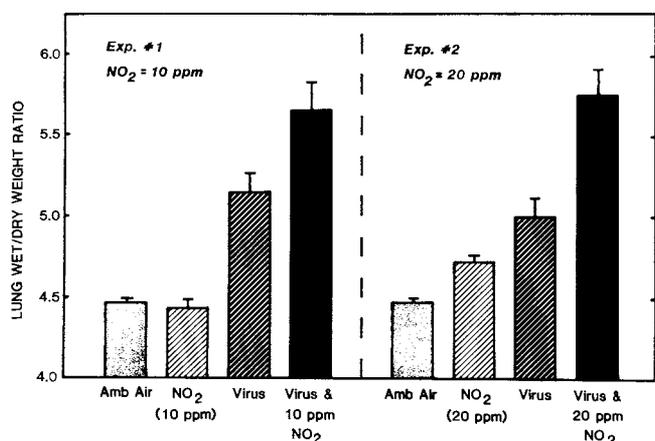


Figure 16. Comparison of infectious virus titers in murine lungs during the course of Sendai virus infection in animals exposed for four hours per day to NO<sub>2</sub>. Mortality occurred with the NO<sub>2</sub>-exposed groups only. Each value represents the pool of five lungs.



**Figure 17.** Comparison of lung wet:dry weight ratios in mice infected eight days previously with Sendai virus and exposed for four hours per day to NO<sub>2</sub>. Each value represents the mean  $\pm$  SE of six to eight determinations.

73.1 percent were alveolar macrophages, 12.3 percent were lymphocytes, and 14.6 percent were PMNs.

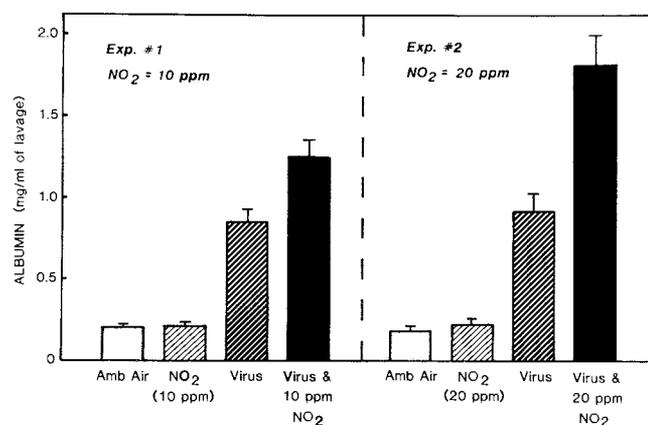
## DISCUSSION

The studies performed herein clearly demonstrate that a four-hour acute exposure to NO<sub>2</sub> adversely affects the bactericidal activity of the murine lung. However, the concentration of NO<sub>2</sub> at which this impairment occurs is dependent on the challenge organism, the exposure protocol, and the immunologic status of the host. The threshold level at which NO<sub>2</sub> exposure caused bactericidal dysfunction was lower when mice were predisposed by treatment with corticosteroids.

Nitrogen dioxide exposures for four hours per day during the course of infection with *L. monocytogenes* had no effect on the number of viable bacteria recovered from the lungs. Nor did NO<sub>2</sub> exposures for four hours per day have any effect on the course of murine Sendai virus infection; rather, exposure enhanced the pathogenesis of the viral pneumonitis.

### PULMONARY ANTIBACTERIAL DEFENSES

In this study, *S. aureus* was used as the indicator organism to probe quantitatively the functional integrity of the alveolar macrophage phagocytic system, and *P. mirabilis* and *K. pneumoniae* were used to probe the dual phagocytic system of the lung, consisting of the resident alveolar macrophages and the PMNs newly recruited into the alveoli (Rehm et al. 1979). In addition, bacterial challenge with *P. pneumotropica* was used to probe the effect of NO<sub>2</sub> exposure on the phagocytic defense mechanisms of the lungs



**Figure 18.** Comparison of lung lavage albumin concentrations in mice infected eight days previously with Sendai virus and exposed for four hours per day to NO<sub>2</sub>. Each value represents the mean  $\pm$  SE of six determinations.

against a pathogen endogenous to the respiratory tract of the experimental host (Brennan et al. 1969; Jakab and Dick 1973).

Pulmonary antibacterial defenses against *S. aureus* are primarily dependent on the alveolar macrophages (Goldstein et al. 1974), whereas lung defenses against gram-negative bacteria are dependent on a dual phagocytic system that consists of both alveolar macrophages and PMNs (Pierce et al. 1977; Rehm et al. 1979, 1980). The peripheral phagocyte is not a constituent of the normal lung's murine cell population, but it can immigrate rapidly to the lung in response to gram-negative bacteria, and thus provide additional phagocytic defense capabilities to the lung (Pierce et al. 1977). Failure of PMNs to immigrate to the lungs is associated with a decrease of pulmonary defenses against gram-negative bacteria but not against *S. aureus* (Rehm et al. 1979; Astry et al. 1983).

Nitrogen dioxide exposure after staphylococcal challenge caused a progressive dose-dependent suppression of bactericidal activity (Figure 2). This impairment was observed in mice exposed to levels of NO<sub>2</sub> of 5 ppm or greater. It is noteworthy that these results are quantitatively similar to data obtained by Goldstein and associates (1973) in an almost identical system. These workers observed a significant defect in the intrapulmonary killing of *S. aureus* at an NO<sub>2</sub> concentration of  $7.0 \pm 0.3$  ppm but not at  $3.8 \pm 0.5$  ppm. In addition, the threshold level observed herein held constant during the three-year study for experiments involving the pulmonary clearance of radiolabeled staphylococci and the pulmonary antibacterial defenses in the predisposed host.

The initial enhancement (followed by the suppression) of pulmonary bactericidal activity against *P. mirabilis*, by exposure to increasing concentrations of NO<sub>2</sub>, was unex-

pected (Figure 3). To gain insight into the possible mechanisms of this modulating effect, the lungs of mice were lavaged in order to quantify the phagocytic cell populations. Exposure of *P. mirabilis*-challenged mice to 10 ppm and 25 ppm of NO<sub>2</sub> increased the auxiliary (PMN) phagocytic cell population of the lungs nearly twofold over that observed with the bacterial challenge alone (Figures 4 and 5). Nitrogen dioxide exposure alone is known to increase the intraalveolar PMNs obtainable by pulmonary lavage (Gardner et al. 1969). The results herein demonstrate that NO<sub>2</sub> acts as a concomitant inflammatory agent in *P. mirabilis*-challenged lungs. Therefore, a possible explanation for the enhanced bactericidal activity against *P. mirabilis* at an NO<sub>2</sub> exposure concentration of 10 ppm may be that the augmented recruitment of phagocytic PMNs into the lungs increased the intrapulmonary killing capabilities. This contention is supported by the observation that recruitment of PMNs into the lungs follows the inhalation of endotoxin (Hudson et al. 1977) and gram-negative bacteria (Rehm et al. 1980; Baseler et al. 1983). Prior induction of intraalveolar PMNs enhances the intrapulmonary killing of organisms (Rylander et al. 1975). Following this line of reasoning, the enhancing effect in bactericidal activity brought about by augmented recruitment of phagocytic cells to the lungs at exposure to 10 ppm of NO<sub>2</sub> was finally overcome at exposure to 20 ppm, most likely through impairments in the intrinsic phagocytic process itself (Acton and Myrvik 1972; Vassallo et al. 1973; Amoroso et al. 1981; Rietjens et al. 1986; Suzuki et al. 1986) or as a consequence of edema.

In order to determine whether or not the phenomenon of NO<sub>2</sub> acting as a coinflammatory agent would hold true after challenge with other gram-negative bacteria, a small study was performed with *K. pneumoniae*. After *K. pneumoniae* challenge,  $13.5 \times 10^5$  phagocytic cells were lavaged from the lungs (Figure 6), compared with an approximately threefold increase ( $37.5 \times 10^5$ ) lavaged from lungs challenged with *P. mirabilis* (Figure 4). Exposure to 10 ppm of NO<sub>2</sub> increased the phagocytic cell yield in *K. pneumoniae*-challenged lungs to  $20.1 \times 10^5$  (Figure 6), compared with  $59.4 \times 10^5$  in *P. mirabilis*-challenged lungs (Figure 4). These data show that NO<sub>2</sub> exposure also acts as a concomitant inflammatory agent after an inhalation challenge with *K. pneumoniae*. However, the magnitude of the recruited phagocytic inflammatory response after a bacterial challenge, and the subsequent exposure to 10 ppm of NO<sub>2</sub>, is significantly less than that observed with *P. mirabilis*. Therefore, the fewer phagocytic cells in the lungs of *K. pneumoniae*-challenged mice may explain why pulmonary antibacterial defenses were suppressed at exposure to 10 ppm of NO<sub>2</sub> in the case of *K. pneumoniae* and enhanced in the case of *P. mirabilis*.

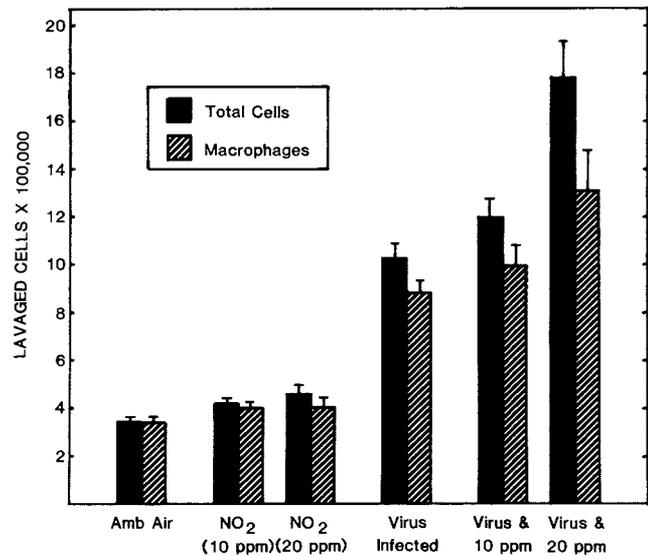


Figure 19. Comparison of total cells and alveolar macrophages lavaged from mice infected eight days previously with Sendai virus and exposed for four hours per day to NO<sub>2</sub>. Each value represents the mean  $\pm$  SE of eight determinations.

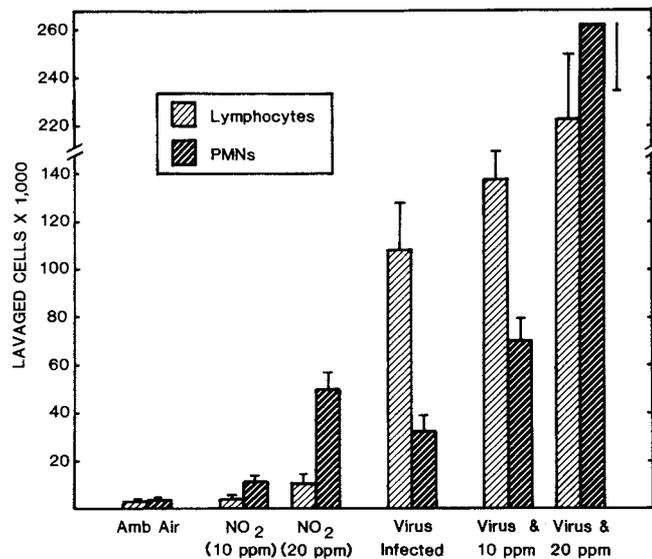


Figure 20. Comparison of total lymphocytes and PMNs lavaged from mice infected eight days previously with Sendai virus and exposed for four hours per day to NO<sub>2</sub>. Each value represents the mean  $\pm$  SE of eight determinations.

The modulating effect of exposure to increasing concentrations of NO<sub>2</sub> was again different when the challenge organism was *P. pneumotropa*. This organism is endogenous to the respiratory tract flora of many rodent colonies (Brennan et al. 1969), including the mice used in this study. Exposure to 5, 10, 15, or 20 ppm of NO<sub>2</sub> had no statistically significant effect on the intrapulmonary killing

of this organism, although at 10 ppm it almost approached significance ( $27.4 \pm 3.0$  percent in NO<sub>2</sub>-exposed versus  $18.6 \pm 3.1$  percent in controls;  $p > 0.05 < 0.1$ ). The bactericidal defect was finally noted at NO<sub>2</sub> exposure levels of 20 and 30 ppm. Pulmonary lavages showed that NO<sub>2</sub> exposure concentrations of 10 ppm impaired the recruitment of the auxiliary PMN phagocytic defenses to the lung upon challenge with this bacterium (Figure 8). Following the line of reasoning established previously with *P. mirabilis*, fewer intraalveolar phagocytic cells may explain the near bactericidal defect against *P. pneumotropica* at NO<sub>2</sub> exposure levels of 10 ppm. In addition, contrasted with *S. aureus*, *P. mirabilis*, and *K. pneumoniae* organisms, another host-defense parameter, the antibody, undoubtedly plays a role in pulmonary resistance against *P. pneumotropica*. Active or passive systemic immunization is known to enhance the intrapulmonary killing of gram-negative bacteria in normal animals (Jakab 1976) and to mitigate the bactericidal defects when pulmonary antibacterial defenses are suppressed (Jakab and Green 1973). Serum antibody transudated into the lungs during the inflammatory process may account for the observation that the final bactericidal defect against *P. pneumotropica* occurred at exposure levels of 25 ppm of NO<sub>2</sub>, whereas the defect against *P. mirabilis* (in those mice that possessed no demonstrable antibody against this organism) occurred at 20 ppm of NO<sub>2</sub>.

The studies detailed above clearly demonstrate that an acute exposure to NO<sub>2</sub> for four hours after a bacterial challenge causes dysfunction in pulmonary antibacterial defenses, but that the level at which this defect occurs depends both on the organism used to probe a specific defense parameter and on the NO<sub>2</sub> concentration (Figure 9). When *S. aureus* was used to probe the functional activity of the alveolar macrophage phagocytic system, the bactericidal defect was initially observed at exposure concentrations of 5 ppm and a clear dose-response relationship was observed with increasing concentrations. When gram-negative bacteria were used to probe the functional activity of both the resident and auxiliary phagocytic defenses of the lung, the results depended on whether or not the challenge organism recruited additional phagocytic defenses to the lungs and whether the bacterium was exogenous or endogenous to the respiratory tract of the host.

The studies in which the mice were exposed to NO<sub>2</sub> and then challenged with the bacteria were performed to determine whether or not exposure sequence played a role. The effect of exposure to NO<sub>2</sub> (for four hours prior to the bacterial challenge) on the pulmonary antibacterial defenses depended on the challenge organism. With *S. aureus*, no bactericidal defect was observed at 5 ppm. However, Goldstein and associates (1973) observed bactericidal dysfunction

against *S. aureus* after a 17-hour exposure to  $2.3 \pm 0.2$  ppm of NO<sub>2</sub> or greater ( $6.6 \pm 0.6$  ppm), but not at  $1.0 \pm 0.1$  ppm. Taken together, these results indicate a time-dose relationship in which time may be the more important factor. Exposure concentrations of 10 ppm clearly impaired pulmonary antistaphylococcal activity. However, this defect was not permanent; intrapulmonary killing of *S. aureus* was again normal in NO<sub>2</sub>-exposed mice that breathed ambient air for 18 hours before the staphylococcal challenge.

Exposure to 20 ppm of NO<sub>2</sub> after bacterial challenge suppressed the intrapulmonary killing of *P. mirabilis*, and at 30 ppm the animals succumbed to the toxic effects of treatment with both NO<sub>2</sub> and the bacterium (Figure 3). In contrast, preexposure to 20 ppm of NO<sub>2</sub> had no significant effect on bactericidal activity, whereas preexposure to 30 ppm enhanced the intrapulmonary killing of *P. mirabilis*. The reason for this enhancement is not known; however, a four-hour preexposure to this high concentration of NO<sub>2</sub> might injure the lung sufficiently (Gregory et al. 1983) to predispose it to a greater and more rapid influx of PMNs upon *P. mirabilis* challenge. The resultant effect would be to provide the lungs with additional phagocytic defenses by increasing the phagocyte:bacteria ratio.

In combination, the studies involving NO<sub>2</sub> exposure both before and after bacterial challenge with *S. aureus* and *P. mirabilis* show that the antibacterial defenses of the lungs are susceptible to the inhibiting effects of short, acute exposures of NO<sub>2</sub> at lower concentrations when NO<sub>2</sub> is administered after the lungs have been seeded with the bacteria. This observation was noted previously in our laboratory; studies with similar exposure protocols that used the cigarette-smoke component, acrolein, showed that the bactericidal defect was induced at a lower concentration when acrolein exposure followed bacterial challenge (C.A. Astry and G.J. Jakab, unpublished observations). In addition, preexposure to NO<sub>2</sub> plus bacterial challenge with our most sensitive bacterial probe, *S. aureus*, show no residual effect at 18 hours after cessation of exposure to 10 ppm of NO<sub>2</sub>.

Finally, since the *S. aureus* challenges did not induce confounding variables brought about by large inflammatory responses into the lungs (Appendix K), an experiment was performed that combined NO<sub>2</sub> exposure (at a level of 2.5 ppm) both four hours before and after staphylococcal challenge in an attempt to determine whether or not a continuous NO<sub>2</sub> exposure protocol (with the exception of the 45 minutes required for the bacterial challenge) would lower the threshold dose for the NO<sub>2</sub>-induced bactericidal defect. This experiment was essentially negative (Appendix N): Exposure to 2.5 ppm of NO<sub>2</sub> both before and after bacterial challenge caused no alterations in the intrapulmonary kill-

ing of *S. aureus*, compared with the control mice that were exposed simultaneously to NO<sub>2</sub> either before or after the staphylococcal challenge. In the context of the studies of Goldstein and coworkers (1973), who observed a suppression of intrapulmonary killing of *S. aureus* after a 17-hour prechallenge exposure to 2.3 ± 0.2 ppm of NO<sub>2</sub> followed by ambient air, the combined data again indicate a time-dose relationship in which the period of exposure appears to be the more important factor.

#### PULMONARY PARTICLE CLEARANCE

Previous studies by Goldstein and colleagues (1973), in which aerosol challenges with <sup>32</sup>P-labeled *S. aureus* were used to evaluate the effect of NO<sub>2</sub> on the rate of physical transport from the murine lungs (as quantified by decline in radiotracer activity), found no effect on <sup>32</sup>P removal at NO<sub>2</sub> exposure levels ranging from 1.9 ± 0.3 ppm to 14.8 ± 0.3 ppm. In our study, we overlapped and expanded these studies and found that the physical removal rates were not altered by exposures to NO<sub>2</sub> concentrations up to 20 ppm. In contrast, as observed previously with unlabeled staphylococcal challenges, NO<sub>2</sub> exposure caused a clear dose-related defect in pulmonary antibacterial defenses. These data emphasize that in terms of protection against infectious agents, in situ bactericidal destruction by the intrapulmonary phagocytic defense system is the dominant mechanism, rather than mechanical removal by the physical translocation systems (Green et al. 1977).

#### PULMONARY ANTIBACTERIAL DEFENSES IN THE PREDISPOSED HOST

Under normal conditions, inhaled bacteria are rapidly killed in murine lungs. However, exposure to environmental contaminants, therapeutic agents, viral infections, and various other stresses suppresses the antibacterial activity of the lungs (reviewed in Huber et al. 1977). To determine if an underlying condition increased the susceptibility of murine lungs to the detrimental effects of NO<sub>2</sub> at levels lower than those observed in normal mice, influenza virus infection and corticosteroid treatment were used as the predisposing factors.

Virus-associated secondary bacterial infections are of important clinical significance, especially during influenza epidemics (Martin et al. 1959; Loosli 1967, 1973; Jarstrand and Tunevall 1974). Experimental studies have shown that during the acute phase of the viral infection, the bactericidal mechanisms of the lung become progressively depressed, with maximal suppression occurring approximately a week after viral infection (Green 1966; Jakab 1974). Thereafter, the antibacterial defenses of the lung become reestablished,

and they are essentially normal by the end of the second week of infection. This impairment of pulmonary antibacterial defenses is associated with dysfunctions of the alveolar macrophage phagocytic system (Couch 1981; Jakab 1981b, 1986). Corticosteroid treatment is also known to suppress the antibacterial defenses of the lungs (Green and Kass 1964a; Pennington 1978; Blackwood and Pennington 1982; Nugent and Pesanti 1982).

In our study, both influenza virus infection and corticosteroid treatment suppressed the intrapulmonary killing of *S. aureus* (Figures 12 and 13). In addition, as observed previously, pulmonary bactericidal activity in noninfected and untreated control animals were suppressed at NO<sub>2</sub> exposure levels of 5 ppm and greater. Nitrogen dioxide exposure superimposed on the virus infection had no effect on the intrapulmonary killing of staphylococci at 5 ppm of NO<sub>2</sub>, but at 10 ppm it significantly contributed to the virus-induced bactericidal defect. In contrast, the combination of corticosteroid treatment and NO<sub>2</sub> exposure significantly impaired the intrapulmonary killing of *S. aureus* at concentrations lower (between 1 and 2.5 ppm) than that observed in normal animals (5 ppm).

The reason that one predisposing factor (corticosteroid treatment) makes the lungs hypersusceptible to the toxic effects of NO<sub>2</sub>, whereas the other predisposing factor (virus infection) does not, is not known. Previously, in similar experiments, we had to use large concentrations of the cigarette-smoke component, acrolein, to demonstrate a combined effect of virus infection and acrolein exposure on pulmonary antibacterial defenses (Jakab 1977; Astry and Jakab 1983). It is possible that differences in the cellular composition of the alveolar regions of the lungs during the time of exposure to NO<sub>2</sub> might have contributed to these results.

Virus infections of the murine lungs result in a dynamic inflammatory response. For example, on the eighth day of the infection, up to six times as many phagocytic cells can be lavaged from virus-infected lungs as from the lungs of noninfected control animals (Wyde et al. 1978, 1982; Warr and Jakab 1983). These additional phagocytes could be available to ingest and kill bacteria.

In contrast to the recruitment of inflammatory cells to virus-infected lungs, corticosteroid treatment reduces the pulmonary cell population (Pennington 1978) and the inflammatory response to the lungs after a bacterial challenge with the gram-negative organism *Pseudomonas aeruginosa* (Pennington 1977), but not after a challenge with staphylococci (Nugent and Pesanti 1982). Therefore, virus infection may provide the necessary phagocyte:bacteria ratio that would not be altered by a superimposed exposure to

5 ppm of NO<sub>2</sub>. In contrast, corticosteroids could reduce alveolar macrophage phagocytic function, thereby predisposing the antibacterial defenses of the lung to a lower concentration of NO<sub>2</sub> than would be true for the lungs of the untreated host.

Although the mechanisms of reduced phagocytic function need to be elucidated, the implications of the corticosteroid studies are clear. An underlying host defect involving lung defenses rendered the lung hypersusceptible to the effects of NO<sub>2</sub>. The influence of an altered host status on lung susceptibility to infection after NO<sub>2</sub> exposure is poorly understood; however, these findings point the way toward the analysis of other populations that may be at high risk (Pennington 1984) when exposed to pollutant gases through the numerous animal models of immunosuppression (Pennington 1985), chronic lung damage (Snider et al. 1986), and old age (Esposito and Pennington 1983).

### BACTERIAL INFECTION

*L. monocytogenes* has been used previously as a test organism to determine the systemic toxicity of benzene (Rosenthal and Snyder 1985) and formaldehyde (Dean et al. 1984) inhalation. In this study, the organism was used to evaluate the pulmonary resistance of the host to the effects of NO<sub>2</sub>. The results show that high levels of exposure to NO<sub>2</sub> (10 and 20 ppm) for four hours per day during the two-week infection period did not alter the infective process in the lungs.

Resistance against *L. monocytogenes* is more complex than that against the other bacterial agents studied herein. Host defenses against *S. aureus* depend solely on the alveolar macrophage phagocytic system (Goldstein et al. 1977a), and resistance to *P. mirabilis* and *K. pneumoniae* depends on the dual phagocytic system of the lungs (both alveolar macrophages and inflammatory PMNs) (Rehm et al. 1979), which, in the case of *P. pneumotropica*, is augmented by a specific antibody. Resistance against *L. monocytogenes* is dependent on alveolar macrophages, PMNs, and cell-mediated immunity. Immediately after infection, this facultative gram-negative intracellular bacterium can be found in alveolar macrophages, and within hours it can be found in inflammatory PMNs (G.A. Warr and G.J. Jakab, unpublished observations). For approximately five days thereafter, the viable bacterial population in the lungs does not appreciably alter. This could be because the organism is not killed or, more likely, because a balance is achieved between intracellular multiplication and killing. Yet, although the proliferation of the organism is controlled, these nonspecific defenses cannot eliminate the organism from the lungs, as specific (immunologic) defenses

are required for the intrapulmonary elimination of *L. monocytogenes* (Truitt and Mackaness 1971; Lefford et al. 1979; Harrington-Fowler et al. 1981; Miyata et al. 1982). One possible mechanism for the elimination of *Listeria* organisms from the lung might be that during the early phases of the infection, the nonspecific phagocytic defenses keep the organism from multiplying extensively in the lungs. Therefore, there is sufficient time for the host to mount the cell-mediated immune response. This specific response consists of T-lymphocytes that interact with the alveolar macrophages through soluble mediators, thus increasing the phagocytic microbicidal activity that subsequently eliminates the organism from the lungs.

An interesting trend appears to be emerging from the data on the various bacteria used in these studies: The more complex the functional resistance mechanism to a bacterium, the higher the dose of NO<sub>2</sub> required to impair defenses against that bacterium. Thus, 5 ppm of NO<sub>2</sub> affects the alveolar macrophage phagocytic system (*S. aureus* studies), whereas the combined phagocytic system involving the alveolar macrophages and PMNs shows an adverse effect at NO<sub>2</sub> concentrations of 10 ppm or greater (*P. mirabilis*, *K. pneumoniae*, and *P. pneumotropica* studies). When cell-mediated immunity is added to the dual phagocytic system (*L. monocytogenes*), an adverse effect on functional resistance against this organism will require in excess of 20 ppm of NO<sub>2</sub>. This also appears to be the case during Sendai virus infection, since the data indicate that exposure to 20 ppm of NO<sub>2</sub> does not alter the infective process, but it does increase the severity of the disease through increased lung pathology (discussed below).

### VIRAL INFECTION

Few studies have been performed of the effects of NO<sub>2</sub> on resistance to viral infection (Henry et al. 1970; Fenters et al. 1973, 1979; Motomiya et al. 1973), as the bulk of the studies have been performed with bacteria. For example, Henry and associates (1970) found that the infection of squirrel monkeys with influenza virus 24 hours before exposure to 10 ppm of NO<sub>2</sub> was fatal to all monkeys within three days. Infected control animals showed symptoms of viral infection, but they did not succumb to the infection. Exposure to 5 ppm of NO<sub>2</sub> after viral infection resulted in death in one of three monkeys.

In this study, we used parainfluenza 1 (Sendai) virus infection to examine the effect of NO<sub>2</sub> exposure on resistance to infection with a pathogen endogenous to the host (Parker et al. 1964; Ward 1974). The rationale for using Sendai virus was that studies of the effects of air pollutants on resistance

to viral infection have used infection with human isolates of influenza virus, or mouse-adapted strains of influenza virus, such as influenza A/PR8/34. These viruses are not natural pathogens to the experimental host; consequently, the relevance of these studies to the effects of air pollution on infection with a human pathogen has been questioned.

The data (Figure 16) show that exposure to NO<sub>2</sub> concentrations of 5, 10, and 20 ppm for four hours per day did not increase infectious pulmonary virus titers. At exposure concentrations of 20 ppm, the mice began to die in increasing numbers from the fourth day of infection. To gain insight into the cause of the deaths, lung wet:dry weight ratios, lung lavage albumin content, and the inflammatory cell response in the lungs were used as indices of pathologic manifestation. In combination, the data show that NO<sub>2</sub> exposure does not alter the growth and elimination of infectious virus in the lungs. This suggests that NO<sub>2</sub> does not affect the infectious process, which involves the control and elimination of the virus infection by nonspecific (interferon) and specific (immunologic) defense mechanisms. However, there is another aspect to consider in pulmonary virus infections, namely, the resultant lung damage. Sendai virus infections result in progressive pathologic changes of murine lungs (Robinson et al. 1968). The studies reported here show that NO<sub>2</sub> increases the severity of the disease process through increased lung damage, rather than through alteration of the infective process.

Whether or not NO<sub>2</sub> exposure can also increase the infective process cannot be established from these data. Virus proliferation in the lungs, and the ensuing lung damage, is virus-dose dependent (Nayak and Kelley 1965; Jakab 1975). We used a dose of virus that resulted in the proliferation of the virus to high titers and caused moderate-to-severe pneumonitis. Whether NO<sub>2</sub> exposure would increase the infective process with a lesser virus inoculum, which would result in lower pulmonary virus titers and a milder pneumonia, remains to be determined.

Finally, it should be remembered that virus infection with either Sendai or influenza A/PR8/34 virus involves pneumonitic processes in the lung parenchyma, whereas the majority of viral infections in humans involve the upper respiratory tract. No adequate models are available in rodents to readily test the hypothesis that NO<sub>2</sub> exposure increases susceptibility to viral infections of the upper respiratory tract. Human volunteer studies must be undertaken to explore this hypothesis, such as those performed by Dr. Thomas Kulle of the University of Maryland, Baltimore, MD, under the auspices of the Health Effects Institute.

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## IMPLICATIONS OF THE FINDINGS

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The goal of this work was to determine the threshold level of acute NO<sub>2</sub> exposure that would induce increased susceptibility to, and increased severity of, bacterial and viral respiratory infections. Physiologic parameters of resistance to respiratory infections were used as endpoints. This aim was accomplished through bacterial and viral challenges of the lungs that provided a composite picture of the dose-response relationship between acute exposures to NO<sub>2</sub> and impairment of a spectrum of respiratory defense parameters.

This work can be viewed as a transitional study between those investigations that used inhalation challenges with lethal infectious agents and those that are more germane to air pollution issues related to public health, namely, infectious agents that may lead to acute and chronic illness. Our approach used inhalation challenges with bacteria that are nonlethal by replication, which allowed us to characterize the effect of exposures to NO<sub>2</sub> through physiologic abnormalities such as suppression of intrapulmonary killing of organisms. The results form the necessary data base upon which specific questions can be formulated for future studies regarding the effect of exposure to NO<sub>2</sub> on resistance to infections of the respiratory tract.

First, the experiments with the gram-positive *S. aureus* and the gram-negative bacteria unraveled some of the confounding variables in the study of resistance mechanisms of the lungs and how they are modulated by exposures to NO<sub>2</sub>.

Second, the experiments with staphylococci showed that the alveolar macrophage phagocytic system, as quantified by the intrapulmonary killing of *S. aureus*, is the defense component of the lungs most susceptible to the adverse effects of NO<sub>2</sub>. A four-hour exposure to 5 ppm of NO<sub>2</sub> impaired the intrapulmonary killing of this organism.

Third, the experiments involving NO<sub>2</sub> exposure for four hours both before and after bacterial challenge with *S. aureus* show that pulmonary defenses are more susceptible to the adverse effect of NO<sub>2</sub> when the lung is already seeded with the organism. When NO<sub>2</sub> exposure both before and after bacterial challenge was examined at 2.5 ppm, the threshold dose for the adverse effect of NO<sub>2</sub> was not reduced.

Fourth, the corticosteroid experiments show an adverse effect of between 1 and 2.5 ppm of NO<sub>2</sub> on the intrapulmonary killing of *S. aureus* when NO<sub>2</sub> exposure is superimposed on immunosuppressed animals, compared with the 5 ppm of NO<sub>2</sub> needed for an adverse effect in

untreated animals. These results demonstrate that the predisposed host is hypersusceptible to the adverse effects of NO<sub>2</sub>. The implication of this finding is the probable existence of a high-risk population (because of immunosuppression, chronic lung damage, or old age) whose altered host status makes them more susceptible to infection after exposure to NO<sub>2</sub> (Green 1972).

Fifth, the studies with Sendai virus clearly show that exposure to NO<sub>2</sub> increases lung damage during virus pneumonia. Although this finding is a short-term sequela of virus infection and NO<sub>2</sub> exposure, long-term lung damage can also result from virus pneumonitis and exposure to air pollutants. For example, mice infected with influenza A/PR8/34 and continuously exposed to 0.5 ppm of ozone showed enhanced development of pulmonary fibrosis (Jakab 1988). Since childhood infections may be associated with increased risk of adult lung disease (Kattan 1979), these experimental studies indicate that concern should be given not only for the effect of NO<sub>2</sub> exposure on the short-term morbidity of the infectious episode, but also for the long-term noninfectious sequelae that may manifest themselves in the epidemic of chronic adult lung disease.

The current U.S. National Ambient Air Quality Standard for NO<sub>2</sub> is 0.05 ppm for the annual arithmetic mean of 24-hour values. Average 24-hour NO<sub>2</sub> concentrations vary widely, and can commonly reach from 0.05 ppm to 0.2 ppm, with some cities reaching short-term spikes as high as 0.5 ppm. The concentration of NO<sub>2</sub> that gave acute effects in this study (5 ppm) is substantially higher than the acceptable standards. However, pure NO<sub>2</sub>, in the absence of copollutants, probably exists only in laboratory situations. Moreover, the effective concentration could be reduced to less than one-half this concentration in immunosuppressed animals, suggesting that defects in host resistance may render subgroups of one population more susceptible to the adverse effects of NO<sub>2</sub>. There is virtually no information on the subgroups that might be implicated. Humans are undoubtedly exposed to NO<sub>2</sub> in complex mixtures of atmospheric contaminants. For this reason, the health effects of air pollution cannot be attributed to a single agent alone, as interactions need to be taken into consideration (Green 1972; Goldstein et al. 1974; Ehrlich et al. 1977; Illing et al. 1980).

In the studies reported here, we have established a model to evaluate the effects of NO<sub>2</sub> on pulmonary resistance mechanisms against infections, and we have provided the necessary data base for the further exploration of this model. The new information presented here indicates a divergence of effects of NO<sub>2</sub> on gram-positive and gram-negative bacteria, as well as on viruses. Our results provide initial clues as to which types of infections might be expected in

an NO<sub>2</sub>-exposed population, as well as which elements of the population might be at increased risk. The results, therefore, provide leads for more in-depth research concerning the defenses of the lung likely to be affected by NO<sub>2</sub>, as well as those that appear more resistant.

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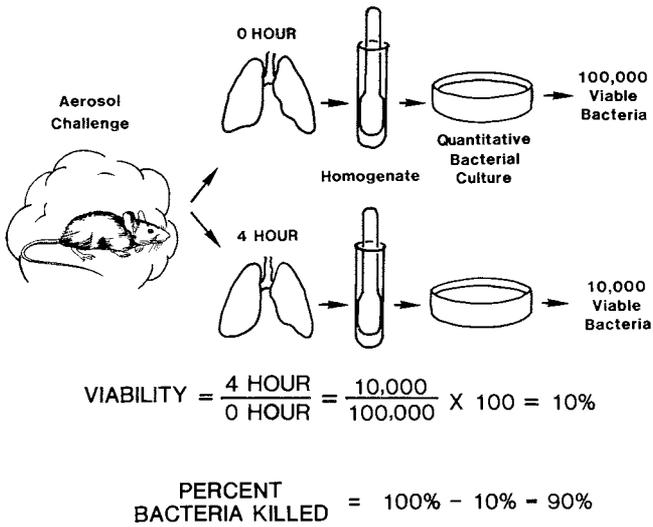
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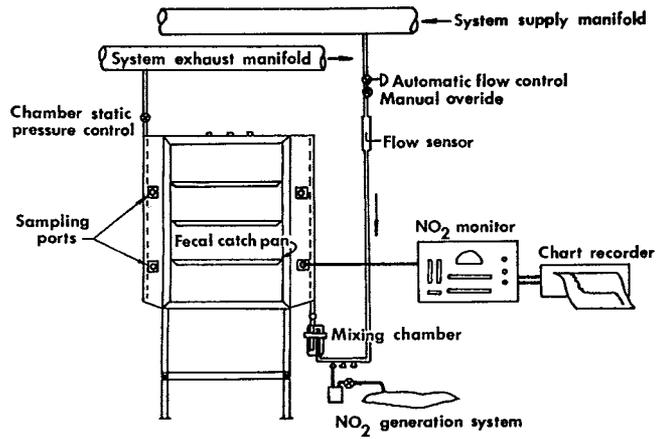
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APPENDIX A. Schematic Representation of the Pulmonary Bactericidal Assay

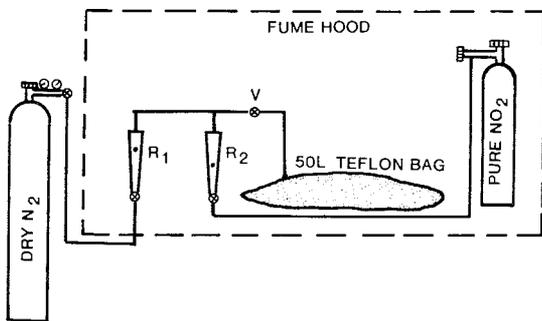


APPENDIX B. Inhalation Chamber System

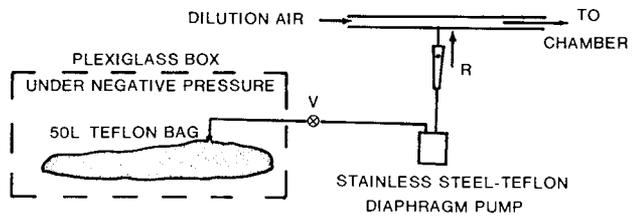


APPENDIX C. Nitrogen Dioxide Bag Fill and Generation System

NO<sub>2</sub> BAG FILL



NO<sub>2</sub> GENERATION



APPENDIX D. Comparison of Intrapulmonary Killing of *Staphylococcus aureus* in Mice Exposed to Various Concentrations of Nitrogen Dioxide for Four Hours After Bacterial Challenge

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>a</sup>		
			0 Hours	4 Hours	
				Control	NO <sub>2</sub> -Exposed
2.5	1	10	100.0 ± 10.7	6.5 ± 0.7	8.3 ± 2.4
	2	10	100.0 ± 8.4	12.8 ± 3.8	6.2 ± 0.5
	Pool	20	100.0 ± 6.6	9.7 ± 2.0	7.3 ± 1.2
4	1	10	100.0 ± 9.5	7.3 ± 1.2	9.0 ± 0.7
	2	10	100.0 ± 7.2	7.3 ± 0.9	12.8 ± 1.9
	Pool	20	100.0 ± 5.8	7.3 ± 0.7	10.9 ± 1.1
5	1	6	100.0 ± 9.9	8.7 ± 0.9	10.0 ± 0.6
	2	6	100.0 ± 8.0	11.4 ± 4.3	29.0 ± 5.0
	3	6	100.0 ± 8.1	6.2 ± 0.7	14.7 ± 2.1
	Pool	18	100.0 ± 4.7	8.8 ± 1.5	17.9 ± 2.6 <sup>b</sup>
10	1	6	100.0 ± 10.9	7.2 ± 1.3	23.1 ± 6.9
	2	6	100.0 ± 11.4	11.6 ± 2.3	41.1 ± 13.9
	3	10	100.0 ± 8.1	7.4 ± 1.3	14.1 ± 2.3
	Pool	22	100.0 ± 5.4	8.5 ± 1.0	23.9 ± 4.8 <sup>c</sup>
15	1	6	100.0 ± 10.1	11.3 ± 1.3	56.7 ± 5.9
	2	8	100.0 ± 8.2	10.5 ± 4.4	41.9 ± 6.9
	Pool	14	100.0 ± 6.2	10.9 ± 2.5	48.2 ± 4.9 <sup>c</sup>

<sup>a</sup> Each value represents the mean ± SE.

<sup>b</sup> p < 0.05.

<sup>c</sup> p < 0.01.

APPENDIX E. Comparison of Intrapulmonary Killing of *Proteus mirabilis* in Mice Exposed to Various Concentrations of Nitrogen Dioxide for Four Hours After Bacterial Challenge<sup>a</sup>

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>b</sup>		
			0 Hours	4 Hours	
				Control	NO <sub>2</sub> -Exposed
5	1	10	100.0 ± 8.8	27.3 ± 1.9	26.4 ± 2.9
	2	10	100.0 ± 8.7	15.0 ± 1.3	17.5 ± 1.6
	Pool	20	100.0 ± 6.0	21.1 ± 1.8	21.9 ± 1.9
10	1	6	100.0 ± 3.2	19.5 ± 2.1	16.6 ± 2.0
	2	6	100.0 ± 6.5	29.1 ± 5.2	17.3 ± 1.6
	3	6	100.0 ± 5.6	26.4 ± 2.1	20.1 ± 2.0
	4	6	100.0 ± 9.8	23.6 ± 3.2	14.5 ± 2.5
	Pool	24	100.0 ± 3.1	24.6 ± 1.7	17.1 ± 1.1 <sup>c</sup>
15	1	6	100.0 ± 8.9	26.6 ± 2.8	20.3 ± 2.2
	2	6	100.0 ± 5.2	26.8 ± 1.5	25.7 ± 2.4
	3	8	100.0 ± 5.4	17.8 ± 1.6	18.2 ± 1.4
	Pool	20	100.0 ± 3.6	23.2 ± 1.5	21.1 ± 1.3
17.5	1	10	100.0 ± 11.2	20.0 ± 2.0	21.3 ± 2.3
	2	10	100.0 ± 10.8	22.1 ± 1.8	23.6 ± 1.7
	Pool	20	100.0 ± 7.6	21.0 ± 1.3	22.4 ± 1.4
20	1	6	100.0 ± 12.8	21.3 ± 1.8	32.7 ± 4.9
	2	6	100.0 ± 14.1	20.9 ± 2.9	38.5 ± 5.5
	3	6	100.0 ± 10.1	26.2 ± 4.1	36.5 ± 5.5
	4	6	100.0 ± 5.1	19.9 ± 3.2	35.6 ± 2.1
	Pool	24	100.0 ± 5.2	22.1 ± 1.5	35.8 ± 2.2 <sup>d</sup>
25	1	10	100.0 ± 12.2	23.4 ± 2.2	39.3 ± 3.8 <sup>d</sup>

<sup>a</sup> Analysis of variance (ANOVA) total p for concentration ≤ 0.0001.

<sup>b</sup> Each value represents the mean ± SE.

<sup>c</sup> p < 0.05.

<sup>d</sup> p < 0.01.

APPENDIX F. Comparison of Cells Retrieved from Murine Lungs by Bronchoalveolar Lavage at Four Hours After Treatment with Either Ambient Air, Nitrogen Dioxide, *Proteus mirabilis*, or *Proteus mirabilis* and Nitrogen Dioxide

Treatment	Total Cell Count Per Lung <sup>a</sup> ( $\times 10^9$ )	Percent <sup>a,b</sup> Macrophages	Percent <sup>a,b</sup> PMNs	Differential Cell Count Per Lung <sup>a</sup>	
				Macrophages ( $\times 10^5$ )	PMNs ( $\times 10^3$ )
Ambient Air	6.3 $\pm$ 0.4	99.5 $\pm$ 0.2	0	6.2 $\pm$ 0.4	0
NO <sub>2</sub> (10 ppm)	8.1 $\pm$ 1.2	97.3 $\pm$ 1.1	1.6 $\pm$ 0.9	7.9 $\pm$ 1.2	7.5 $\pm$ 3.1
<i>P. mirabilis</i>	37.5 $\pm$ 2.0	9.2 $\pm$ 1.5	88.7 $\pm$ 1.8	3.5 $\pm$ 0.7	3324.7 $\pm$ 186.1
<i>P. mirabilis</i> + 10 ppm NO <sub>2</sub>	59.4 $\pm$ 4.3	7.9 $\pm$ 1.4	90.1 $\pm$ 1.4	4.7 $\pm$ 0.9	5359.3 $\pm$ 418.7
Ambient Air	5.9 $\pm$ 0.4	99.1 $\pm$ 0.4	0.4 $\pm$ 0.2	5.8 $\pm$ 0.4	1.7 $\pm$ 1.2
NO <sub>2</sub> (25 ppm)	6.3 $\pm$ 0.4	94.9 $\pm$ 0.8	3.8 $\pm$ 0.7	6.0 $\pm$ 0.4	23.8 $\pm$ 4.5
<i>P. mirabilis</i>	38.4 $\pm$ 3.3	9.4 $\pm$ 1.2	88.4 $\pm$ 1.4	3.7 $\pm$ 0.6	3385.3 $\pm$ 276.2
<i>P. mirabilis</i> + 25 ppm NO <sub>2</sub>	54.4 $\pm$ 4.0	3.1 $\pm$ 0.2	93.4 $\pm$ 1.6	1.7 $\pm$ 0.2	5076.8 $\pm$ 391.8

<sup>a</sup> Each value represents the mean  $\pm$  SE of eight determinations.

<sup>b</sup> Lymphocytes were  $<$  2 percent.

APPENDIX G. Comparison of Intrapulmonary Killing of *Klebsiella pneumoniae* in Mice Exposed to 10 ppm of Nitrogen Dioxide for Four Hours After Bacterial Challenge

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>a</sup>		
			0 Hours	4 Hours	
				Control	NO <sub>2</sub> -Exposed
10	1	6	100.0 $\pm$ 10.8	30.4 $\pm$ 3.5	39.4 $\pm$ 2.8
	2	6	100.0 $\pm$ 10.0	23.7 $\pm$ 2.5	54.9 $\pm$ 4.3
	3	6	100.0 $\pm$ 10.8	30.1 $\pm$ 2.1	42.5 $\pm$ 6.0
	Pool	18	100.0 $\pm$ 5.7	28.1 $\pm$ 1.7	45.6 $\pm$ 3.0 <sup>b</sup>

<sup>a</sup> Each value represents the mean  $\pm$  SE.

<sup>b</sup>  $p <$  0.001.

APPENDIX H. Comparison of Cells Retrieved from Murine Lungs by Bronchoalveolar Lavage Four Hours After Treatment with Either Ambient Air, Nitrogen Dioxide, *Klebsiella pneumoniae*, or *Klebsiella pneumoniae* and Nitrogen Dioxide

Treatment	Total Cell Count Per Lung <sup>a</sup> ( $\times 10^5$ )	Percent <sup>a</sup> Macrophages	Percent <sup>a</sup> PMNs	Differential Cell Count Per Lung <sup>a</sup>	
				Macrophages ( $\times 10^5$ )	PMNs ( $\times 10^3$ )
Ambient Air	3.8 $\pm$ 0.3	99.5 $\pm$ 0.3	0.4 $\pm$ 0.2	3.8 $\pm$ 0.3	1.6 $\pm$ 0.8
NO <sub>2</sub> (10 ppm)	4.8 $\pm$ 0.3	95.5 $\pm$ 0.7	3.0 $\pm$ 0.4	4.6 $\pm$ 0.3	14.3 $\pm$ 2.3
<i>K. pneumoniae</i>	13.5 $\pm$ 1.3	29.5 $\pm$ 2.6	67.5 $\pm$ 3.0	3.9 $\pm$ 0.5	918.2 $\pm$ 104.5
<i>K. pneumoniae</i> + 10 ppm NO <sub>2</sub>	20.1 $\pm$ 2.2	29.1 $\pm$ 2.4	67.1 $\pm$ 2.5	5.9 $\pm$ 1.0	1337.9 $\pm$ 143.3

<sup>a</sup> Each value represents the mean  $\pm$  SE of eight determinations.

APPENDIX I. Comparison of Intrapulmonary Killing of *Pasteurella pneumotropica* in Mice Exposed to Various Concentrations of Nitrogen Dioxide for Four Hours After Bacterial Challenge<sup>a</sup>

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>b</sup>		
			0 Hours	4 Hours	
				Control	NO <sub>2</sub> -Exposed
5	1	10	100.0 ± 11.3	11.8 ± 1.7	14.7 ± 2.5
	2	6	100.0 ± 21.3	22.0 ± 2.4	20.7 ± 2.9
	3	10	100.0 ± 18.5	30.2 ± 5.4	23.8 ± 5.1
	Pool	26	100.0 ± 9.3	21.2 ± 2.7	19.6 ± 2.6
10	1	6	100.0 ± 17.7	30.2 ± 5.8	39.7 ± 4.2
	2	6	100.0 ± 6.8	19.5 ± 6.5	34.4 ± 6.5
	3	8	100.0 ± 6.3	11.3 ± 2.7	21.9 ± 3.8
	4	10	100.0 ± 6.6	17.1 ± 3.2	20.2 ± 3.7
	Pool	30	100.0 ± 5.7	18.6 ± 3.1	27.4 ± 3.0
15	1	10	100.0 ± 9.0	20.3 ± 2.3	28.7 ± 5.2
	2	10	100.0 ± 6.0	25.2 ± 6.2	22.0 ± 3.3
	Pool	20	100.0 ± 5.2	22.7 ± 3.2	25.3 ± 3.1
20	1	6	100.0 ± 11.3	13.7 ± 2.5	23.9 ± 1.4
	2	6	100.0 ± 17.7	19.6 ± 8.3	23.3 ± 6.5
	3	6	100.0 ± 21.3	10.3 ± 3.2	13.1 ± 3.0
	4	10	100.0 ± 13.0	31.0 ± 4.1	35.1 ± 8.3
	Pool	28	100.0 ± 7.4	20.4 ± 2.8	25.5 ± 3.6
25	1	10	100.0 ± 12.4	11.8 ± 2.4	25.3 ± 3.6
	2	10	100.0 ± 15.4	22.2 ± 3.4	38.7 ± 5.1
	Pool	20	100.0 ± 9.6	17.0 ± 2.3	32.0 ± 3.4 <sup>c</sup>
30	1	10	100.0 ± 9.6	14.6 ± 2.6	29.7 ± 5.9
	2	10	100.0 ± 10.4	25.1 ± 3.6	44.7 ± 6.2
	Pool	20	100.0 ± 6.9	19.4 ± 2.5	37.2 ± 4.5 <sup>c</sup>

<sup>a</sup> ANOVA total p for concentration ≤ 0.001.

<sup>b</sup> Each value represents the mean ± SE.

<sup>c</sup> p < 0.01.

APPENDIX J. Comparison of Cells Retrieved from Murine Lungs by Bronchoalveolar Lavage Four Hours After Treatment with Either Ambient Air, Nitrogen Dioxide, *Pasteurella pneumotropica*, or *Pasteurella pneumotropica* and Nitrogen Dioxide

Treatment	Total Cell Count Per Lung <sup>a</sup> (× 10 <sup>5</sup> )	Percent <sup>a</sup> Macrophages	Percent <sup>a</sup> PMNs	Differential Cell Count Per Lung <sup>a</sup>	
				Macrophages (× 10 <sup>5</sup> )	PMNs (× 10 <sup>3</sup> )
Ambient Air	6.3 ± 0.6	99.5 ± 0.3	0.3 ± 0.2	6.3 ± 0.6	1.7 ± 1.1
NO <sub>2</sub>	7.0 ± 0.5	97.4 ± 0.6	1.7 ± 0.4	6.8 ± 0.4	11.9 ± 0.3
<i>P. pneumotropica</i>	43.6 ± 4.7	9.9 ± 1.3	89.5 ± 1.4	4.3 ± 0.8	3893.4 ± 417.5
<i>P. pneumotropica</i> + 10 ppm NO <sub>2</sub>	35.0 ± 3.0	7.1 ± 1.1	92.5 ± 1.1	2.4 ± 0.4	3246.7 ± 296.1

<sup>a</sup> Each value represents the mean ± SE of eight determinations.

APPENDIX K. Comparison of Cells Retrieved from Murine Lungs by Bronchoalveolar Lavage at Four Hours After Treatment with Either Ambient Air, Nitrogen Dioxide, *Staphylococcus aureus*, or *Staphylococcus aureus* and Nitrogen Dioxide

Treatment	Total Cell Count Per Lung <sup>a</sup> ( $\times 10^5$ )	Percent <sup>a</sup> Macrophages	Percent <sup>a</sup> PMNs	Differential Cell Count Per Lung <sup>a</sup>	
				Macrophages ( $\times 10^5$ )	PMNs ( $\times 10^3$ )
Ambient Air	6.3 $\pm$ 0.6	98.8 $\pm$ 0.2	0.9 $\pm$ 0.2	6.2 $\pm$ 0.6	5.8 $\pm$ 1.5
NO <sub>2</sub> (5 ppm)	7.2 $\pm$ 0.6	99.6 $\pm$ 0.2	0.1 $\pm$ 0.1	7.2 $\pm$ 0.6	0.6 $\pm$ 0.6
<i>S. aureus</i>	8.2 $\pm$ 0.8	96.8 $\pm$ 0.6	2.2 $\pm$ 0.4	7.9 $\pm$ 0.8	18.2 $\pm$ 3.4
<i>S. aureus</i> + 5 ppm NO <sub>2</sub>	7.2 $\pm$ 0.7	96.6 $\pm$ 0.6	1.9 $\pm$ 0.5	7.0 $\pm$ 0.7	11.4 $\pm$ 2.4

<sup>a</sup> Each value represents the mean  $\pm$  SE of eight determinations.

APPENDIX L. Comparison of Intrapulmonary Killing of *Staphylococcus aureus* in Mice Exposed to Various Concentrations of Nitrogen Dioxide for Four Hours Before Bacterial Challenge<sup>a</sup>

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>b</sup>			
			Control		NO <sub>2</sub> -Exposed	
			0 Hours	4 Hours	0 Hours	4 Hours
5	1	8	100.0 $\pm$ 7.9	9.2 $\pm$ 1.4	100.0 $\pm$ 10.8	11.1 $\pm$ 1.2
	2	8	100.0 $\pm$ 12.6	17.6 $\pm$ 1.8	100.0 $\pm$ 7.2	13.5 $\pm$ 1.5
	Pool	16	100.0 $\pm$ 7.2	13.4 $\pm$ 1.5	100.0 $\pm$ 6.2	12.3 $\pm$ 1.0
10	1	8	100.0 $\pm$ 7.4	15.7 $\pm$ 1.7	100.0 $\pm$ 12.4	18.7 $\pm$ 1.7
	2	8	100.0 $\pm$ 13.6	13.4 $\pm$ 1.3	100.0 $\pm$ 14.2	24.8 $\pm$ 1.7
	3	8	100.0 $\pm$ 8.6	11.1 $\pm$ 1.9	100.0 $\pm$ 12.6	21.1 $\pm$ 3.2
	Pool	24	100.0 $\pm$ 5.7	13.4 $\pm$ 1.0	100.0 $\pm$ 7.2	21.5 $\pm$ 1.4 <sup>c</sup>

<sup>a</sup> ANOVA total p concentration  $\leq$  0.0001.

<sup>b</sup> Each value represents the mean  $\pm$  SE.

<sup>c</sup> p < 0.01.

APPENDIX M. Comparison of Intrapulmonary Killing of *Proteus mirabilis* in Mice Exposed to Various Concentrations of Nitrogen Dioxide for Four Hours Before Bacterial Challenge

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>a</sup>			
			Control		NO <sub>2</sub> -Exposed	
			0 Hours	4 Hours	0 Hours	4 Hours
20	1	8	100.0 $\pm$ 13.4	31.1 $\pm$ 3.2	100.0 $\pm$ 6.8	37.3 $\pm$ 5.1
	2	8	100.0 $\pm$ 11.7	30.7 $\pm$ 4.5	100.0 $\pm$ 10.3	30.3 $\pm$ 5.4
	Pool	16	100.0 $\pm$ 8.6	30.9 $\pm$ 2.7	100.0 $\pm$ 6.0	33.8 $\pm$ 3.7
30	1	8	100.0 $\pm$ 10.7	28.8 $\pm$ 4.7	100.0 $\pm$ 11.6	15.7 $\pm$ 2.1
	2	8	100.0 $\pm$ 21.2	26.4 $\pm$ 4.7	100.0 $\pm$ 11.5	16.4 $\pm$ 2.6
	Pool	16	100.0 $\pm$ 11.5	27.6 $\pm$ 3.2	100.0 $\pm$ 7.9	16.1 $\pm$ 1.6 <sup>b</sup>

<sup>a</sup> Each value represents the mean  $\pm$  SE.

<sup>b</sup> p < 0.01 between four-hour control and four-hour NO<sub>2</sub>-exposed.

APPENDIX N. Comparison of Intrapulmonary Killing of *Staphylococcus aureus* in Mice Exposed to 2.5 ppm Nitrogen Dioxide for Four Hours Before and After Bacterial Challenge

Treatment	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>a</sup>	
			0 Hours	4 Hours
Air-Bacteria-Air	1	8	100.0 ± 7.7	10.6 ± 1.3
	2	8	100.0 ± 9.4	12.8 ± 1.6
	Pool	16	100.0 ± 6.4	11.8 ± 1.1
Air-Bacteria-NO <sub>2</sub>	1	8		11.2 ± 1.3
	2	8		10.3 ± 1.3
	Pool	16		10.8 ± 0.9
NO <sub>2</sub> -Bacteria-Air	1	8	100.0 ± 12.3	9.1 ± 1.1
	2	8	100.0 ± 10.2	10.3 ± 1.0
	Pool	16	100.0 ± 7.7	9.7 ± 0.8
NO <sub>2</sub> -Bacteria-NO <sub>2</sub>	1	8		10.2 ± 1.0
	2	8		9.8 ± 1.4
	Pool	16		10.0 ± 0.8

<sup>a</sup> Each value represents the mean ± SE.

APPENDIX O. Comparison of Intrapulmonary Killing of *Staphylococcus aureus* in Mice Infected Eight Days Previously with Influenza A/PR8/34 Virus That Were Exposed to Various Concentrations of Nitrogen Dioxide for Four Hours After Bacterial Challenge<sup>a</sup>

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>b</sup>					
			0 Hours		4 Hours		Virus-Infected <sup>c</sup>	Infected + NO <sub>2</sub> -Exposed
			Control	Virus-Infected	Control	NO <sub>2</sub> -Exposed		
5	1	5	100.0 ± 9.0	100.0 ± 13.9	6.1 ± 1.9	13.1 ± 2.9	34.3 ± 11.3	36.0 ± 10.3
	2	5	100.0 ± 15.6	100.0 ± 12.1	13.0 ± 2.8	16.1 ± 2.1	49.1 ± 9.4	42.5 ± 6.1
	3	5	100.0 ± 17.3	100.0 ± 21.7	11.7 ± 2.8	16.6 ± 3.1	39.1 ± 6.0	49.4 ± 11.3
	4	5	100.0 ± 13.6	100.0 ± 15.5	12.0 ± 1.5	19.1 ± 3.1	44.7 ± 7.9	44.8 ± 5.4
	Pool	20	100.0 ± 6.5	100.0 ± 7.4	10.7 ± 1.2	16.2 ± 1.4	41.8 ± 4.3	43.2 ± 4.1
10	1	5	100.0 ± 11.7	100.0 ± 11.4	7.8 ± 0.7	22.3 ± 4.4	33.0 ± 6.6	41.7 ± 3.0
	2	5	100.0 ± 6.8	100.0 ± 21.6	11.2 ± 1.8	24.0 ± 2.9	44.2 ± 9.0	59.8 ± 6.4
	3	5	100.0 ± 13.2	100.0 ± 16.4	11.2 ± 2.6	18.2 ± 3.0	52.2 ± 5.5	77.2 ± 11.4
	4	5	100.0 ± 13.2	100.0 ± 18.0	10.0 ± 1.8	29.1 ± 7.7	47.2 ± 6.6	62.4 ± 13.4
	Pool	20	100.0 ± 5.3	100.0 ± 7.9	10.0 ± 0.9	23.4 ± 2.4 <sup>d</sup>	44.2 ± 3.6	60.3 ± 5.2 <sup>e</sup>

<sup>a</sup> ANOVA total p for NO<sub>2</sub> concentrations ≤ 0.0001; ANOVA total p for experimental groups ≤ 0.005.

<sup>b</sup> Each value represents the mean ± SE.

<sup>c</sup> Each value is significantly different (p ≤ 0.01) from those for the four-hour noninfected control group.

<sup>d</sup> p < 0.01 between the control and NO<sub>2</sub>-exposed groups.

<sup>e</sup> p < 0.01 between virus-infected and infected and NO<sub>2</sub>-exposed groups.

APPENDIX P. Comparison of Intrapulmonary Killing of *Staphylococcus aureus* in Mice Treated with Corticosteroids for Four Days That Were Exposed to Various Concentrations of Nitrogen Dioxide for Four Hours After Bacterial Challenge<sup>a</sup>

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Percent of Initial Viable Bacteria Remaining <sup>b</sup>							
			0 Hours				4 Hours			
			Control	Steroid- Treated		Control	NO <sub>2</sub> -Exposed	Steroid- Treated	Steroid + NO <sub>2</sub> -Exposed	
1	1	5	100.0 ± 6.7	100.0 ± 7.1		13.2 ± 1.6	14.9 ± 2.7	18.2 ± 1.9	21.2 ± 0.8	
	2	5	100.0 ± 11.8	100.0 ± 11.2		8.8 ± 1.1	9.4 ± 0.7	16.3 ± 0.3	19.2 ± 2.1	
	3	5	100.0 ± 2.9	100.0 ± 3.8		9.0 ± 0.8	9.0 ± 0.8	18.2 ± 0.7	23.2 ± 2.5	
	4	5	100.0 ± 10.1	100.0 ± 9.4		6.5 ± 0.5	6.3 ± 0.7	14.6 ± 1.7	20.3 ± 2.2	
	Pool	20	100.0 ± 3.9	100.0 ± 3.8		9.4 ± 0.7	9.9 ± 1.0	16.8 ± 0.7 <sup>c</sup>	21.0 ± 1.0	
2.5	1	5	100.0 ± 8.8	100.0 ± 6.3		11.1 ± 2.0	12.3 ± 1.8	17.7 ± 1.0	23.3 ± 2.8	
	2	5	100.0 ± 21.0	100.0 ± 9.3		11.3 ± 2.3	9.9 ± 2.0	20.0 ± 2.5	27.8 ± 3.6	
	3	5	100.0 ± 10.9	100.0 ± 11.7		7.3 ± 1.3	6.7 ± 1.0	22.6 ± 4.5	29.9 ± 5.3	
	4	5	100.0 ± 10.4	100.0 ± 8.4		7.6 ± 1.1	9.6 ± 1.2	15.1 ± 2.0	21.0 ± 1.0	
	Pool	20	100.0 ± 6.3	100.0 ± 4.2		9.3 ± 0.9	9.6 ± 0.8	18.9 ± 1.4 <sup>c</sup>	25.5 ± 1.8 <sup>d</sup>	
5	1	5	100.0 ± 15.8	100.0 ± 6.9		7.6 ± 1.3	9.7 ± 1.0	21.0 ± 2.2	44.7 ± 7.9	
	2	5	100.0 ± 12.6	100.0 ± 1.2		10.6 ± 1.2	15.6 ± 2.3	23.8 ± 3.3	35.1 ± 1.2	
	3	5	100.0 ± 12.0	100.0 ± 18.1		10.4 ± 0.9	14.9 ± 1.4	17.2 ± 2.9	27.2 ± 2.4	
	4	5	100.0 ± 4.7	100.0 ± 12.3		9.9 ± 2.7	13.8 ± 2.7	20.5 ± 2.0	39.2 ± 4.9	
	Pool	20	100.0 ± 5.5	100.0 ± 5.8		9.6 ± 0.8	13.5 ± 1.0	20.6 ± 1.3 <sup>c</sup>	36.5 ± 3.0 <sup>d</sup>	
10	1	5	100.0 ± 9.9	100.0 ± 8.4		9.5 ± 0.7	20.4 ± 2.2	20.1 ± 1.7	48.7 ± 6.6	
	2	5	100.0 ± 20.9	100.0 ± 9.0		10.1 ± 1.8	21.1 ± 5.9	18.7 ± 1.8	45.2 ± 2.2	
	Pool	10	100.0 ± 10.9	100.0 ± 5.8		9.8 ± 0.9	20.8 ± 3.0 <sup>e</sup>	19.4 ± 1.2 <sup>c</sup>	46.9 ± 3.3 <sup>d</sup>	

<sup>a</sup> ANOVA total p for NO<sub>2</sub> concentrations ≤ 0.0001; ANOVA total p for treatment groups ≤ 0.001.

<sup>b</sup> Each value represents the mean ± SE.

<sup>c</sup> Each value is significantly different (p < 0.01 or less) from those for the four-hour nontreated control group.

<sup>d</sup> p < 0.05 or less between steroid-treated and steroid-treated + NO<sub>2</sub>-exposed groups.

<sup>e</sup> p < 0.05 or less between control and NO<sub>2</sub>-exposed groups.

APPENDIX Q. Comparison of Lung Wet:Dry Weight Ratios in Mice Infected with Sendai Virus Eight Days Previously That Were Exposed for Four Hours Per Day to Either 10 ppm or 20 ppm of Nitrogen Dioxide

NO <sub>2</sub> Conc. (ppm)	Run No.	n	Noninfected		Virus-Infected	
			Control	NO <sub>2</sub> -Exposed	Control	NO <sub>2</sub> -Exposed
10	1	4	4.525 ± 0.023	4.349 ± 0.089	5.105 ± 0.340	5.563 ± 0.294
	2	4	4.420 ± 0.110	4.487 ± 0.202	5.168 ± 0.215	5.729 ± 0.189
	Pool	8	4.472 ± 0.056	4.418 ± 0.106	5.136 ± 0.187 <sup>a</sup>	5.646 ± 0.165 <sup>b</sup>
20	1	6	4.465 ± 0.045	4.740 ± 0.093	5.009 ± 0.113 <sup>a</sup>	5.747 ± 0.156 <sup>b</sup>

<sup>a</sup> p < 0.01 between noninfected and virus-infected control groups.

<sup>b</sup> p < 0.01 between virus-infected controls and virus-infected NO<sub>2</sub>-exposed groups.

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 APPENDIX R. Comparison of Cells Lavaged from Lungs of Mice That Were Infected with Sendai Virus Eight Days Previously and Exposed to Either Ambient Air or Nitrogen Dioxide at 10.0 ppm or 20 ppm for Four Hours Per Day
 

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Treatment	Total Cell Count Per Lung <sup>a</sup> ( $\times 10^5$ )	Percent <sup>a</sup>			Differential Cell Count Per Lung <sup>a</sup>		
		Macrophages	Lymphocytes	PMNs	Macrophages ( $\times 10^5$ )	Lymphocytes ( $\times 10^3$ )	PMNs ( $\times 10^3$ )
Ambient Air	3.4 $\pm$ 0.3	98.8 $\pm$ 0.4	0.5 $\pm$ 0.2	0.7 $\pm$ 0.3	3.4 $\pm$ 0.3	1.7 $\pm$ 0.7	2.5 $\pm$ 0.8
NO <sub>2</sub> (10 ppm)	4.2 $\pm$ 0.2	96.4 $\pm$ 0.5	0.9 $\pm$ 0.3	2.7 $\pm$ 0.5	4.1 $\pm$ 0.2	3.8 $\pm$ 1.3	11.5 $\pm$ 1.7
NO <sub>2</sub> (20 ppm)	4.7 $\pm$ 0.3	87.3 $\pm$ 1.1	2.1 $\pm$ 0.4	10.6 $\pm$ 0.9	4.1 $\pm$ 0.3	9.9 $\pm$ 2.2	49.8 $\pm$ 6.2
Sendai	10.3 $\pm$ 0.9	86.6 $\pm$ 1.7	10.4 $\pm$ 1.6	3.0 $\pm$ 0.3	8.9 $\pm$ 0.8	107.1 $\pm$ 19.0	30.9 $\pm$ 4.2
Sendai + 10 ppm NO <sub>2</sub>	12.1 $\pm$ 0.9	82.2 $\pm$ 1.6	11.8 $\pm$ 1.1	6.0 $\pm$ 0.6	9.9 $\pm$ 0.8	142.8 $\pm$ 10.6	72.6 $\pm$ 6.3
Sendai + 20 ppm NO <sub>2</sub>	17.8 $\pm$ 1.7	73.1 $\pm$ 1.8	12.3 $\pm$ 0.7	14.6 $\pm$ 1.1	13.0 $\pm$ 1.3	218.9 $\pm$ 25.3	259.9 $\pm$ 25.4

<sup>a</sup> Each value represents the mean  $\pm$  SE of eight determinations.

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 ABOUT THE AUTHOR
 

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**George J. Jakab** is a professor in the Department of Environmental Health Sciences at the Johns Hopkins School of Hygiene and Public Health. He received his Ph.D. in medical microbiology in 1970 from the University of Wisconsin (Madison, WI) and his postdoctoral training in the Department of Medicine at the University of Vermont. Dr. Jakab has conducted research and published articles on pulmonary defense mechanisms against infectious agents and the interaction of pulmonary infections and atmospheric contaminants on acute and chronic lung diseases.

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 PUBLICATIONS RESULTING FROM  
THIS RESEARCH
 

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

Hemenway DR, Jakab GJ. 1987. The monitoring and generation of standard atmospheres of nitrogen dioxide for inhalation studies: Problems and solutions. *Appl Ind Hyg* 2:18-23.

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**INTRODUCTION**

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The Health Effects Institute issued a Request for Applications (RFA 83-2) soliciting proposals on "Nitrogen Oxides and Susceptibility to Respiratory Infections" in the summer of 1983. In September 1983, Dr. George J. Jakab of the Johns Hopkins School of Hygiene and Public Health, Baltimore, MD, proposed a project entitled "Nitrogen Dioxide-Induced Increased Susceptibility to and Severity of Respiratory Infections." In March 1984, the HEI approved the three-year project, with modifications, and authorized total expenditures of \$339,945. The project began in October 1984, and the final report was accepted by the Health Review Committee in October 1987. The Health Review Committee Report is intended to place the Investigator's Report in perspective as an aid to the sponsors of the HEI and to the public.

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**THE CLEAN AIR ACT**

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

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

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards. The current standards include primary and secondary standards for nitrogen dioxide. Those standards were last reviewed in 1985. Also, under Section 166 of the Act, in February 1988,

the EPA published regulations to prevent significant deterioration of air quality due to emissions of nitrogen dioxide. Research of the type described here can increase understanding of the effects of nitrogen dioxide on lung tissue, and thus contribute indirectly to the appropriateness of the existing standards and to ongoing and future regulatory initiatives.

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**BACKGROUND**

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The nitrogen dioxide that is present in urban environments is derived largely from vehicular sources. Morning rush-hour traffic generates high concentrations of nitric oxide (NO), which in the presence of sunlight and oxygen (O<sub>2</sub>), is converted to nitrogen dioxide (NO<sub>2</sub>) (Ehrlich 1980). The current one-hour National Ambient Air Quality Standard for nitrogen dioxide is 0.053 parts per million (ppm) averaged over one year. Although the nitrogen dioxide standard is being met generally, typical long-term ambient concentrations of nitrogen dioxide range from 0.001 ppm in isolated rural areas to hourly peaks in urban areas that can exceed 0.3 ppm (U.S. Environmental Protection Agency 1985). Nitrogen dioxide generated indoors by appliances such as gas stoves reaches average levels of 0.025 ppm, although peaks as high as 0.2 to 0.4 ppm may be reached (Samet et al. 1987). Nitrogen dioxide is known to cause serious biologic effects at high levels of exposure. There is also evidence to suggest that exposure to nitrogen dioxide leads to increased susceptibility to respiratory infections (see reviews by U.S. Environmental Protection Agency 1982; Morrow 1984; Samet et al. 1987).

Under normal conditions, the lung has the capability to maintain sterile conditions, and it disposes of particulates and microorganisms by constantly removing or detoxifying any such agents that are deposited on the lung surfaces (Gardner 1982). This is accomplished through the cooperative action of both specific and nonspecific components of host defense mechanisms, which include transport mechanisms for clearance of foreign particles, bacteria, and viruses; phagocytic cells (alveolar macrophages and polymorphonuclear leukocytes); and immune secretions, such as interferons and immunoglobulins. Toxicological evidence indicates that oxidants such as nitrogen dioxide penetrate beyond the nasopharyngeal region, and can alter the normal functioning of the major pulmonary defenses (Gardner 1984). Data from animal studies suggest that such alterations of pulmonary defenses may make them vulnerable to secondary insults, such as infection (Gardner 1982; Pennington 1988).

Clinical studies on the effect of nitrogen dioxide exposure on respiratory infections are limited because of ethical and

practical considerations. A number of epidemiological studies have investigated this relation between nitrogen dioxide exposure and respiratory infections; however, taken as a whole, the results of epidemiological studies have been inconsistent and inconclusive (Samet et al. 1987; Pennington 1988). The relation between nitrogen dioxide exposure and respiratory infections has also been explored in a number of animal systems. Green (1984) has argued that an appropriate animal model for testing the environmental pollutant and microorganism disease relationship should fulfill several criteria. First, most human infectious episodes generally result in morbidity, rather than mortality. Therefore, in an animal model, the measurement of minor changes in symptomatology and physiology may be more pertinent than the measurement of mortality. Second, endogenous microorganisms are the source of most respiratory infections in humans. Therefore, autochthonous rather than exogenous microbial flora should be used in experimental studies. Finally, the animal model ought to measure the physiologic derangement of the host defenses that precedes infection, such as rates of physical removal and intrinsic killing of bacteria, rather than pathologic measures of defense.

Most animal studies have fulfilled these criteria only to a limited extent. In one in vivo model, animals are exposed to inhaled nitrogen dioxide, then infected with highly virulent microorganisms, generally by aerosol inhalation. The effects of nitrogen dioxide exposure on the lung defenses against infection are assessed by decreased survival of the infected animals. In a typical study, McGrath and Oyervides (1985) noted a four-fold increase in mortality in mice acutely exposed (three days, 24 hours per day) to 5 ppm nitrogen dioxide, followed by bacterial challenge with *Klebsiella pneumoniae*. Monkeys exposed to influenza virus and 5 to 10 ppm nitrogen dioxide had an increased mortality rate, compared to virus-exposed animals that did not receive nitrogen dioxide (Henry et al. 1970; Fenters et al. 1971; Ehrlich and Fenters 1973). Because of the reliance on mortality outcomes, however, caution is necessary in extrapolating the results to risks in humans.

Perhaps a more appropriate in vivo approach involves an assessment of decreased capacity to kill bacteria in the lungs of animals exposed to nitrogen dioxide. A review by Jakab (1980) concluded that, generally, these studies show impaired killing of bacteria by lung phagocytes and increased infectivity of bacterial pathogens in nitrogen-dioxide-exposed animals. Goldstein and colleagues (1973) challenged mice with aerosolized *Staphylococcus aureus*, followed by acute exposure to various concentrations of nitrogen dioxide. The number of viable bacteria in the lungs of the treated animals was compared to that of the air-exposed animals. Bactericidal capacity was reduced in groups exposed to 3.8 ppm or more nitrogen dioxide. In general, all the in vivo studies have used

high levels of nitrogen dioxide (more than 3.8 ppm) in order to produce an effect on the measured outcome (Pennington 1988); people are rarely exposed to such high levels of nitrogen dioxide.

Other studies have focused on the role of specific components of host defense mechanisms in vitro. Macrophages, recovered by bronchoalveolar lavage or tracheal epithelium explants, were functionally evaluated after either previous nitrogen dioxide exposure in the whole animal or direct nitrogen dioxide exposure of the cells in vitro. Following in vitro exposure for 30 minutes, macrophages showed reduced bactericidal capacity and suppressed biochemical secretions at nitrogen dioxide levels as low as 0.1 ppm (Voison et al. 1977). Amoroso and coworkers (1981) found dose-dependent decreases in superoxide anion radical production in alveolar macrophages isolated from rats exposed to 6.1 to 17.0 ppm nitrogen dioxide for two to three hours. This effect is implicated as playing a role in antibacterial activities. Mochitate and colleagues (1986) noted mildly enhanced activities of certain enzymes (such as glucose-6-phosphate dehydrogenase and glutathione peroxidase of the peroxidative metabolic pathway, and succinate-cytochrome c reductase of the mitochondrial respiratory system), as well as increased numbers of macrophages, when rats were exposed to 4 ppm nitrogen dioxide for 10 days. However, Lefkowitz and coworkers (1984) found no significant changes in interferon levels, a measure of immune system responsiveness, in mice exposed to 5.0 ppm nitrogen dioxide for seven days.

An alternative to the models described above is the use of virulent bacteria and virus strains in intact animals, focusing on infectious outcome as the endpoint. Animals with normal pulmonary defenses are capable of defending the lung against minor infectious incidents. If any of the animal's several pulmonary defenses is significantly altered, prolonged microbial viability and enhanced establishment of infection would be expected. With this approach, subtle alterations in the immune system of the host can be detected at exposure concentrations that produce no (or minimal) other overt toxicological effects (Gardner 1982). Moreover, by using microorganisms that produce disease through different pathways, animal models can be used to evaluate the role of specific components of the defense system.

Dr. Jakab reports here on the effects of nitrogen dioxide on mechanisms of defense against respiratory infection, using gram-negative and gram-positive bacteria and an endogenous virus to examine how susceptibility to infection and host responsiveness to infection may be modified. Gram-negative and gram-positive bacteria produce different types of toxins eliciting specific host defense mechanisms. Also, antibacterial defense mechanisms differ from viral defenses. In general, gram-positive bacteria produce toxins that are released by

by lysis of the bacterial cells by macrophages, whereas gram-negative pathogenic bacteria produce fever and an inflammatory reaction (cellular injury and increased capillary permeability) by inducing the release of endogenous agents (pyrogens) from neutrophils (Robbins and Coltran 1979).

Dr. Jakab used the gram-positive bacteria *S. aureus* to probe the effect of nitrogen dioxide on the functional integrity of alveolar macrophages, and the gram-negative bacteria *Proteus mirabilis* to elucidate the contribution of recruited polymorphonuclear leukocytes to the defense system. *K. pneumoniae*, a gram-negative organism that is a respiratory pathogen, was selected to determine whether nitrogen dioxide acted as a coinflammatory agent with the pathogenic bacteria, and *Pasteurella pneumotropica* was used to probe the effect of nitrogen dioxide exposure on the defense mechanisms of the lung against an organism that is endogenous to the respiratory tract of the experimental animal model. The murine respiratory virus parainfluenza 1 (Sendai) was used to determine whether or not nitrogen dioxide exposure increased the severity of a viral respiratory infection that is endogenous to the host.

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## GOALS AND OBJECTIVES

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The broad objective of this investigation was to examine the influence of exposure to nitrogen dioxide on functional parameters that might influence both susceptibility to infection and host responsiveness to infection. The lung's defense mechanisms against bacterial and viral infections were assessed with respect to their modification by exposure to a range of concentrations of nitrogen dioxide. Dose-response studies were performed to determine the concentration of nitrogen dioxide exposure that would result in increased susceptibility to, or increased severity of, viral and bacterial infections in mice, using physiological parameters of host resistance to respiratory infections as endpoints.

The specific objective was to seek answers to the following questions: (1) what is the threshold concentration for a single four-hour exposure to nitrogen dioxide that would alter the defense mechanisms against bacterial infections; (2) what is the influence of the order of exposure to nitrogen dioxide and bacterial challenge, that is, exposure to nitrogen dioxide for four hours before or after the bacterial challenge; (3) what is the effect of nitrogen dioxide exposure on resistance to long-term bacterial infection; (4) does nitrogen dioxide exposure alter resistance to viral infections; and (5) is there an interaction of nitrogen dioxide exposure with the altered resistance to bacterial infections in animals predisposed by infection with Sendai virus, or by immunosuppression with corticosteroid treatment?

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## SUMMARY OF INVESTIGATOR'S CONCLUSIONS

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The principal findings of this study are:

1. The intrapulmonary killing of *S. aureus* was impaired in mice exposed to a concentration of 5 ppm nitrogen dioxide. Challenge by other bacterial species required higher levels of nitrogen dioxide to perturb pulmonary defenses.
2. The adverse effect of nitrogen dioxide on lung antibacterial activity occurred at lower concentrations when the exposure to the irritant gas followed bacterial challenge, as compared to when it preceded the challenge.
3. No effect was observed in mice exposed to 10 or 20 ppm nitrogen dioxide and challenged with *Listeria monocytogenes*, an organism that produces a more chronic infectious process.
4. Exposure to nitrogen dioxide for four hours per day, in conjunction with *S. aureus* challenge, during the course of Sendai virus infection did not alter the bacterial number in the lungs of mice but did enhance the lung pathology associated with the infection.
5. Impairment of intrapulmonary killing of *S. aureus* occurred at a lower concentration, 2.5 ppm, when mice that had been immunosuppressed with corticosteroid were exposed to nitrogen dioxide. Bacterial infection in mice predisposed with Sendai virus was not altered with nitrogen dioxide exposure.

Overall, the data suggest that the mechanisms of resistance to pulmonary infection by gram-positive and gram-negative bacteria are affected differently by nitrogen dioxide exposure.

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## TECHNICAL EVALUATION

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### ASSESSMENT OF METHODS AND STUDY DESIGN

The methods selected for use in this study are standard techniques that are well established. The investigator is very familiar with them and used the techniques appropriately. The concentrations of nitrogen dioxide studied in this investigation ranged from 1 to 30 ppm; however, not all experiments included this full range (see Table 1). The endpoint in experiments involving bacterial challenge was the number of viable bacteria remaining four hours after the bacterial challenge, which either immediately preceded or immediately followed a four-hour exposure to nitrogen dioxide. Two exceptions to this regimen were the *L. monocytogenes* experiment, which used an exposure to nitrogen dioxide of four hours per day for 14 days, and the Sendai experiment, which used an exposure of four hours per day for 10 days. In the viral infection

**Table 1.** Nitrogen Dioxide Exposures for Each Experimental Condition

Challenge Organism	NO <sub>2</sub> Exposure (ppm)									
	1	2.5	4	5	10	15	17.5	20	25	30
<i>S. aureus</i>		X	X	X	X	X				
<i>S. aureus</i> + corticosteroid	X	X		X	X					
<i>S. aureus</i> + Sendai virus				X	X					
<sup>32</sup> P- <i>S. aureus</i>					X	X		X		
<i>P. mirabilis</i>				X		X	X	X	X	
<i>K. pneumoniae</i>					X					
<i>P. pneumotropica</i>				X	X			X	X	X
<i>L. monocytogenes</i>					X			X		
Parainfluenza 1 virus (Sendai)				X	X			X		

studies, the virus titer in lung homogenates was used as an endpoint. Further attributes of infection were noted by differential cell counts, lung wet:dry weight ratios, and albumin in lung lavages. Particle clearance mechanisms were studied by following the disposal of <sup>32</sup>P-labeled bacteria.

The experimental design is well described in the Methods section, and is summarized in tabular form at the end of that section to serve as a guide to the reader. However, it is not clear how animals were allocated to exposure treatment, nor is a formal calculation of the statistical power to detect expected outcomes presented. Appropriate statistical tests were used for data analysis, although a statement of rationale for the choice of Duncan's statistical test would have been useful. The report contains frequent reference to dose-response relationships, yet lacks any formal analyses for trends. Such analyses, using linear or nonlinear regression models, would have provided more precise information on dose-response relationships, and would have strengthened considerably the inferences made from the extensive data that were obtained.

## INTERPRETATION OF RESULTS

The lowest level of nitrogen dioxide at which statistically significant levels of intrapulmonary killing of *S. aureus* were noted is 5 ppm nitrogen dioxide exposure for four hours, and 2.5 ppm nitrogen dioxide with concurrent corticosteroid treatment; Dr. Jakab contends that these represent the "threshold" levels of nitrogen dioxide. However, the determination of a threshold is heavily dependent on study design and analysis. The focus of the current study was to gain an understanding of the mechanisms of host defense; it was not designed specifically to maximize information pertinent to

the threshold and dose-response questions. In addition, the reported "threshold" values were determined qualitatively from the graphs presented in the Investigator's Report. The method of choice for a reliable determination of threshold values is to subject the data to statistical analysis using one of the several models. Therefore, the "threshold" values reported by Dr. Jakab should be interpreted with caution. Furthermore, one must clearly appreciate that threshold values determined in animal studies do not predict the threshold for humans. Again, this study was not designed specifically to elucidate interspecies differences, and the study should be evaluated with this in mind.

The sequence-of-exposure studies in the *S. aureus* system provide mechanistic data on the exposure-outcome relationship: The nitrogen-dioxide-induced impairment in antibacterial activity occurred at lower concentrations when exposure followed bacterial challenge than when it preceded the challenge. These results, along with those of Goldstein and coworkers (1973), who found effects at  $2.3 \pm 0.2$  ppm nitrogen dioxide after 17 hours, suggest that in the sequence-dose relationship, the order of exposure may override the effect of concentration. These are valuable mechanistic implications that have bearing on the design of future infectivity studies.

When the lung was challenged with Sendai virus, the investigator found increased virus-associated lung damage after exposure to nitrogen dioxide (as indicated by increased lung wet:dry weight ratios, albumin content, and influx of inflammatory polymorphonuclear leukocytes); he interprets this observation as suggesting long-term consequences for the lung when exposure and infection coexist. Thus, the author hypothesizes that childhood infections, some of which may be related to nitrogen dioxide exposures, might enhance the risk of lung disease in adults. Such conjectures, although apparently based on other recent observations in the investigator's laboratory, are not really addressed in the investigator's report. There is some evidence that supports the view that episodes of infection and inflammation in early life might indeed influence pulmonary function in later life (Lebowitz et al. 1987; Voter et al. 1988); however, these studies are preliminary, and require confirmation based on larger study samples and longer periods of observations.

In addition to the experimental features discussed above, the outcome of infection depends upon physiological factors, such as clearance mechanisms, cellular defenses triggered in the host (for example, recruitment of macrophages), and host immunity. Dr. Jakab has evaluated the contribution of some of these factors in respiratory infections in mice. In *S. aureus* infections, physical removal rates were found not to be impaired at any nitrogen dioxide concentration tested, which is in agreement with previous work by Goldstein and

colleagues (1973), who tested nitrogen dioxide levels of 2 to 15 ppm. Dr. Jakab concludes that the effects observed at 5 ppm support a bactericidal defect in the phagocytic system, rather than an effect on the transport system.

The author found a biphasic dose-response relationship for bactericidal activity against *P. mirabilis*: At 10, 15, and 17.5 ppm nitrogen dioxide bactericidal activity was enhanced, whereas at 20 and 25 ppm nitrogen dioxide it was suppressed (no effect was noted at 5 ppm). The investigator has explained these results by suggesting that at the intermediate concentrations of nitrogen dioxide, the lungs recruit increased numbers of polymorphonuclear leukocytes which then enhance bactericidal activity. Exposure to 20 ppm or higher concentrations of nitrogen dioxide overcomes this defense mechanism and results in an impairment of bactericidal activity of the recruited cells, allegedly by impairment of intrinsic phagocytic processes; however, upon repeating the experiment with *K. pneumoniae*, another gram-negative bacteria, 10 ppm of nitrogen dioxide failed to enhance bactericidal activity. The author explained this result by stating that this organism does not recruit sufficient numbers of polymorphonuclear leukocytes to make enhancement of recruitment at 10 ppm important. These interpretations are supported by limited experiments only, and the conclusions regarding mechanisms must be recognized as somewhat speculative. Nevertheless, the experiments suggest that, in mice, an acute exposure to nitrogen dioxide for four hours after bacterial challenge causes dysfunctions in pulmonary antibacterial defenses that may differ qualitatively at different nitrogen dioxide exposure concentrations, and that may be dependent upon the particular organism used to probe specific defense parameters.

The investigator emphasizes the particular importance of the experiment in animals in which immunosuppression was induced by corticosteroid treatment, and suggests that there may be high-risk individuals in the population who are particularly susceptible to nitrogen dioxide enhancement of susceptibility to pulmonary infections. However, the data from these experiments are very limited, and are still far from confirming that nitrogen dioxide exposure is a risk factor for human populations.

The levels tested in this study (see Table 1) are relatively high as compared to typical human exposures (see Background section of this report). However, we do not know if the actual lung dose in mice and humans is the same even with identical exposures to nitrogen dioxide. The known differences between the structure of the nasal cavity in humans and rodents (Patra 1986) suggest that a higher proportion of nitrogen dioxide may be absorbed in the upper airways of mice resulting in a lower proportionate dose to the lung tissue. Therefore, it is possible that the actual dose to the lower respiratory tract of mice, particularly at the lower levels administered by Dr. Jakab, may not be so far away from those in humans.

In the discussion section of the report, Dr. Jakab makes the following interesting speculation, which may prove to be an important guideline for future research in this area: "From the data with all the bacteria employed in these studies, an interesting trend appears to be emerging, that the more complex the functional resistance mechanism, the higher the dose of nitrogen dioxide required to impair defenses against that bacteria." Thus, while 5 ppm nitrogen dioxide affects the alveolar macrophage phagocytic system (*S. aureus* studies), the combined phagocytic system involving the alveolar macrophages and polymorphonuclear leukocytes is only affected adversely at nitrogen dioxide concentrations of 10 ppm or more (*P. mirabilis*, *K. pneumoniae*, and *P. pneumotropica* studies). The results with *L. monocytogenes* involves cell-mediated immunity as well as the dual phagocytic system, and adverse effects of nitrogen dioxide on functional resistance against this organism requires exposures in excess of 20 ppm nitrogen dioxide.

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#### REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

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The investigator used animal and infection models to better understand pulmonary defense mechanisms, in order to allow more knowledgeable judgments concerning human dose-response and organism-specific response relationships.

The results from this study point to additional questions that need to be pursued and, in particular, to studies that would further clarify the temporal relations of exposure and response. Since most of Dr. Jakab's research was conducted with a single four-hour exposure to nitrogen dioxide, it leaves open the question of whether or not more prolonged exposures would result in respiratory effects at lower nitrogen dioxide exposures than the 5 ppm reported here. Further research to determine the comparability of alveolar concentrations of nitrogen dioxide achieved at varying exposure concentrations in mice and humans would facilitate analysis of the human health significance of these murine studies.

The role of corticosteroid in increasing susceptibility to bacterial infection, by producing effects at lower nitrogen dioxide concentrations, is presumed to be related to suppression of immune system function. Further studies of the role of the immune system in modulating interactions between nitrogen dioxide and bacteria would appear to be a valuable direction for future research. The present investigation clearly suggests that there are broad differences in the mechanisms through which nitrogen dioxide can alter lung defenses against infection or the pathological consequences of infection, or both, at high levels of nitrogen dioxide. It remains for future work to explore whether or not there are conditions under which this holds at lower concentrations of nitrogen dioxide. Acquisition of further insight into chemotactic or intracellular bactericidal mechanisms that explain these broader influ-

ences would be a valuable goal for research. This kind of information is needed to explain why different exposures to different concentrations of nitrogen dioxide can result in varied, qualitative responses to infectious agents (as was observed with *P. mirabilis*). It is important that these molecular or cellular studies also be conducted with a dose-response protocol, and that they be corroborated with the kind of physiologic mechanism experiments conducted in the present investigation.

The studies presented here, within the limits of the design used, determined nitrogen dioxide levels that produce enhanced infectious outcome in a laboratory animal model. Extrapolation of these data to human exposures can best be aided by developing mechanistic criteria in the laboratory animal models that can be applied in human clinical or epidemiologic studies. This investigation has made progress toward this end.

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**Special Reports**

<b>Title</b>	<b>Publication Date</b>
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	Supplement January 1988

**Research Reports**

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

# LIST OF HEI PUBLICATIONS



## Research Reports

<b>Report No.</b>	<b>Title</b>	<b>Principal Investigator</b>	<b>Publication Date</b>
18	Respiratory Infections in Coal Miners Exposed to Nitrogen Oxides	M. Jacobsen	July 1988
19	Factors Affecting Possible Carcinogenicity of Inhaled Nitropyrene Aerosols	R.K. Wolff	July 1988

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