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Murine Respiratory Mycoplasmosis: A Model to Study Effects of Oxidants

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Research Report Number 47

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Murine Respiratory Mycoplasmosis: A Model to Study Effects of Oxidants

Jerry K. Davis[1], Maureen Davidson, and Trenton R. Schoeb

ABSTRACT

Previous studies have shown that exposure to nitrogen dioxide at concentrations of 5 and 10 parts per million[2] (ppm) decreases intrapulmonary killing of *Mycoplasma pulmonis*, and that this decrease is related to increased lung lesions and mortality. The specific objectives of the present study were to titrate the effects of nitrogen dioxide on pulmonary clearance of *M. pulmonis*, determine the mechanisms by which this organism is killed within the lungs, and determine the target that the nitrogen dioxide affects.

Pathogen-free C57BL/6N mice were exposed to 0, 0.5, 1, 2, or 5 ppm of nitrogen dioxide (contamination with other oxides of nitrogen compounds was 5% or less) for four hours and then immediately were exposed to aerosols of viable, radiolabeled *M. pulmonis* strain UAB CT. One-half of the animals in each group were killed immediately after exposure to the infectious aerosols, and the rest were killed 24 hours later. The amount of radioactivity and the number of viable *M. pulmonis* were determined for each group. Exposure to less than 5 ppm of nitrogen dioxide had no effect on intrapulmonary killing of *M. pulmonis*, although exposure to 1 ppm of nitrogen dioxide did increase mechanical removal.

We were unable to develop a completely in vitro mycoplasma killing method. However, we were able to demonstrate the in vitro killing of *M. pulmonis* that had been allowed to associate with alveolar macrophages in vivo. Thus, mouse lungs contain unidentified factors that allow cells to kill *M. pulmonis*. Furthermore, we obtained evidence that suggests that prior exposure to nitrogen dioxide abrogates killing in these experiments.

We also have shown that exposure to nitrogen dioxide does not increase the protein content of bronchoalveolar lavage fluid. Using immunofluorescence, more than 95% of the cells recovered by lavage were macrophages; with double-label immunofluorescence, more than 98% of the cell-associated mycoplasmas were on or in alveolar macrophages. In assessing the cytological parameters of lung lavage cells from mice exposed to nitrogen dioxide, *M. pulmonis*, or both, we found that both insults affected the viability of recovered macrophages. Viability immediately after exposure as measured by trypan blue exclusion or by fluorescein diacetate uptake, was 89% ± 4% and 88% ± 4% in the control group, respectively; 56% ± 19% and 64% ± 11% in the group receiving *M. pulmonis* alone; 23% ± 7% and 48% ± 9% in the group receiving nitrogen dioxide alone; and 16% ± 6% and 25% ± 6% in the group receiving both *M. pulmonis* and 10 ppm nitrogen dioxide exposures. Significant effects on macrophage viability were seen after exposure to 5 and 10 ppm nitrogen dioxide, but not after exposure to 2 ppm nitrogen dioxide. Furthermore, macrophage viability in the groups exposed to 10 ppm nitrogen dioxide did not completely return to normal until seven days after exposure, at which time intrapulmonary killing also returned to normal. It is possible that macrophages are not actually killed in vivo, but rather damaged, and thus are less able to withstand the stress of pulmonary lavage. However, the effect of nitrogen dioxide exposure in relation to intrapulmonary killing of *M. pulmonis* appears to be decreased alveolar macrophage function.

INTRODUCTION

Considerable epidemiologic evidence links exposure to nitrogen dioxide (NO_2) with increased susceptibility to, or severity of, infectious respiratory disease in humans (Florey et al. 1979; Speizer et al. 1980; Ekwo et al. 1983; Pengelly et al. 1984; Honicky et al. 1985; Lindvall 1985; Samet 1989). However, the epidemiologic evidence concerning the relation between NO_2 exposure and increased susceptibility to infection is inconclusive because most studies have used parameters of "acute respiratory illness" that may not be related to infectious episodes. Also, data from these studies are inconsistent because of differences in experimental designs, measurement methods, sample sizes and compositions, exact mixtures of pollutants in the environments, and adjustments made for the socioeconomic status of subjects (Lindvall 1985).

In animal studies, NO_2 exposure affects mortality due to infectious agents (Ehrlich 1966, 1980; Henry et al. 1969; Brownstein et al. 1977; Gardner 1980; Jakab 1981; McGrath

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

and Oyervides 1982), pulmonary clearance of infectious agents (Ehrlich 1966; Goldstein et al. 1973; Jakab 1987a,b), alveolar macrophage numbers (Sherwin et al. 1968; Mochitate et al. 1986), phagocytic activity (Sone et al. 1983; Suzuki et al. 1986), enzyme activities (Vassallo et al. 1973; Wright et al. 1982; Mochitate et al. 1986), superoxide anion production (Amoruso et al. 1981; Suzuki et al. 1986), bacterial killing (Vassallo et al. 1973; Amoruso et al. 1981), and tumoricidal activity (Sone et al. 1983). Various investigators also have evaluated the effects of NO_2 on surfactant (Haagsman and van Golde 1985; Seeger et al. 1985), interferon concentrations (Lefkowitz et al. 1984), immunologic responses (Hidekazu and Shimizu 1980; Schnizlein et al. 1980; Fujimaki et al. 1981; Hillam et al. 1983; Azoulay-Dupois et al. 1985; Lefkowitz et al. 1986), prostaglandin metabolism (Chaudhari et al. 1979), physiologic and metabolic responses (Fenters et al. 1973; Mustafa and Tierney 1978; DeNicola et al. 1981; Wright and Mavis 1981), and lung structure (Hine 1970; Dowell et al. 1971; Stephens et al. 1972; Guidotti 1980; Crapo et al. 1984).

Because of differences in the microorganisms, animal species, and experimental designs used by various investigators, there is little direct experimental evidence to link the well-characterized biologic effects of NO_2 with increased susceptibility of the host to a naturally occurring respiratory disease. Only a few investigators have studied the effects of NO_2 exposure on respiratory disease induced by a natural respiratory pathogen in the natural host (Brownstein et al. 1977; Jakab 1988). For many infectious agents, disease and lesion production are the result of a long evolutionary relationship, with concomitant subtle interactions between the host and microorganism. Thus, the host-parasite interactions may be abnormal in species that are not the normal host for the microbial agent. Furthermore, assumptions are usually made that the changes in mortality due to the infectious agent are due to changes in pulmonary clearance, and that these, in turn, result from changes in alveolar macrophage function. Again, there is little experimental evidence to link these effects. There actually are reasons to doubt these assumptions, particularly the link between pulmonary clearance and alveolar macrophages. Bacterial killing in the lungs is not solely dependent upon alveolar macrophage function even with *Staphylococcus aureus* (Juers et al. 1976; LaForce et al. 1978; Toews et al. 1979; Nugent and Pesanti 1982; Onofrio et al. 1983; Oishi et al. 1986) or *Klebsiella pneumoniae* (Toews et al. 1979), two organisms commonly used to assess the effects of NO_2 and other pollutants on infectious respiratory disease. Other factors important for pulmonary microbicidal activity against bacteria and fungi include alveolar lining material (Juers et al. 1976; LaForce et al. 1978), the presence of neutrophils in the lungs (Lipscomb et al. 1983; Onofrio et al. 1983), opsonic activities of fibronectin, serum components, and complement (Heidbrink et al. 1982; Chudwin et al. 1985; Toews et al. 1985; Oishi et al. 1986), natural killer cells (Hidore and Murphy 1986; Williams et al. 1987), and extracellular substances (Coonrod and Yoneda 1983; Coonrod et al. 1984; Nugent and Fick 1987). Thus, there are several potential targets for NO_2 effects that have not been examined experimentally.

In many previous studies of the effects of NO_2 on the respiratory tract, the health status of the animals was unknown. Some previous studies of the effects of oxidants on susceptibility to respiratory disease and the mechanisms involved may have been confounded by the presence of *M. pulmonis* or other infectious agents. Over 60% of the "barrier-maintained" rodent colonies in the United States are infected with *Mycoplasma* sp. or other pathogens (Cassell et al. 1981; Lindsey 1986). Both mycoplasmas and viruses can alter host responses necessary for defense against infectious disease and neoplasia (Barthold 1986; Brownstein 1986; Cassell et al. 1986; Hickman 1986; Lindsey et al. 1986; Bennett et al. 1987). Thus, the effects of NO_2 on infectious disease and immunologic responses need to be reexamined in animals known to be free of all pathogens that may affect the results.

Although naturally occurring murine respiratory mycoplasmosis is potentially devastating to respiratory research with mice or rats, when it is experimentally induced it provides excellent animal models to study respiratory disease induced by an infectious agent in its natural host (Cassell 1982). *Mycoplasma pulmonis* alone can produce all the lesions of the naturally occurring respiratory disease (Cassell et al. 1973; Lindsey and Cassell 1973; Davis and Cassell 1982; Davis et al. 1982), but many intrinsic and extrinsic factors can influence markedly the disease expression and lesion severity. These factors include age, genetics, nutrition, and environment (Broderson et al. 1976; Brownstein et al. 1977; Cassell 1982; Schoeb et al. 1982; Davis et al. 1985b; Davidson et al. 1988a,b). Extensive data on the interactions of mouse phagocytes and *M. pulmonis* are available (Jones and Hirsch 1971; Jones et al. 1972; Jones 1975; Howard and Taylor 1979, 1984; Davis et al. 1980a; Taylor and Howard 1980b, 1981). When the peritoneal cavity of mice is used as a test site, exudates rich in macrophages enhance the clearance of *M. pulmonis*, but no enhancement occurs with neutrophil-rich exudates (Howard and Taylor 1984). In vitro, *M. pulmonis* can be killed by both macrophages and neutrophils, but killing requires the presence of a specific antibody (Jones and Hirsch 1971; Jones et al. 1972; Jones 1975; Taylor and Howard 1980a,b). Without the presence of an antibody, the organisms attach to phagocytes in vitro, but no killing occurs (Jones and Hirsch 1971; Jones 1975). The

mechanism of killing is generally thought to be intracellular. However, it has been suggested that extracellular macrophage killing of *M. pulmonis* can occur and may be mediated through the complement cleavage product, C3a (Taylor-Robinson et al. 1978). *Mycoplasma pulmonis* infection also increases natural killer cell activity in murine lungs (W.C. Lai, University of Texas Health Science Center, Dallas, Texas; personal communication). Thus, natural killer cells also may be involved in pulmonary clearance of this organism. Other attractive features of this model are the development of lesions in both the upper and lower respiratory tract, the absence of systemic disease in immunocompetent animals, the reproducibility and mutability of the experimental infection, and the ability to quantify objectively various parameters of disease (Lindsey and Cassell 1973; Cassell et al. 1974; Cassell and Davis 1978; Davis et al. 1980b, 1982, 1985a,b, 1986; Davis and Cassell 1982).

Previously, we identified two mouse strains that differ in their susceptibility to murine respiratory mycoplasmosis, C57BL/6N and C3H/HeN, the former strain being the more resistant of the two (Davis et al. 1985a; Parker et al. 1987). We also established an aerosol procedure for the exposure of mice to *M. pulmonis* (Davis et al. 1986) and showed that a single four-hour exposure to NO_2 enhanced murine respiratory mycoplasmosis in both strains of mice (Parker et al. 1989). Both 10 and 15 ppm of NO_2 significantly increased the mortality of C57BL/6N mice. Exposure of both strains to NO_2 at 10 ppm significantly decreased the gross pneumonia dose 50% (PD_{50}); NO_2 exposure at 5 ppm decreased the PD_{50} only in the C3H/HeN strain. Nitrogen dioxide exposure did not alter significantly the incidence or severity of nasal passage lesions in either strain, but did increase the incidence of lung lesions in C57BL/6N mice and the severity of lung lesions in both strains. In addition, exposure of C57BL/6N mice to 10 ppm NO_2 increased the number of colony forming units (CFUs) of *M. pulmonis* that could be recovered from the lung.

On the basis of these results, pulmonary clearance was assessed with C57BL/6N mice that were exposed to 0, 5, or 10 ppm of NO_2 for four hours before being exposed to aerosols of radiolabeled *M. pulmonis* (Parker et al. 1989). The resistant mouse strain was chosen to simplify evaluation of the NO_2 effect. There was little difference between the number of disintegrations per minute (dpm) in the lungs of mice exposed to NO_2 and those of control mice. However, there were more organisms and a higher CFU:dpm ratio in the lungs of mice exposed to NO_2 than in the lungs of the control animals. This effect was demonstrated with both 5 and 10 ppm NO_2. These results clearly show that exposure to NO_2 decreases mycoplasmacidal mechanisms operative in the lungs of C57BL/6N mice. Also, changes in mycoplasmacidal activity occurred before changes in inflammatory cell numbers, suggesting that the differences between mice exposed to NO_2 and control mice were in preexisting mycoplasmacidal mechanisms. There were no differences in antibody titers between control mice and those exposed to NO_2. Significant killing of *M. pulmonis* within eight hours of infection and before the development of adaptive immunity contrasted strongly with all of the in vitro evidence that suggests a specific antibody is necessary for macrophages to kill *M. pulmonis*. Thus, either macrophages are not the major nonspecific defense mechanism or other opsonins can replace specific antibody. Regardless of the mechanisms, this study established a direct experimental link between decreased pulmonary clearance after NO_2 exposure, increased numbers of organisms in the lungs, increased pulmonary lesions, and increased mortality (Parker et al. 1989). This provides strong evidence that increased mortality resulting from NO_2 enhancement of *M. pulmonis* infection is, at least in part, due to changes in pulmonary clearance.

AIMS

The overall goal of the present study was to determine the mechanism or mechanisms by which exposure to NO_2 alters intrapulmonary killing of *M. pulmonis*. The specific objectives were: (1) to determine the effects of low doses of NO_2 on pulmonary clearance; (2) to provide a direct experimental link between NO_2 exposure, decreased pulmonary clearance due to defects in intrapulmonary killing, and alveolar macrophage function; (3) to develop an in vitro assay capable of demonstrating killing of *M. pulmonis*; and (4) to determine the target for the NO_2 effect. These objectives remained essentially unchanged throughout the project, but the methods by which they were achieved were modified. To ensure that the dose-response relations were accurate, it was necessary to add an additional objective: (5) to establish improved quality control methods for NO_2 exposure.

METHODS

ANIMALS

The animals used in these experiments were 8- to 10-week-old C57BL/6N mice from breeding colonies maintained at the University of Alabama at Birmingham (UAB); they were free of all known murine pathogens. In experiments to evaluate the virulence of the stock culture of *M.*

pulmonis, 8- to 10-week-old C3H/HeN mice were also used. Breeding colonies of both strains were maintained in shoe box cages inside Trexler-type plastic film isolators (Germ-Free Supply Division, Standard Safety Equipment Co., Palatine, IL). Mice were maintained in isolators before they were used in experiments. After exposure to NO_2 or *M. pulmonis*, mice were housed in sterile microisolator cages (Lab Products, Maywood, NJ). All cages were provided with sterile hardwood-chip bedding (PJ Murphy Forest Products, Rochelle Park, NJ), sterile food (Agway, Inc., Syracuse, NY) and water ad libitum. The breeding colonies were monitored periodically for the presence of mycoplasmas by enzyme-linked immunosorbent assay for IgG and IgM antibodies to *M. pulmonis* and *Mycoplasma arthritidis* (Cassell et al. 1981; Cassell and Brown 1983; Davidson et al. 1983). In addition, the colonies were monitored for other murine pathogens by fecal cultures, necropsy, serologic tests for rodent viruses, and histological examination of all organs from retired breeders. Serologic tests for rodent viruses on sera from retired breeders were conducted by Charles River Laboratories (Wilmington, MA) for pneumonia virus of mice, reovirus-3, Theiler's GDVII, polyoma virus, Sendai virus, minute virus of mice, ectromelia, mouse adenovirus, mouse hepatitis virus, and lymphocytic choriomeningitis virus. No murine pathogens have been detected in these colonies for the past five years. All experiments were conducted according to the National Institutes of Health report, "Guide for the Care and Use of Laboratory Animals" (Committee on Care and Use of Laboratory Animals of the Institute of Animal Resources 1985).

EXPOSURE OF ANIMALS TO NITROGEN DIOXIDE

Mice were exposed to NO_2 in whole body exposure chambers with a chamber volume of 257 L (Parker et al. 1989). For most experiments, groups of 6 to 12 mice were randomly selected and housed together for two to three days before exposure to allow for social adaptation. Inside the exposure chamber, mice were housed in eight cages located at the same level; all mice in the same cage were from the same social group. The maximum number of mice in each individual cage was five, but the number usually ranged from two to four. In some quality control experiments (Appendix A), the chamber was loaded to capacity (80 mice, with 5 housed per cage) with two tiers of cages. The chambers were operated at negative pressure compared with the room, with an air flow rate of 64 L/minute (15 volumes/hour). Nitrogen dioxide concentrations within the chamber and in the ambient air were measured by a Beckman Model 952a $NO/NO_2/NO_x$ Analyzer (U.S. Environmental Protection Agency Designated Identification Number RFNA-0179–034). The NO_x channel of the analyzer was calibrated weekly using known concentrations of nitric oxide (NO), and the weekly calibration curve was checked with span gases containing known concentrations of NO_2. Calibration checks were run daily, both before and after exposures, using 0.0 and 9.0 ppm NO and the span gas (or gases) closest in concentration to the planned exposure concentration. Subsequent to the institution of improved quality control methods, all cylinders were checked for purity. No NO_x contaminants were found in cylinders of ultrapure air or in cylinders of NO used for calibration. All cylinders of NO_2 contained small amounts (3% to 5%) of NO. Nitric oxide contamination in cylinders used to generate the concentrations inside the exposure chamber averaged 4% ± 0.1%. This level of contamination was verified using infrared spectrophotometry by Dr. H. Ken Dillon, Department of Environmental Health Sciences, UAB.

For the sake of consistency with our previous study (Parker et al. 1989), each four-hour exposure time was calculated from the time the chamber reached the target concentration of NO_2. However, time-weight averages of NO_2 concentrations were also calculated. Animals in one chamber were exposed for four hours to various concentrations of NO_2. Control animals in the other chamber were exposed to ambient air free of NO_2. Four hours was chosen as the exposure time to minimize food and water deprivation of the animals. Because of the high metabolic rate of mice, it is recommended that this species not be deprived of food and water for more than three to four hours (Tuffrey 1987); and water should not be provided inside the exposure chamber because this would introduce an extraneous variable in the experiments. In addition, our previous study showed that exposure to NO_2 for four hours is effective in exacerbating mycoplasma disease and decreasing intrapulmonary killing (Parker et al. 1989). The NO_2 concentrations used for measuring NO_2 effects on pulmonary clearance were 0.5, 1, 2, and 5 ppm, with the latter dose used as a positive control (Parker et al. 1989). In most experiments we used 5 ppm of NO_2 because this was the lowest dose that induced a decrease in intrapulmonary killing of *M. pulmonis* in our previous study (Parker et al. 1989), but we used 10 ppm of NO_2 in some mechanistic experiments to magnify the biologic effect. Quality control experiments (Appendix A) showed that there were no significant differences in NO_2 concentrations at different locations within the exposure chamber. A Drager Model 21 ammonia (NH_3) detection system was used to determine whether or not NH_3 was present in the chambers during an animal exposure. No NH_3 was detected during any exposure.

All exposure chambers are located in a room that meets the standards set by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) for hous-

ing laboratory animals. The room has individual environmental controls for temperature, for the light-dark cycle, and for air exchange, and is negative in air pressure compared with the hall. The room temperature is set at 75°F, and the air is changed 15 times per hour, without using recirculated air. The electrical system for the room is on the same backup emergency power system as that used for the animal facility and is tested periodically by the UAB Animal Resources Program. For any given day, temperature variation inside the chamber is minimum, but temperatures may vary from 23° to 26°C (73° to 79°F) on different days, which is within the limits for the room temperature control. The chamber is designed so that the controls that allow gas flow to the chamber shut off if the chamber is not vented correctly or if the chamber is not negative in pressure relative to the room. The chamber also includes instruments to measure air flow and temperature inside the chamber, and the chamber's air pressure in relation to the air pressure in the room. The concentration of NO_2 inside the chamber is measured automatically at seven different points by an adjustable switchbox that samples the air for five minutes at each probe.

MICROORGANISMS

The UAB CT strain of *M. pulmonis* was used in all experiments. UAB CT was isolated from a mouse with murine respiratory mycoplasmosis and identified as a pure culture of *M. pulmonis* by immunofluorescence (Del Guidice et al. 1967). A single stock culture, frozen at −70°C in 1-mL aliquots, was used for all experiments. An abbreviated study of biological endpoints using susceptible C3H/HeN mice confirmed the virulence of the stock (Davis et al. 1986; Davidson et al. 1988b). The culture had a PD_{50} slightly less than 10^4 CFU (four of the six C3H/HeN mice developed gross lesions at this dose) and a 50% lethal dose (LD_{50}) of approximately 10^6 CFU. These endpoints were similar to those of stocks used in our previous study (Parker et al. 1989). In addition, the new stock induced microscopic lesions in 100% of both C3H/HeN and C57BL/6N mice.

For each experiment, freshly grown organisms were prepared by inoculating frozen stock cultures into mycoplasma broth A (Davidson et al. 1988b) and harvesting the cultures in late log phase by centrifugation at 10,000 × *g* for 30 minutes. Radiolabeled *M. pulmonis* was prepared by inoculating 10 mL of stock cultures into 500 mL of mycoplasma broth A containing 3.6 mCi of a mixture of ^{35}S-L-methionine and ^{35}S-L-cysteine (Trans-^{35}S Label®, ICN Biomedicals, Inc., Costa Mesa, CA) and harvesting the organisms as stated above. The dpm:CFU ratio for the organisms used in all experiments ranged from 1:100 to 1:1,000. For aerosolization, *M. pulmonis* cultures were pelleted, washed three times with sterile 0.9% sodium chloride (NaCl), and suspended in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) (Davis et al. 1980a) containing only *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES, Research Organics, Inc., Cleveland, OH). Control aerosols were generated with a sterile RPMI-1640 medium.

A clinical isolate of *Candida albicans* was obtained from the Microbiology Laboratory at the UAB University Hospital. The stock culture, grown in brain-heart infusion broth (Baltimore Biological Laboratories, Rockville, MD), was prepared and frozen at −70°C. Overnight cultures prepared by inoculating frozen stocks into brain-heart infusion broth were washed in phosphate-buffered saline (PBS) and resuspended in PBS for use in experiments.

QUANTIFICATION OF *MYCOPLASMA PULMONIS*

Numbers of *M. pulmonis* were determined as described previously (Davis et al. 1980a; Schoeb et al. 1982). Briefly, serial tenfold dilutions of a sample (*M. pulmonis* culture, lung homogenate, or cell culture homogenate) were plated on mycoplasma agar plates and incubated at 37°C for seven days. Then the number of colonies was counted.

QUANTIFICATION OF RADIOACTIVITY

To determine radioactivity, samples (*M. pulmonis* cultures, lung homogenates, or cell culture homogenates) were solubilized (Protosol, New England Nuclear, Boston, MA), bleached with 30% hydrogen peroxide (H_2O_2) if needed (for lung homogenates), and counted with Aquasol (New England Nuclear) in a Wallac 1410 liquid scintillation counter (Pharmacia LKB Nuclear, Inc., Gaithersburg, MD).

AEROSOL INFECTIONS WITH *MYCOPLASMA PULMONIS*

Mice were exposed to aerosols containing viable *M. pulmonis* in a nose-only exposure chamber as described previously (Davis et al. 1986). The maximum amount of time between the end of NO_2 exposures and exposure to *M. pulmonis* aerosols was approximately 30 minutes. *Mycoplasma pulmonis* aerosols were generated with a model 45 nebulizer (DeVilbiss Health Care Division, Somerset, PA). The nose-only chamber has 72 ports, 36 on each side, to which animal tubes are attached via brass fittings with O-rings. Typically, half of the animals to be exposed to *M. pulmonis* aerosols were previously exposed to NO_2, and half were controls. Previous experiments have shown no significant variation in the aerosol dose delivered to the different exposure ports in this chamber (Davis et al. 1986).

The chamber was operated with a nebulizer air flow of 5.3 L/minute at 5.0 psi, with the nebulizer containing 5 mL of a *M. pulmonis* suspension (10^8 to 10^9 CFU/mL), which was replaced every five minutes throughout the exposure. The diluting air flow was 20 L/minute. During each exposure, a seven-stage Mercer-style impactor (Intox Products, Albuquerque, NM) (Mercer 1973) was placed at the level of the animals in the exposure chamber, and was used to collect a 15-minute aerosol sample (1 L/minute). The size distribution data were determined by a plot of the percentage (mass) less than that of the effective cutoff particle diameter against the log of the effective cutoff particle diameter. The least-squares regression line from these data was used to obtain the median mass aerodynamic diameter (MMAD) of the particles and the geometric standard deviation (σ_g) of the size distribution.

PULMONARY CLEARANCE EXPERIMENTS

Some of the mice were killed immediately after exposure to aerosols of radiolabeled *M. pulmonis* (within 30 minutes of the end of the aerosol run); the remaining mice were killed later, at time points dependent on the experimental design. Mice were killed with an overdose of ketamine (Quad Pharmaceuticals, Indianapolis, IN) and xylazine (Haver-Mobay Corporation, Shawnee, KA), at a ratio of 0.75 mg xylazine to 100 mg ketamine. This anesthetic combination is not known to depress phagocyte function. Lungs were aseptically collected, minced in 1 mL of broth A, and sonicated at a rate known to release, but not kill, cell-bound mycoplasmas (Schoeb et al. 1982; Davis et al. 1985a). A 0.1-mL sample of the sonicated lung was cultured for enumeration of *M. pulmonis*, and the rest of the lungs were prepared for liquid scintillation counting (Parker et al. 1987, 1989; Davidson et al. 1988a).

Pulmonary clearance was calculated by a modification of the original method described by Green and Goldstein (1966). Changes in mycoplasma numbers were defined as change in log CFU in the lung between end of exposure (time zero) and the later time points. This is expressed as: ΔlogCFU = logCFU (at time *t* for each animal) − mean logCFU (mean of animals in the appropriate group at time zero). Physical clearance of radioactive components for each group of mice was defined as change in dpm from zero hour values, which is expressed as: Δdpm = dpm (at time *t* for each animal) − (mean dpm of animals in the appropriate group at time zero). A modification of the radioactive ratio method of Green and Goldstein (1966) was used to calculate the percentage of mycoplasmas killed at later time points relative to the end of exposure. This is expressed as: % change = ([CFU:dpm in lungs of each animal at later times] − [mean CFU:dpm in lungs of the same group at time zero]) ÷ (mean CFU:dpm in lungs of the same group at time zero) × 100 (Davidson et al. 1988a). Thus, an activity value equal to 100 indicated an absence of killing of organisms in the lungs. An activity value less than 100 indicated killing of the organism within the lungs, and an activity value greater than 100 indicated proliferation of organisms. This method of expressing data was chosen to minimize daily variation among exposures, which is unavoidable when freshly grown organisms are used for each experiment.

BRONCHOALVEOLAR LAVAGE PROCEDURE

Mice were anesthetized with ketamine and xylazine. The trachea was exposed, and a sterile 19-gauge intravenous catheter (Deseret Medical, Becton-Dickinson, Inc., Sandy, UT) was inserted approximately 5 mm into the lumen. For initial in vitro experiments, the collection fluid consisted of PBS (pH 7.3) containing 0.2% anhydrous dextrose and 500 U/L of zinc-free heparin. In experiments in which the noncellular fraction of lavage samples was used, the collection fluid was 0.9% NaCl without preservatives or additives. Initial in vivo–in vitro experiments used RPMI-1640 medium that contained 2.5% of 1 M HEPES, 1% L-glutamine (GIBCO), and 2.5% of an 8% solution of bovine serum albumin (Sigma, St. Louis, MO) in PBS with the pH adjusted to 7.3 with 7.5% sodium bicarbonate ($NaHCO_3$) (RPMI-BA). After the initial in vivo–in vitro experiments, other experiments were done to compare the viability of alveolar macrophages after collection in one of the following media: pyrogen-free 0.9% NaCl; 0.9% NaCl containing 1% dextrose (0.9% NaCl-G); PBS; RPMI-1640 containing 1% L-glutamine, 20% fetal bovine serum (FBS) (GIBCO), and 2.5% of 1 M HEPES (GIBCO) with the pH adjusted to 7.3 with 7.5% $NaHCO_3$ (RPMI-S); and RPMI-BA. The viability of *M. pulmonis* in each fluid was also determined. Regardless of the collection fluid, lavage was done using three separate 1-mL washes per mouse. Usually, samples were pooled in groups of six animals.

Lavage samples were separated into cellular and noncellular fractions by centrifugation at 400 × *g* for 10 minutes at 4°C. The cell-free nature of the noncellular fraction was checked by microscopic examination. Concentrated noncellular fractions were prepared using either Amicon filters (cutoff 10,000 Da) or a vacuum centrifugation evaporator (Savant SpeedVac®, Farmingdale, NJ). The fractions were stored at −70°C and reconstituted with sterile water to 1/10 of the original volume.

IN VITRO KILLING OF *MYCOPLASMA PULMONIS* BY ALVEOLAR MACROPHAGE CULTURES

Mouse alveolar macrophage cultures were established either from the cellular fraction of lavage samples or from total lung mononuclear cells as described previously (Davis et al. 1980a). The cellular fraction was collected as described above, and total lung mononuclear cells were prepared by mechanical disaggregation of the lungs (Davis et al. 1980a). Cells were suspended to 10^6/mL in RPMI-BA, and amounts of 0.3 mL of the cell suspension were added to glass vials (15 × 45 mm) and incubated for 15 minutes at 37°C to allow attachment. Nonadherent cells were removed by gentle washing with fresh medium, and cultures were infected by replacing RPMI-BA with 1 mL of RPMI-S medium containing approximately 3×10^6 viable *M. pulmonis*. In experiments in which specific anti-*M. pulmonis* serum (indirect immunofluorescence titer 1,024 or more against *M. pulmonis*) was used, 15% FBS and 5% of the test serum replaced 20% FBS in RPMI-S. Similarly, 15% FBS and 5% of the concentrated noncellular fraction was used to determine whether or not unidentified factors in the lavage samples promoted killing by alveolar macrophages.

After infection, cultures were centrifuged at 800 × *g* at 4°C for 20 minutes, and then incubated for 15 minutes at 37°C to promote attachment of mycoplasmas to the cells. The cultures were washed five times with RPMI-1640 medium, and the medium was replaced with RPMI-S. Vials were capped loosely to allow for gas diffusion after infection. Some vials were processed immediately after washing, and other vials were processed after various periods of continued incubation. The number of viable mycoplasmas in each vial was determined (Davis et al. 1980a). Our previous study has shown that mouse macrophage cultures prepared in this manner can kill *M. pulmonis* in the presence of specific antisera and can inhibit the growth of *Staphylococcus epidermidis* (Davis et al. 1980a).

In in vivo–in vitro experiments, C57BL/6N mice were exposed for 30 minutes to aerosols containing viable *M. pulmonis*. Some of the mice were used to measure intrapulmonary (in vivo) killing. In initial experiments, the remaining mice were used for bronchoalveolar lavage either immediately after aerosol exposure to *M. pulmonis* or four hours later. In later experiments, the four-hour time point was omitted. Pulmonary lavage was performed on each mouse using either cold (5°C) RPMI-BA or 0.9% NaCl (separate experiments), and samples were pooled in groups of 10 to give a minimum of two pools at each time point in each experiment. Cold lavage fluid was used to decrease mycoplasma killing during processing, even though the use of cold fluid represented an additional stress factor for alveolar macrophages (Senior et al. 1981). Each pool was separated into cellular and noncellular fractions by centrifugation. Cultures were established with separated cells alone, supernatants alone, and with recombined cells and supernatants. After they were washed, separated cells were resuspended in RPMI-BA. The only difference between these cultures and the in vitro cultures was that unattached cells and mycoplasmas were not removed in the in vivo–in vitro experiments. Instead, the number of organisms was determined in each culture immediately after the culture was established and four hours later. In initial experiments, the lungs of mice used for lavage also were processed for determining the amount of intrapulmonary killing by the residual material in the lungs.

In all but the most time-dependent in vitro or in vivo–in vitro experiments, the viability of the starting cell population was determined by trypan blue exclusion (Davis et al. 1980b), and functional capability was monitored by the phagocytosis of yeast (Stewart et al. 1975) or latex beads (Werb and Cohn 1972). The number of cells that attached per vial was determined by the lysis of the attached cells and the enumeration of the released nuclei (Rabinovitch et al. 1977). The numbers of attached cells in the in vitro cultures and the in vivo–in vitro cultures established from normal animals were similar ($2.5 \pm 0.5 \times 10^5$ and $3.0 \pm 0.16 \times 10^5$, respectively). For in vivo–in vitro cultures established from animals exposed to NO_2, the concentration of cells in the starting population was adjusted to approximately 10^7 cells/mL so that the number of attached viable cells per vial would be similar ($9.5 \pm 0.2 \times 10^4$), even though the viability of the starting population was less (17% ± 5% viable cells from NO_2-exposed animals vs. 87% ± 6% viable cells from control mice).

QUANTIFICATION OF PROTEIN IN LAVAGE SAMPLES

To determine whether or not infection with *M. pulmonis* or infection combined with NO_2 exposure induced transudation of serum proteins into the lungs, 30 mice were exposed to 10 ppm of NO_2 and aerosols of *M. pulmonis*. Ten additional mice were exposed to ultrapure air. Ten of the exposed mice and all of the control mice were killed immediately after exposure. Ten mice exposed to NO_2 and *M. pulmonis* were killed four hours after exposure, and the remaining mice were killed 24 hours after exposure. Bronchoalveolar lavage was performed on each mouse. Each lavage sample was separated into cellular and noncellular fractions; and the noncellular fractions were frozen at −70°C. All samples were analyzed for protein content by the BioRad Bradford micro-method protein assay (BioRad, Rockville Centre, NY) in a single run, using a standard curve prepared from assaying known amounts of bovine se-

rum albumin in 0.9% NaCl. Previous investigators have shown that increased protein in lavage fluid is a sensitive indicator of serum exudation (Sherwin and Richters 1971; Sherwin and Carlson 1973; Hatch et al. 1986).

CHARACTERIZATION OF CELLS IN LAVAGE SAMPLES

To determine whether or not there were changes in the cell composition of lavage samples within four hours after exposure to *M. pulmonis*, 30 C57BL/6N mice were exposed to aerosols of viable *M. pulmonis*. Fifteen mice exposed to aerosols and 15 control mice were killed immediately after exposure; the remainder were killed four hours after exposure, and bronchoalveolar lavage was done on each mouse. The samples from each group were pooled, and the cellular fraction was isolated and resuspended to 10^6 cells/mL in PBS containing 10% FBS. Samples of the pooled cells from each group were stained with the following reagents: fluorescein isothiocyanate (FITC)–labeled rabbit antimouse lymphocyte serum (Accurate Chemical and Scientific Corp., Westbury NY) for labeling mature and immature lymphocytes; FITC-labeled antimouse thymocyte serum (Accurate Chemical and Scientific Corp.) for labeling T lymphocytes; FITC-labeled antimouse immunoglobulin (Cederlane Laboratories, Limited, Hornby, Ontario, Canada) for labeling B-lymphocytes; rat monoclonal antibody to mouse macrophages (Accurate Chemical and Scientific Corp.), followed by FITC-labeled goat antirat IgG (Sigma Chemical Co.) to stain macrophage populations; or fluorescent 0.41-μm latex beads (Seradyn, Indianapolis, IN) to stain phagocytic macrophages. Each fraction also was treated with rabbit anti–*M. pulmonis* serum followed by rhodamine isothiocyanate (RITC)–labeled swine antirabbit immunoglobulin (Sigma Chemical Co.) for labeling *M. pulmonis*, so that the relation of organisms to each cell population could be determined. In preliminary experiments, splenocytes were stained with each antiserum at various serial dilutions to determine the working dilution of each antiserum, and all of the antisera were checked in double-label experiments against RITC-labeled antimouse immunoglobulin serum to ensure specificity at the working dilution. The method for staining cells in suspension and the examination of stained populations was previously described (Davis 1980b). In brief, cells were examined by alternate-phase and immunofluorescence microscopy, and a total of 200 nucleated cells were counted.

CELL VIABILITY MEASUREMENTS

To determine the effects of NO_2 and *M. pulmonis* on the viability of alveolar macrophages, the cellular fraction of lavage samples was isolated from mice that had been exposed to various concentrations of NO_2, aerosols of viable *M. pulmonis*, or both, and portions of each were allowed to react with either trypan blue (Sigma Chemical Co.) or fluorescein diacetate (Sigma Chemical Co.). Trypan blue exclusion depends on an intact plasma cell membrane and the prevention of pinocytosis (Mishell and Shiigi 1980). In these experiments, cells were incubated at 4°C before being mixed with 0.18% trypan blue solution in PBS and were read within two minutes of mixing (Davis et al. 1980b). In contrast, fluorescein diacetate is taken in by all cells but is only hydrolysed inside live cells to give the characteristic green fluorescence (Mishell and Shiigi 1980).

STATISTICAL ANALYSIS

Data were analyzed using SYSTAT (Systat Inc., Evanston, IL) with an IBM Personal Computer (Wilkinson 1988). Nonparametric data were analyzed by the Kruskal-Wallis test or by the Friedman analysis of variance (ANOVA) by ranks (Conner 1971). Parametric data were analyzed either by Student's *t* test, or by the ANOVA, depending upon the experimental design and the number of groups to be compared (Dowdy and Wearden 1983). The Bonferroni correction (Wilkinson 1988) was used when more than two groups of nonparametric data were compared, or when planned comparisons were made between groups of parametric data. When all possible pairwise comparisons between groups of parametric data had been made, either Tukey's test or the Tukey-Kramer test was used, depending upon whether the groups had equal or unequal numbers, respectively (Wilkinson 1988). Mycoplasma CFU data or pulmonary clearance data were converted to logarithms for analysis.

RESULTS

TITRATION OF NITROGEN DIOXIDE EFFECTS ON PULMONARY CLEARANCE OF *MYCOPLASMA PULMONIS*

Four groups of 12 pathogen-free C57BL/6N mice, 8 to 10 weeks old, were used to measure pulmonary clearance. Each group of 12 was subdivided so that 6 mice were exposed to room air and the other 6 to one of four concentrations of NO_2 (0.5, 1, 2, or 5 ppm) in ultrapure air. All mice were also exposed to aerosols of viable, radiolabeled *M. pulmonis*. We killed six of the mice in each group (three exposed mice and three control mice) immediately after infection, and killed the remaining six mice 24 hours after infection, and calculated pulmonary clearance for each exposure group. The 24-hour period was used because our previous study had shown that the maximum difference

between control animals and animals exposed to NO_2 occurred during this time (Parker et al. 1989). The entire experiment was repeated until data had been collected from a total of 32 mice exposed to 0.5 ppm of NO_2 (18 before we instituted improved quality control procedures for delivering NO_2), 38 had been exposed to 1 ppm NO_2 (18 before improved quality control procedures), 91 exposed to 2 ppm (10 before improved quality control procedures), 34 exposed to 5 ppm NO_2 (8 before improved quality control procedures), and 105 exposed to room air (11 before improved quality control procedures). From 2 to 5 samples per exposure group were not available for a variety of reasons, including: asphyxiation of mice during aerosol infection, bacterial or fungal contamination of mycoplasma cultures, or accidental loss of sample during sample preparation. The number of animals in the room air control group was increased because control mice had to be sham-exposed with each NO_2 exposure group, and the 2 ppm group was repeated eight additional times to verify the lack of any significant difference between this group and controls.

The MMAD for these experiments averaged 1.85 ± 0.22 μm, and the σ_g averaged 2.43 ± 0.42. There was no significant difference in any parameter or in any exposure group between experiments run before or after improved quality control procedures were instituted. Therefore, all experimental results for each exposure were pooled. There were significant differences in ΔlogCFU, Δdpm, and the percentage of change in mycoplasma numbers ($p < 0.001$ for each) between the different exposure groups (Table 1). For both ΔlogCFU and the percentage of change in mycoplasma numbers, only the 5-ppm exposure group was significantly different from the control group. This shows that intrapulmonary killing was decreased in mice exposed to 5 ppm NO_2. The only group with a significant change in Δdpm was the 1 ppm exposure group (Table 1), indicating increased mechanical removal of organisms.

EFFECTS OF ALVEOLAR MACROPHAGES ON *MYCOPLASMA PULMONIS* IN VITRO

Our original in vitro study on interactions between macrophages and mycoplasmas used CD-1 mice (Davis et al. 1980a). Our previous in vivo studies used C57BL/6N mice (Parker et al. 1987, 1989). One possible explanation for the apparent differences in the results from the two studies is genetic differences in macrophage activity between the two mouse strains. Therefore, we repeated the in vitro study using C57BL/6N mice. Alveolar macrophage cultures from C57BL/6N mice were infected with *M. pulmonis* in the presence of normal or immune rabbit sera. Macrophage cultures contained at least 85% viable cells and were at least 90% positive for opsonized yeast phagocytosis. Rabbit serum was used because mouse alveolar macrophages exert the maximal mycoplasmacidal effect in vitro in the presence of immune rabbit serum (Davis et al. 1980a). Additional controls consisted of *M. pulmonis* incubated in the presence of sera without macrophages.

No significant mycoplasmacidal effect occurred with either normal or immune serum in the absence of alveolar macrophages (ΔlogCFU = 0.6 ± 0.02 and 0.45 ± 0.09 at two and four hours of culture, respectively). Alveolar macrophages also could not kill *M. pulmonis* in the absence of immune serum, but they could kill it in the presence of rabbit anti-*M. pulmonis* serum (Figure 1). These results confirm the previous study done in CD-1 mice (Davis et al. 1980a). In addition, the total population of lung mononu-

Table 1. Effects of Exposure to Various Doses of Nitrogen Dioxide on Pulmonary Clearance of *Mycoplasma pulmonis* in C57BL/6N Mice[a]

NO_2 (ppm)[b]	ΔlogCFU	Δdpm	% Change in Mycoplasmas
0 (n = 105)	−2.36 ± 1.19	−154 ± 154	−81 ± 52
0.5 (n = 32)	−2.86 ± 1.49	−352 ± 261	−97 ± 6
1 (n = 38)	−2.95 ± 1.23	−461 ± 344[c]	−96 ± 11
2 (n = 91)	−2.24 ± 0.86	−177 ± 116	−87 ± 42
5 (n = 34)	−0.59 ± 0.38[c]	−122 ± 246	12 ± 1.04[c]

[a] Values are expressed as the mean ± SD for each group. Negative numbers indicate a decrease in the parameter with respect to the end of exposure to *M. pulmonis*; positive numbers indicate an increase in the parameter.

[b] Nitrogen dioxide concentrations based on four hours of continuous exposure. Corresponding time-weight averages of NO_2 concentrations for the 0.5, 1, 2, and 5 ppm exposures are 0.46 ppm/minute, 0.93 ppm/minute, 1.9 ppm/minute, and 4.6 ppm/minute, respectively; n = the total number of mice exposed to each concentration.

[c] Within each column, ANOVA indicated that a significant difference existed between means ($p < 0.001$). The t test with the Bonferroni correction was used to determine which means were different from the control value ($p < 0.05$).

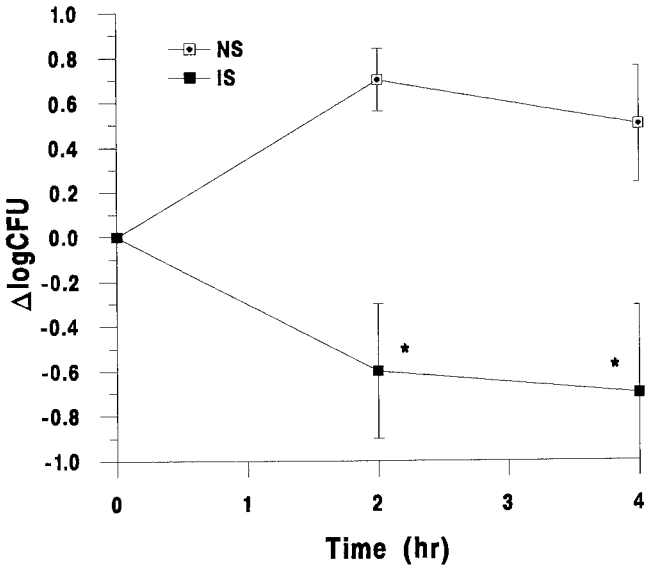

Figure 1. In vitro effect of macrophages from C57BL/6N mice on *M. pulmonis*. Cultures contained either 5% normal (NS) or 5% anti-*M. pulmonis* (IS) rabbit serum in medium. Numbers are the mean ± SD for ΔlogCFU from the time of addition of *M. pulmonis* to 7 or 8 cultures. An asterisk (*) indicates that values for the cultures containing macrophages and IS were significantly different ($p < 0.001$) from cultures containing macrophages and NS.

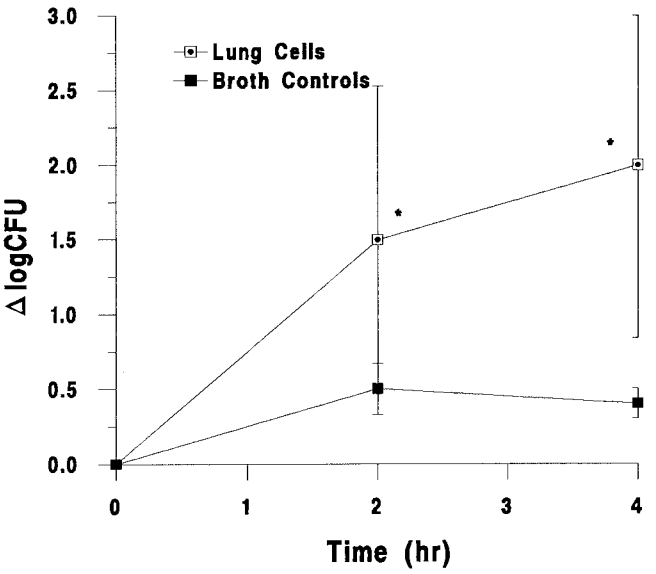

Figure 2. In vitro effect of total lung mononuclear cells from C57BL/6N mice on *M. pulmonis*. Cultures contained either total lung mononuclear cells or were broth controls without cells. Numbers represent mean ± SD for ΔlogCFU from the time of addition of *M. pulmonis* to three cultures per time point without cells and six cultures per time point with cells. An asterisk (*) indicates that values for cultures containing cells were significantly different ($p < 0.001$) from the broth control cultures.

clear cells isolated from C57BL/6N mouse lungs did not kill *M. pulmonis* in vitro (Figure 2). In fact, the presence of these cells allowed for increased *M. pulmonis* growth.

EFFECTS OF THE NONCELLULAR FRACTION OF LAVAGE SAMPLES ON *MYCOPLASMA PULMONIS* IN VITRO

To examine the possibility that extracellular substances are responsible for the killing of *M. pulmonis* in lungs, lung lavage was done on C57BL/6N and C3H/HeN mice, and the noncellular fraction was prepared by centrifugation. Both *M. pulmonis* and *C. albicans* (the positive control) were cultured for two hours in the presence of lung lavage fluids. As reported by others (Nugent and Fick 1987), unconcentrated lung lavage fluid killed *C. albicans* but did not kill *M. pulmonis* (Table 2). There was a trend for concentrated noncellular fractions of lavage fluid to kill *M. pulmonis* (Table 3) regardless of the concentration method (SpeedVac® or Amicon filtration). However, these results were not statistically significant due to the extreme variability between concentrated samples prepared on the same day. Hypertonic saline (9% NaCl) did not kill either *M. pulmonis* or macrophages, thus suggesting that killing was mediated by factors derived from the lung, not by hypertonicity of the concentrated noncellular fractions. When used at 5% concentration (1:20 dilution) in a cell culture medium, concentrated noncellular fractions did not promote the killing of *M. pulmonis* by alveolar macrophages (Table 3).

Table 2. In Vitro Effects of Bronchoalveolar Lavage Fluid on *Candida albicans* and *Mycoplasma pulmonis*[a]

Organism	Medium	Time (hours) 1	Time (hours) 2
Candida albicans	Lavage fluid from C3H mice	−0.8 ± 0.36[b]	−1.0 ± 0.61[b]
	Lavage fluid from C57 mice	−0.4 ± 0.24	−0.7 ± 0.24[b]
Mycoplasma pulmonis	Lavage fluid from C3H mice	−0.3 ± 0.47	−0.1 ± 0.23
	Lavage fluid from C57 mice	0.3 ± 0.29	−0.7 ± 1.38

[a] Results are expressed as the mean ± SD of the ΔlogCFU from the addition of lavage fluid to the cultures (time zero); $n = 5$ for each group.

[b] Significant difference ($p < 0.03$) from time zero.

Table 3. In Vitro Effects of Concentrated Lavage Fluid and Concentrated Lavage Fluid Plus Alveolar Macrophages on *Mycoplasma pulmonis*[a]

Cultures	ΔlogCFU
Concentrated fluid[b]	−1.2 ± 2.0
5% Concentrated lavage fluid with alveolar macrophages	0.1 ± 0.29
9% NaCl (control)	−0.1 ± 0.1

[a] Values are expressed as mean ± SD for ΔlogCFU from the addition of lavage fluid to cultures for each group based on 11 or 12 cultures per group in two or three separate experiments. The data exhibited considerable heteroscedasticity and therefore were analyzed by nonparametric methods. The ΔlogCFU was not significantly different when using the nonparametric Kruskal-Wallis test.

[b] There were no significant differences in the effects of concentrated lavage fluid based on the method of preparation.

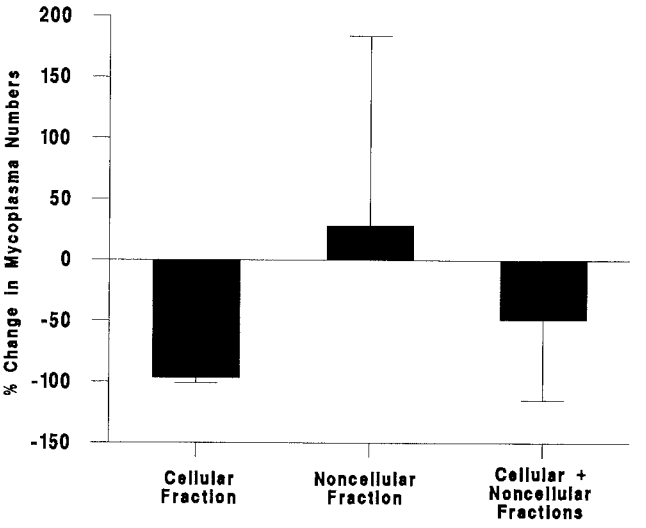

Figure 3. In vivo–in vitro killing of *M. pulmonis* after aerosol inoculation of C57BL/6N mice and collection of bronchoalveolar lavage using RPMI-BA medium. Bars represent the mean ± SD of the percentage of change in mycoplasma CFUs after four hours in culture. Mycoplasma numbers decreased significantly ($p < 0.001$) in cultures containing the cellular fraction of lavage fluid and in cultures containing both the cellular and noncellular fractions of the lavage fluid. n = 6 cultures for each group. Companion ΔlogCFUs were −2.21 ± 1.31, −0.31 ± 0.77, and −0.87 ± 1.0 for cultures containing cellular fraction only, fluid fraction only, and cellular and fluid fractions, respectively.

EFFECTS OF CELLS FROM BRONCHOALVEOLAR LAVAGE SAMPLES ON KILLING OF *MYCOPLASMA PULMONIS* IN IN VIVO–IN VITRO EXPERIMENTS

Initial in vivo–in vitro experiments used cells from bronchoalveolar lavage samples collected in RPMI-BA. Because of the slightly decreased viability of macrophages, experiments were conducted to determine the best lavage fluid and incubation fluid. The best compromise was performing lavage with 0.9% NaCl, and incubating the cultures in RPMI-BA (Table 4). This combination best preserved the viability of both alveolar macrophages and *M. pulmonis*, without allowing extensive growth of the latter in the collection fluid. Furthermore, the maximum recovery of lavaged cells can be achieved with a medium such as saline, which is free of divalent cations (Senior et al. 1981).

Cells from bronchoalveolar lavage were capable of killing *M. pulmonis* when they were allowed to associate with the mycoplasmas in vivo. Killing was assayed in vitro after lavage was performed with RPMI-BA or 0.9% NaCl. When bronchoalveolar lavage was done with RPMI-BA, maximal killing was seen in cultures established immediately after exposure, specifically in cultures containing cells only (Figure 3). Mycoplasmacidal effects in these cultures were approximately equal to those seen in companion in vivo pulmonary clearance experiments (the percentage of change in mycoplasma numbers in vivo was 95% ± 9%, $p < 0.001$).

Table 4. Effects of Various Fluids Used for Bronchoalveolar Lavage on *Mycoplasma pulmonis* and Alveolar Macrophages[a]

Fluid	Viability of Alveolar Macrophages		*M. pulmonis* ΔlogCFU
	At 0 Hours	At 4 Hours	at 4 Hours
RPMI-BA	90 ± 2^{bc}	85 ± 15de	0.3 ± 0.7^{g}
RPMI-S	88 ± 13^{bc}	99 ± 1^{d}	1.7 ± 0.4^{h}
0.9% NaCl	87 ± 6^{bc}	79 ± 12^{ef}	−0.2 ± 0.5^{gi}
0.9% NaCl-G	99 ± 1^{b}	68 ± 9^{f}	−0.8 ± 0.2^{i}
PBS	81 ± 8^{c}	72 ± 8^{ef}	−0.5 ± 0.4^{i}

[a] Values are expressed as the mean ± SD for percentage of macrophage viability or ΔlogCFU.

[b,c,d,e,f,g,h,i] The ANOVA indicated a significant difference between the means in each column ($p \leq 0.004$). Tukey's test was used to determine which means within each column were significantly different. Within each column, means with the same superscript are not significantly different; those with different superscripts are significantly different ($p < 0.05$) from each other.

Less killing was seen in cultures that contained both the noncellular and cellular fractions, and no killing was seen in the noncellular fraction alone. There was a trend (not statistically significant) for mycoplasma killing in cultures established four hours after exposure (the percentages of change in mycoplasma numbers were −83% ± 33%, −15% ± 67%, and −8% ± 101% for cultures containing the cellular fraction, the noncellular fraction, and both fractions, respectively). Not all the mycoplasmacidal effects were removed from the lungs by lavage, as shown by the mycoplasmacidal activity of the residual material in the lung (the percentage of change in mycoplasma numbers was −84% ± 58%, $p < 0.001$). The MMAD for these experiments was 2.91 ± 0.69 μm, and σ_g was 2.82 ± 0.05. In contrast, when 0.9% NaCl was used as the lavage fluid, maximal killing was seen in the cultures that contained both the cellular and noncellular fractions (Figure 4). Mycoplasmacidal activity in in vivo controls for these experiments was −93 ± 11% change in mycoplasma numbers, and the ΔlogCFU was −1.5 ± 0.82. The MMAD for these experiments was 1.9 ± 0.62 μm, and the σ_g was 2.29 ± 0.14.

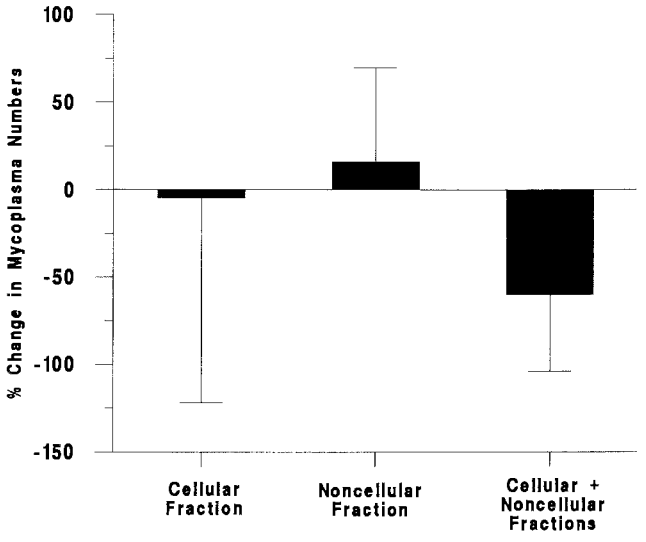

Figure 4. In vivo–in vitro killing of *M. pulmonis* after aerosol inoculation of C57BL/6N mice and bronchoalveolar lavage using 0.9% NaCl. Bars represent the mean ± SD of the percentage of change in mycoplasma CFUs after four hours in culture. Mycoplasma numbers decreased significantly ($p < 0.02$) in cultures containing both the cellular and noncellular fractions of lavage fluid. $n = 12$ cultures for each group. Companion ΔlogCFUs were −0.02 ± 0.88, −0.1 ± 1.03, and −0.6 ± 0.47 for cultures containing cellular fraction only, fluid fraction only, and cellular and fluid fractions, respectively.

EFFECTS OF NITROGEN DIOXIDE ON IN VIVO–IN VITRO INTERACTIONS BETWEEN CELLS FROM LAVAGE SAMPLES AND *MYCOPLASMA PULMONIS*

It proved technically impossible to compare mycoplasma killing in both control animals and animals exposed to NO_2 in the same experiment because of the inability to process twice as many animals within the same time frame. Thirty-two animals were exposed to 10 ppm of NO_2 for four hours before being exposed to aerosols containing viable *M. pulmonis*. The time-weight average for NO_2 exposure was 9.3 ppm/minute. The MMAD for these experiments averaged 1.7 ± 0.10 μm, and the σ_g was 1.37 ± 0.11. Eight control mice and eight mice exposed to NO_2 were used to measure in vivo intrapulmonary killing. The lungs of the other mice exposed to NO_2 were used to measure killing in the cellular fraction of lavage fluid, with all bronchoalveolar lavage done immediately after exposure.

Mycoplasma pulmonis grew in the lungs of animals exposed to NO_2 (the percentage of change in mycoplasma numbers was 950% ± 404%, ΔlogCFU was 1.0 ± 0.17, $p <$ 0.005 for both), but was killed in the lungs of control mice (the percentage of change in mycoplasma numbers was −95% ± 0.02%, ΔlogCFU was −1.3 ± 0.17, $p < 0.005$ for both values). In cultures containing only the noncellular fraction from animals exposed to NO_2, the killing of *M. pulmonis* was statistically significant (Figure 5), but probably was not biologically significant, especially because the

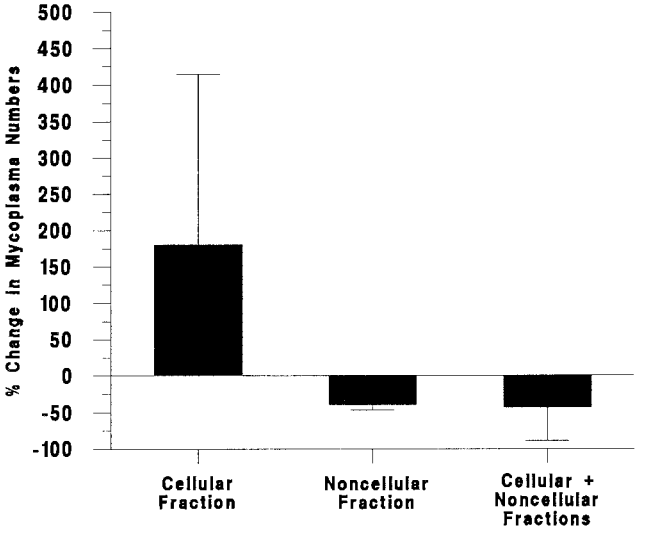

Figure 5. In vivo–in vitro killing of *M. pulmonis* after aerosol inoculation of C57BL/6N mice exposed to 10 ppm NO_2. Bars represent the mean ± SD of the percentage of change in mycoplasma CFUs after four hours in culture. Mycoplasma numbers decreased significantly ($p < 0.005$) in cultures containing the noncellular fraction only. n = 4 cultures per group. Companion ΔlogCFUs were 1.5 ± 1.59, −0.2 ± 0.08, and −0.5 ± 0.73 for cultures containing cellular fraction only, fluid fraction only, and cellular and fluid fractions, respectively.

organisms grew in vivo. The slight in vitro killing effect may be related to the release of intracellular enzymes from damaged macrophages. The data in Figures 4 and 5 cannot be directly compared because the samples from control mice and those from mice exposed to NO_2 could not be evaluated on the same day. Thus, the differences between the two experiments can only be regarded as suggestive evidence that NO_2 exposure abrogates killing in the in vivo–in vitro model.

CHARACTERIZATION OF THE NONCELLULAR FRACTION OF LAVAGE SAMPLES

To determine, at least in a preliminary fashion, whether or not *M. pulmonis* induced serum transudation into the lungs during the time that maximal pulmonary killing was seen, we analyzed the protein content of pooled lavage fluid from mice exposed to *M. pulmonis* and of pooled lavage fluid from control mice. At least within the initial 24 hours after exposure, no differences in protein content were found between the two groups (Table 5). The MMAD for this experiment was 1.27 μm, and the σ_g was 1.27.

CHARACTERIZATION OF CELLS FROM LAVAGE SAMPLES

Although other studies found no overall increase in the number of inflammatory cells within the lungs of C57BL/6N mice for the first eight hours after exposure to aerosols of *M. pulmonis* (Parker et al. 1989), the possibility remained that there was a change in the compartmentalization of host defensive cells within the lung. There could have been a shift in lymphocytes or other cell types from the interstitial compartment to the alveolar and airway compartments. This possibility was explored by the immunofluorescent examination of cells recovered by lavage from animals exposed to aerosols of *M. pulmonis*. There was no difference in the number of cells recovered by lavage from mice immediately after exposure to *M. pulmonis* or from mice lavaged four hours after exposure, and more than 95% of the cells in both groups were alveolar macrophages (Table 6). Double-labeling immunofluorescent experiments were conducted to determine which cell types were associated with *M. pulmonis* antigen, both immediately after exposure and four hours later. Although mycoplasmas that were not associated with cells were predominant at both time points, almost all of the cell-associated organisms were associated with alveolar macrophages. We subjectively noted that mycoplasma antigen was present in larger clumps and that more antigen per cell was present in cells collected by lavage four hours after exposure than in those collected immediately after exposure.

Table 5. Protein in Bronchoalveolar Lavage Fluid Collected from C57BL/6N Mice After Exposure to Aerosols of *Mycoplasma pulmonis*

M. pulmonis Aerosol	Time After Exposure (hours)	Protein (μg/mL)
–	0[a]	75 ± 20
+	0	62 ± 32
+	4	87 ± 35
+	24	99 ± 51

[a] Values are expressed as the mean ± SD of the protein content of the lavage fluid from 10 C57BL/6N mice for each group. The ANOVA indicated no significant differences between groups.

EFFECTS OF NITROGEN DIOXIDE AND *MYCOPLASMA PULMONIS* ON CELLS FROM LAVAGE SAMPLES

In examining the effects of NO_2 exposure on the in vivo–in vitro model, we noticed that the viability of cells recovered by bronchoalveolar lavage was consistently lower in cultures from animals that had been exposed to NO_2. We therefore analyzed the viability of lavaged cells recovered by bronchoalveolar lavage for the first 24 hours after exposure to either 10 ppm NO_2, *M. pulmonis*, or both agents. The time-weight average for NO_2 exposure was 9.3 ppm/minute. The MMAD for this experiment was 1.73 μm, and the σ_g was 2.39. Viability was assessed by both trypan blue exclusion and fluorescein diacetate uptake. Although there was excellent correlation (r = 0.98) between the two methods, fluorescein diacetate uptake gave higher values in all groups except controls (average difference 11%). However, fluorescein diacetate uptake was more difficult to interpret because of the difficulty in detecting dead cells and trypan blue exclusion alone was chosen for additional experiments.

Exposure to either insult significantly reduced the viability of macrophages (Figure 6), but the effect of exposure to NO_2 was greater than the effect of exposure to *M. pulmonis*. Furthermore, the viability of macrophages after exposure to NO_2 did not return to normal within 24 hours, whereas the viability of macrophages after exposure to *M. pulmonis* did. Furthermore, subjective examination suggested that there were increased numbers of ciliated epithelial cells in samples collected from mice exposed to NO_2. The abrogation of the NO_2 effect 24 hours after exposure in mice exposed to both NO_2 and *M. pulmonis* is probably due to the recruitment of inflammatory cells into the lungs of infected animals (Parker et al. 1989).

Table 6. Characterization of Cells Collected by Bronchoalveolar Lavage from Mice Exposed to *Mycoplasma pulmonis* Aerosol

Antiserum and Histochemistry	Results[a]
0 Hours	
Antimacrophage serum	> 95% of total cells stained.
Anti-T-cell serum	< 1% of total cells stained.
Anti-B-cell serum	2% of total cells stained.
Anti-*M. pulmonis* serum[b]	Many free mycoplasmas; some mycoplasmas in clumps; many mycoplasmas stained on or in macrophages; < 0.01% of B cells stained; no T-cells stained.
Wright-Giemsa stain	All cells were mononuclear; > 95% were morphologically compatible with macrophages; many mycoplasmas on or in macrophages.
4 Hours	
Antimacrophage serum	> 95% of total cells stained.
Anti-T-cell serum	1% of total cells stained.
Anti-B-cell serum	1% of total cells stained.
Anti-*M. pulmonis* serum	Many free mycoplasmas; many mycoplasmas in large clumps; almost all macrophages stained; rare B-cells stained; no T-cells stained.
Wright-Giemsa stain	All cells were mononuclear; > 95% were morphologically compatible with macrophages; many mycoplasmas on or in macrophages; many large clumps of mycoplasmas; more mycoplasmas phagocytosed than at zero hours.

[a] All results are based on the examination of 200 cells per sample.

[b] Anti-*M. pulmonis* serum was used in conjunction with antimacrophage serum, anti-T-cell serum, and anti-B-cell serum for double labeling of mycoplasmas and specific cell populations.

To determine the range of NO_2 doses that would affect alveolar macrophages, mice were exposed to 0, 2, 5, or 10 ppm NO_2, and the cellular fraction of bronchoalveolar lavage samples was collected. Time-weight averages for NO_2 concentrations were 0, 1.9, 4.6, and 9.3 ppm/minute, respectively. Exposure to both 5 and 10 ppm NO_2 significantly reduced the viability (trypan blue exclusion) of recovered alveolar macrophages when compared with controls and to mice exposed to 2 ppm NO_2 (Figure 7). Exposure to 2 ppm did not affect macrophage viability. To determine how long macrophage viability remained depressed after NO_2 exposure, mice were exposed to 10 ppm NO_2, or aerosols of viable *M. pulmonis*, or both NO_2 and *M. pulmonis*. Viability measurements (trypan blue exclusion) on cells from bronchoalveolar lavage samples were made on the day of exposure and one, three, and seven days later. The time-weight average for NO_2 exposure was 9.3 ppm/minute. The results are shown in Figure 8. Viability of cells from mice exposed to NO_2 alone did not return to normal until seven days after exposure. Our subjective assessment of cellular morphology suggested that macrophage size was increased three days after exposure.

RECOVERY OF PULMONARY CLEARANCE MECHANISMS AFTER EXPOSURE TO NITROGEN DIOXIDE

Mice were exposed to 10 ppm of NO_2 (the time-weight average for NO_2 exposure was 9.3 ppm/minute), and pulmonary clearance experiments were conducted one, three, and seven days after exposure. Because we previously had not examined the possibility of changes in mechanical transport at time points later than four hours after exposure to NO_2, the component parts of pulmonary clearance (mechanical transport and intrapulmonary killing) were not examined specifically in these preliminary experiments. Pulmonary clearance mechanisms remained depressed in animals exposed to NO_2 until seven days after exposure (Figure 9).

DISCUSSION AND CONCLUSIONS

Along with our previous study (Parker et al. 1989), the present experiments offer compelling evidence that exposure of mice to concentrations of at least 5 ppm of NO_2

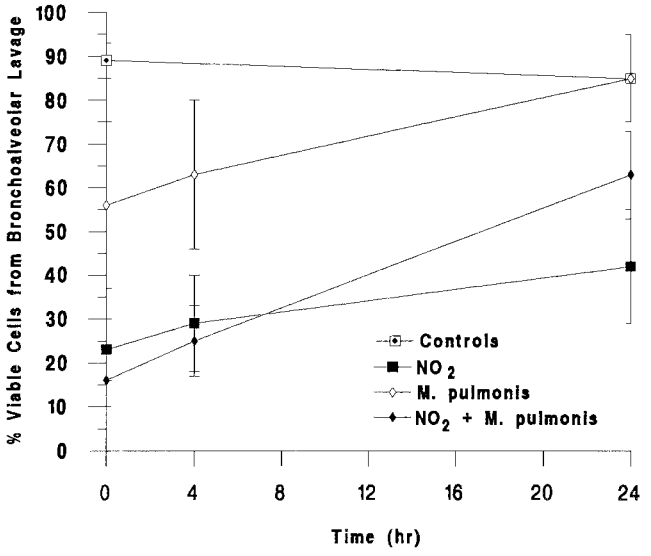

Figure 6. Percentage of viable cells recovered by lavage as measured by trypan blue exclusion after exposure to 10 ppm NO_2, viable *M. pulmonis*, or both. Numbers represent the mean ± SD of percentage of viability, based on counting 200 cells per sample in two separate experiments. n = 5 or 6 samples per group. The three-factor ANOVA indicated that NO_2, *M. pulmonis*, and time all significantly affected viability ($p < 0.001$ for all factors), and that there were significant interactions between NO_2 and time ($p < 0.001$), *M. pulmonis* and time ($p < 0.001$), and between NO_2 plus *M. pulmonis* and time ($p < 0.05$). Multiple means comparison was done by the Tukey-Kramer test to determine the relative effects of the various treatments and to determine which time point means for each treatment group were significantly different from controls. Nitrogen dioxide had significantly greater effect on viability than did *M. pulmonis* ($p < 0.05$). Twenty-four hours after infection, there was no significant difference between the viability of cells from control mice and from mice exposed to *M. pulmonis*. Viability of bronchoalveolar lavage cells remained depressed in mice exposed to NO_2 ($p < 0.001$), but exposure to *M. pulmonis* significantly abrogated ($p < 0.05$) the NO_2 effect.

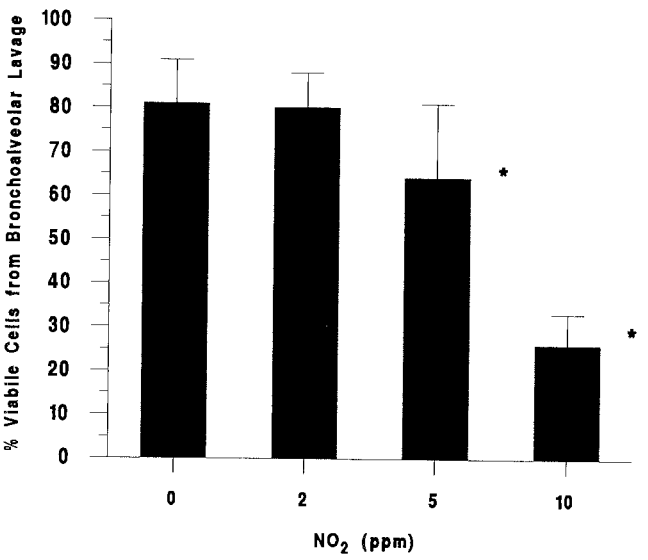

Figure 7. Effects of different concentrations of NO_2 on viability of cells from lavage samples. Bars represent the mean ± SD of the percentage of viable cells. n = 10 to 12 mice per group in two experiments. The ANOVA indicated that there was no significant difference between duplicate experiments, but there were significant differences in cell viability at NO_2 concentrations of 5 and 10 ppm ($p < 0.001$) compared with controls. Tukey's test was used to determine which means were different. Asterisks (*) indicate that cell viability in both the 5 and 10 ppm group were significantly different ($p < 0.003$) from controls and from each other.

can affect the incidence and severity of respiratory disease due to *M. pulmonis*. Previous experiments provided direct experimental links between decreased pulmonary clearance related to decreased intrapulmonary killing, increased numbers of *M. pulmonis* organisms in the lungs, an increased incidence of both gross and microscopic lesions, and an increased incidence of death in infected mice (Parker et al. 1989). The present study shows that decreased intrapulmonary killing was related to decreased alveolar macrophage function in animals exposed to NO_2. Although an increased susceptibility to *M. pulmonis* after exposure to NO_2 has been shown by other investigators (Brownstein et al. 1977; Nisizawa et al. 1988), our studies provide a complete chain of evidence in which all levels of assessment from lethality to the cellular level have been evaluated. Differences in concentrations of NO_2 required to demonstrate the exacerbation of *M. pulmonis* infection between the present study and those of other investigators

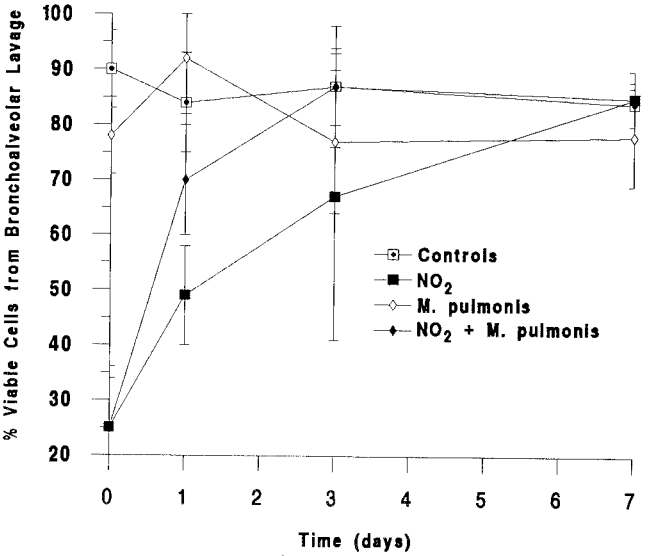

Figure 8. Recovery of viability of cells from lavage samples after exposure to 10 ppm NO_2 and *M. pulmonis*. Data points represent the means ± SD of the percentage of viable cells at different time points. n = 5 or 6 mice per group. The three factor ANOVA indicated that *M. pulmonis*, NO_2, and time all significantly affected viability ($p < 0.001$) and that significant interactions existed between NO_2 and time, *M. pulmonis* and time, and NO_2 plus *M. pulmonis* and time ($p < 0.001$ for all comparisons). Multiple means comparisons were done by the Tukey-Kramer test. The *M. pulmonis* group was significantly different from controls only at day 0 ($p < 0.05$). The NO_2 group was significantly different from controls on days 0, 1, and 3. The NO_2 plus *M. pulmonis* group was significantly different from controls only on days 0 and 1.

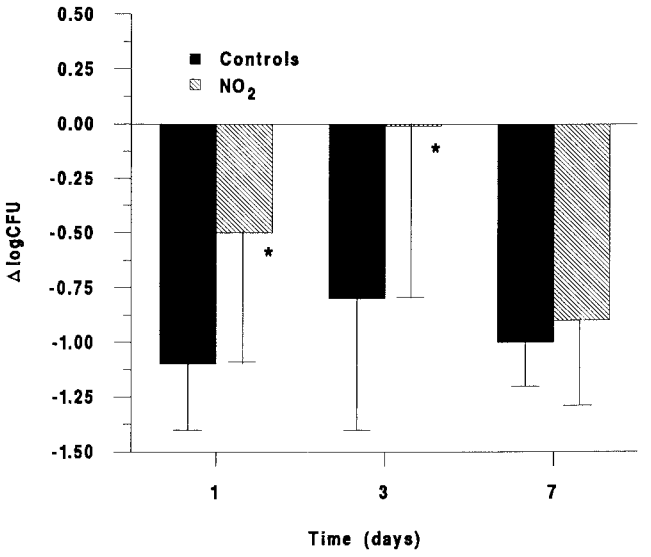

Figure 9. Recovery of pulmonary clearance following exposure of C57BL/6N mice to 10 ppm NO_2. Bars represent the mean ± SD of ΔlogCFU. n = 6 mice per group. An asterisk (*) indicates that the value for the group exposed to NO_2 is significantly different from that of the control group for the same day ($p < 0.02$).

may be related to the strain of mice, the strain of *M. pulmonis* used, the dose of organisms employed, or combinations of these factors.

The conclusion that decreased alveolar macrophage function is related to decreased intrapulmonary killing of *M. pulmonis* is based on the following evidence: maximal killing in the in vivo–in vitro system required the presence of cells recovered by lavage (Figures 3 and 4); exposure of mice to 10 ppm NO_2 appeared to decrease mycoplasmacidal mechanisms in in vivo–in vitro experiments (Figures 4 and 5); the vast majority of cells in lavage fluid were alveolar macrophages (Table 6); and the vast majority of cell-associated mycoplasmas were in association with macrophages (Table 6). Indications that alterations in the ability of alveolar macrophages to kill *M. pulmonis* are caused by NO_2 include: (a) a decrease in macrophage viability following exposure to NO_2 (Figure 6), (b) a correspondence between the lowest dose that affects the viability of lavaged cells and decreases the intrapulmonary killing of *M. pulmonis* (Figure 7 and Table 1), and (c) a parallel between the recoveries of the viability of lavaged cells and the intrapulmonary killing of *M. pulmonis* (Figures 8 and 9).

In a variety of both in vivo and in vitro experiments, other investigators also have concluded that alveolar macrophages are a target for NO_2 exposure (Valand et al. 1970; Acton and Myrvik 1972; Goldstein et al. 1973; Myrvik 1973; Vassallo et al. 1973; Goldstein et al. 1974; Katz and Laskin 1976; Brownstein et al. 1977; Ehrlich et al. 1979; Sherwood et al. 1981; McGrath and Oyervides 1982; Goldstein 1984; Voisin and Aerts 1984; Suzuki et al. 1986; Jakab 1987a,b, 1988; Miller et al. 1987; Schlesinger 1987a,b; Hooftman et al. 1988; Rose et al. 1988; Frampton et al. 1989). In both our studies and in those of other investigators, physical transport mechanisms were relatively resistant to the detrimental effects of NO_2. Low concentrations can increase particle clearance (Vollmuth et al. 1986), as we observed in mice exposed to concentrations of 1 ppm of NO_2. However, even multiple exposures of up to 20 to 30 ppm of NO_2 do not adversely affect mucociliary clearance in mice (Creasia 1981; Ranga and Kleinerman 1981; Jakab 1987a), which also agrees with our results.

Additional studies will be required to determine exactly how NO_2 interferes with the killing of *M. pulmonis* and other organisms. Many reported changes in macrophage physiology (Myrvik 1973; Voisin et al. 1976, 1977, 1979; Voisin and Aerts 1984; Miller et al. 1987), cell morphology (Voisin et al. 1976, 1977, 1979; Voisin and Aerts 1984), enzyme content (Voisin et al. 1976, 1977, 1979; Amoruso et al. 1981; Wright et al. 1982; Voisin and Aerts 1984), monokine production (Lefkowitz et al. 1984; Frampton et al. 1989), response to monokines (Greene and Schneider 1978), and phagocytic ability (Hooftman et al. 1988) are probably the indirect results of exposure to NO_2 or other oxidants, and may be related to cell damage and not to the initiating event. Nitrogen dioxide and other oxidants cause changes in the physical state of lipids in the superficial lipid domains of the pulmonary endothelial cell plasma membrane that result in a decrease in the fluidity of the membrane. The membrane changes are thought to interfere with receptor-ligand interactions (Patel and Block 1988; Patel et al. 1988). Similar changes in the plasma cell membranes occur after in vitro exposure of alveolar macrophages to NO_2 (Voisin et al. 1976, 1977, 1979; Voisin and Aerts 1984) and may be related to the formation of lipid peroxides that can be cytotoxic (Sagai et al. 1984, 1987; Sagai and Ichinose 1987).

From the present study, it is impossible to determine whether macrophage viability truly is decreased in vivo, or whether cells are just damaged and thus more susceptible to the stresses involved in lavage. Other investigators also have reported decreases in the viability of alveolar macrophages after exposure to NO_2 (Gardner et al. 1969; Ehrlich et al. 1979; Guth and Mavis 1985) or have shown changes in the enzyme content of lavage fluid that could be related to cellular damage (Guth and Mavis 1985, 1986). In general, these studies used either higher concentrations of NO_2 or longer exposure periods than those used in the present study. Other indications that NO_2 may reduce cell viability at relatively low concentrations come from studies in which

mouse macrophages were exposed in vitro to concentrations of 0.01 to 2 ppm NO_2 (Voisin et al. 1974, 1987; Voisin and Aerts 1984). However, other investigators did not find decreases in viability in alveolar macrophages exposed in vitro to NO_2 at concentrations up to 15 ppm (Pinkston et al. 1988). The differences in both in vivo and in vitro responses among various studies may be related to the differences in the levels of protective enzymes (Ichinose et al. 1982; Voisin et al. 1987) or in the levels of free radical scavengers in either the host or the culture system (Sagai et al. 1984, 1987; Guth and Mavis 1986; Rietjens et al. 1986).

It is possible that the alveolar macrophages of mice used in the present study are more susceptible to the effects of NO_2 than those in many other studies, simply because the mice are free of pathogens. Our data indicate that exposure to *M. pulmonis* induces changes in macrophage viability that are similar but less dramatic than changes induced by NO_2 exposure. However, the recovery of macrophage viability begins as early as 24 hours after exposure to *M. pulmonis*. With *M. pulmonis*, it is likely that the recovery of viable macrophage numbers is related to the recruitment of cells into the lungs, because this corresponds to the increase in inflammatory cell numbers seen in both normal C57BL/6N mice and C57BL/6N mice exposed to NO_2 after infection with *M. pulmonis* (Parker et al. 1987, 1989). Recruited macrophages may not possess the same capabilities that the native population possesses, and there is insufficient data to determine whether or not the population of macrophages within the lungs ever returns to the preinfected state. Thus, there may be changes in alveolar macrophage populations in mice naturally infected with low numbers of mycoplasmas, and all subpopulations may not be equally susceptible to damage by oxidants.

Changes in the cytology of cells recovered by lavage after exposure to NO_2 were noted in the present study, but were not systematically evaluated. Similar changes in the cytology of alveolar macrophages after NO_2 exposure have been seen in other studies (Mochitate et al. 1986; Hibbs et al. 1988; Hooftman et al. 1988). After either long-term exposure (Gregory et al. 1983; Last et al. 1984; Last and Warren 1987) or during recovery from high-dose exposure (Kondo 1989), alveolar macrophage numbers may increase beyond normal levels. Neither the acute nor chronic effects of NO_2 exposure on various macrophage subpopulations have been evaluated, but given the central role of macrophages in immunity, it is possible that effects on macrophage subpopulations may be involved in the reported changes in immune mechanisms after exposure to NO_2 (Fenters et al. 1971; Hidekazu and Shimizu 1980; Schnizlein et al. 1980; Fujimaki et al. 1981; Joel et al. 1982; Hillam et al. 1983; Lefkowitz et al. 1984; Azoulay-Dupois et al. 1985; Nisizawa et al. 1988).

Although our data suggest that damage to the alveolar macrophage per se is at least one mechanism by which exposure to NO_2 alters intrapulmonary killing of *M. pulmonis*, alveolar macrophages may not be the only target of NO_2. We were unable to establish a completely in vitro model of intrapulmonary killing of *M. pulmonis*. The data obtained with concentrated lavage fluid suggest that an appreciable amount of killing of *M. pulmonis* is mediated by noncellular factors (Table 3), and the in vivo–in vitro experiments indicate that unidentified factors present in the lungs are required for cells recovered by lavage to kill *M. pulmonis* (Figures 3 and 4). The specific molecular mechanism used to kill the organisms in concentrated lavage fluid has not been identified; this factor may be similar to other factors present in lavage fluid that are capable of killing other organisms (Coonrod and Yoneda 1983; Coonrod et al. 1984; Nugent and Fick 1987). The exact requirements enabling alveolar macrophages to kill *M. pulmonis* also are uncertain. On the basis of analogy with macrophage killing of *M. pulmonis* and other mycoplasmas in vitro when specific immune serum is present (Jones and Hirsch 1971; Jones et al. 1972; Jones 1975; Howard and Taylor 1979, 1984; Davis et al. 1980a; Taylor and Howard 1980b, 1981), it is likely that lavage fluid contains a nonspecific opsonin. Nonspecific opsonins have been identified for other pulmonary pathogens (Juers et al. 1976; LaForce et al. 1978; Heidbrink et al. 1982; Chudwin et al. 1985; Toews et al. 1985; Oishi et al. 1986), and exposure to NO_2 can alter surfactant concentrations in the lungs (Seeger et al. 1985; Miller and Cottrell 1987). Surfactant is thought to be a nonspecific opsonin for some bacteria (Juers et al. 1976; LaForce et al. 1978). However, it is also possible that alveolar macrophages recovered through lavage after exposure to aerosols of *M. pulmonis* were activated through the effects of the organism on other cell types, probably natural killer cells or lymphocytes in the lungs. These other mechanisms by which macrophages are activated may be additional targets for NO_2 exposure. Alterations in these mechanisms may in turn, be influenced by changes in alveolar macrophage function. Multiple targets for NO_2 exposure are suggested by Jakab (1987a), who showed that different concentrations of NO_2 are required to affect the intrapulmonary killing of different bacteria.

Although there was some contamination with NO of the NO_2 used in the present study, this is unlikely to have been an important factor. Even in long-term experiments, the effects of NO on the respiratory tract of mice were less than those of NO_2 (Nakajima et al. 1979), and no histological or ultrastructural effects were seen in rats continuously exposed to 2 ppm of NO for six weeks (Azoulay et al. 1977). The maximum NO concentration that would have been present in our 5-ppm exposures was only 0.25 ppm. Nitro-

gen dioxide in the lungs is rapidly oxidized to NO_3^-; NO first is oxidized to NO_2^- and then changed to NO_3^- (Bompart et al. 1982). Therefore, the end result in vivo is exposure to the same agent. Furthermore, our results are similar to results obtained by other investigators in regard to the acceleration of particle clearance at concentrations of 1 to 2 ppm of NO_2, a decrease to normal physical removal at higher concentrations (Vollmuth et al. 1986), and concentration effects on the decrease in intrapulmonary killing (Jakab 1987a,b, 1988; Rose et al. 1988). This is true even though different mechanisms of generating NO_2 exposure concentrations were used, and different organisms were studied. Decreased intrapulmonary killing of *S. aureus* occurred only after exposure to 5 ppm of NO_2, unless the mice were first treated with corticosteroids (Jakab 1988). Exposure of mice to 5 ppm of NO_2 was required to enhance susceptibility to murine cytomegalovirus (Rose et al. 1988). In humans, exposure to 3 ppm of NO_2 did not increase susceptibility to the influenza virus (Goings et al. 1989). Only under conditions of continuous exposure for long periods of time has increased susceptibility to infectious disease in mice been demonstrated at lower exposure concentrations of NO_2 (Miller et al. 1987), although damage to macrophages and changes in macrophage function can be demonstrated in vitro at lower concentrations (Voisin et al. 1974, 1987; Voisin and Aerts 1984; Frampton et al. 1989). However, there are several indications that NO_2 can be synergistic with other pollutants (Graham 1989), including ozone (Last et al. 1983; Mustafa et al. 1984), sulfuric acid mists (Schlesinger 1987a, 1989; Schlesinger and Gearhart 1987; Schlesinger et al. 1987), ferrous sulfate aerosols (Sherwood et al. 1981), and respirable aerosols (Last et al. 1983, 1984; Last and Warren 1987), and information about exacerbation of infectious disease by mixtures of pollutants is sketchy at best.

The present study also extends our knowledge of nonspecific host defense mechanisms operative in mycoplasmal diseases. The pulmonary clearance experiments and the in vivo–in vitro model are the first demonstrations of macrophage killing of any mycoplasma in the absence of a specific antibody. Although the specific factor or factors necessary for macrophage killing of mycoplasmas were not identified, the present study shows that the required factors are present in the naive lung and are not derived from serum transudates. Furthermore, the ability of *M. pulmonis* to damage directly the alveolar macrophages has not been demonstrated previously. This may be an important virulence factor in mycoplasmal disease.

This study demonstrates the usefulness of murine respiratory mycoplasmosis as a model to study the effects of pollutants on resistance to infectious disease. It also highlights the need for a better understanding of the action of NO_2 and other pollutants on alveolar macrophages, and it suggests that other potential targets need to be examined. The *M. pulmonis* model would be useful for many of these studies. The present study also shows that endogenous *M. pulmonis* infection must be excluded from future studies of the effects of pollutants on macrophage physiology and function and of the mechanisms of lung injury.

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APPENDIX A. Quality Control Methods for Nitrogen Dioxide Exposure

Procedures that were evaluated in developing improved quality control methods for NO_2 exposure were: (1) calibration of the NO_2 analyzer; (2) the timing of the NO_2 exposures; (3) the verification and stability of the calibration and generation gases; (4) the regional concentrations of NO_2 inside the exposure chamber; and (5) the possible presence of NH_3 within the exposure chamber.

CALIBRATION OF THE NITROGEN DIOXIDE ANALYZER

Procedures outlined in the Beckman analyzer manual were used to verify that the Beckman Model 952a $NO/NO_2/NO_x$ instrument was connected properly and that the circuitry was zeroed properly and working correctly. This included verification of the meter mechanical zero, the cooling system, the potentiometers, and the catalyst converter. Some minor adjustments were made in potentiometer settings, but the instrument operated within acceptable limits as described in the manual. The Beckman analyzer incorporates an internal atmospheric dump for adjusting flow rate changes due to atmospheric pressure changes, and proper gas flow rates are obtained using a vacuum pump connected to the analyzer. The proper procedure for adjusting the valves used to set the flow rate is outlined in the Beckman Manual and was used for all calibrations. The analyzer was modified so that the voltage output of the NO channel was five times the voltage output of the NO_x channel. This increased the ability to detect NO contamination in NO_2 calibration gases. Thus, both the meter and the strip chart recordings show a NO concentration that is five times higher than the true concentration in the gas mixture being analyzed; i.e., a 4% contamination with NO is shown as a 20% contamination. Because of this modification, this analyzer cannot be used to read directly the NO_2 channel because all chemiluminescence analyzers calculate NO_2 concentrations by subtracting the NO channel from the NO_x channel.

Initially, the analyzer was calibrated with the NO channel, and NO concentrations were set with a bubble flowmeter. The calibration curve was fine-tuned with the NO_x channel of the analyzer and NO_2 span gases. Using the procedure outlined in the Beckman manual, the NO channel was calibrated using 91 ppm NO in nitrogen, or, in later experiments, using 10 ppm NO in nitrogen. First, the NO and zero calibration gases were checked for any contamination with other NO_x compounds by delivering undiluted standard gas to the analyzer and reading the NO and NO_x channels for 30 minutes each. The zero gas read zero on both channels. For the NO standard, the NO and NO_x channels gave identical readings, indicating no contamination of the NO standard with other NO_x compounds. All further readings during the calibration were made using the NO_x channel. A standard amount of NO was then delivered by adjusting the gas flow rates using a bubble flowmeter, and a standard curve was constructed by setting the flowmeter to deliver 9, 8, 7, 6, 5, 2, and 1 ppm NO. Because of instrument drift, we found it necessary to recalibrate the instrument weekly. For each calibration run, correlation coefficients were at least 0.95, or the instrument was recalibrated. As long as bubble flowmeters and the high NO standard (91 ppm) were used to generate the initial standard curve, accuracy below 5 ppm could be improved by using the NO_2 span gases to adjust the multipoint titration curve. After the curve was generated using NO, all of the span gases were read, and the concentration versus meter reading was plotted. If necessary, the span control potentiometer was adjusted slightly so that the meter reading of the NO_x channel was in agreement with the calculated reading from the primary NO curve. Using this fine-tuning procedure, correla-

tion coefficients of at least 0.98 in the 1 to 20 ppm NO_2 range could be achieved. To simplify and speed up the calibration procedure, and to improve the accuracy of the initial NO calibration, a mass flow controller (Aalbrog Instruments and Controls Inc., Monsey, NY) was obtained. This controller, in combination with a lower NO concentration (10 ppm), allowed calibration using the NO channel, which did not require fine tuning. Readings of span gases in the range of the exposure used for experiments verified that the calibration was correct.

Calibration checks were run daily, both before and after exposures, using 0 and 9 ppm NO, and the span gas (or gases) closest in concentration to the planned exposure concentration. To test the reliability of the mass flow controller for delivery of concentrated NO_2 to the chamber, different settings for the mass flow controllers for various concentrations of NO_2 were determined from the calibration curve. The valves were repeatedly opened and closed over a 24-hour period, and the concentrations of NO_x were determined. The NO_x concentrations were always within 1% of the calculated values.

There was minimal variation within each day in analyzer readings when known concentrations of NO or NO_2 were analyzed (Table A.1), but up to a 5% variation was found on different days, even when using the same calibration standards. Thus, it was necessary to recalibrate the analyzer weekly.

Table A.1. Between- and Within-Day Variation in Oxides of Nitrogen Analyzer Reading

	Gas Cylinder		
Day[a]	Zero	9 ppm NO[b]	7.1 ppm NO_2[b]
1	0	9.4	7.1
	0	9.5	7.1
	0	9.4	7.1
3	0	9.4	7.3
	0	9.4	7.3
	0	9.4	7.4
5	0	9.3	7.3
	0	9.4	7.3
	0	9.4	7.2
8[c]	0	9.9	7.5

[a] The analyzer was calibrated on Monday (Day 1), and three different readings were taken using the same calibration cylinders on Monday, Wednesday, and Friday. As can be seen, the readings are consistent within each day and actually varied only minimally during the first five days.

[b] Data are NO_x concentrations of standard gases.

[c] The analyzer was left running over the weekend, and by day 8 (the following Monday), it required recalibration due to instrument drift.

TIMING OF NITROGEN DIOXIDE EXPOSURES

Theoretically, the time required for the equilibration of the empty chamber to 99% of the desired concentration would be 18.5 minutes. In preliminary experiments, this was correct for an empty chamber. However, when the chamber was loaded with 36 (the maximum number used in an NO_2 exposure) to 80 mice, it took approximately 20 minutes to reach equilibrium. For the sake of consistency with our previous study (Parker et al. 1989), all exposure times (four hours) were calculated from the time the chamber reached the target concentration of NO_2, but the time-weight averages of NO_2 concentrations were also calculated.

VERIFICATION AND STABILITY OF THE CALIBRATION AND GENERATION GASES

We obtained NO and NO_2 standard gases, and ultrapure zero air from three different suppliers (Scott Specialty Gases, Plumstedville, PA; Tamm Medical Gases, Birmingham, AL; and Scott Marrin, Riverside, CA). Prior to July of 1989, only the supplier's analysis was available. However, all cylinders of standard NO_2 span gases and all cylinders of high concentrations of NO_2 used to generate chamber concentrations that were received after July of 1989 were checked for concentrations of NO and NO_x both at the supplier and the University. For the gases used to generate exposure concentrations, this analysis was verified by infrared spectrophotometry (Miran 1A Infrared Analyzer, The Foxboro Co., Foxboro, MA) by Dr. H. Ken Dillon, Department of Public Health, University of Alabama. He also checked our gas mixtures against the NO_2 samples of the National Institute of Standards and Technology, obtained from the Birmingham office of the Environmental Protection Agency.

All cylinders were used within six months of arrival at the University. All cylinders, including those used before July of 1989, were returned to the manufacturer for reanalysis of remaining contents. Based on the manufacturers' reanalyses, there was no evidence of the breakdown of NO_2 within the cylinders, regardless of the source or time of use. In addition, the Beckman analyzer was used to monitor the NO content of each cylinder during each experiment after July of 1989. This procedure verified that cylinders from Tamm Medical Gases and Scott Marrin were stable for at least six months. Although our policy was to discard any cylinder that showed an increase in NO content, this was never necessary. Nevertheless, we continued to discard cylinders older than six months.

Invariably, the ultrapure zero air and NO standards from all suppliers were free of contamination with other gases. However, all of the standard NO_2 span gases and cylinders with high concentrations of NO_2 were contaminated with NO. The total NO_x concentrations in cylinders of NO_2 invariably agreed with the certified analysis, and the contamination with NO in NO_2 from all suppliers was 3 to 5% (Table A.2). In the cylinders actually used to deliver chamber concentrations of NO_2 used in exposure experiments, NO contamination was always less than 5% (chemiluminescence method). Thus, the maximum concentrations of NO at exposure levels of 0.5, 1, 2, and 5 ppm NO_2 were 0.025, 0.05, 0.10, and 0.25 ppm, respectively.

REGIONAL CONCENTRATIONS OF NITROGEN DIOXIDE WITHIN THE EXPOSURE CHAMBER

To evaluate the possibility that NO_2 concentrations were not uniform throughout the chamber, we sampled nine sites inside the chamber (including all eight corners and the geometric center of the cube-shaped exposure area, which is where the original sampling probe was located). The samples were taken during exposures to 15.6 and 5.0 ppm NO_2 without mice, and at 5.0 and 2.0 ppm NO_2 with the chamber loaded with 80 mice (more than two times the normal animal density used in any experimental exposure). These concentrations represent the highest concentration that can be delivered from concentrated 2,000 ppm NO_2 at the normal chamber flow rate of 64 L/minute, the lowest concentration at which an NO_2 effect on pulmonary clearance was seen, and the next lowest concentration normally used. The maximum number of mice that could be loaded into the exposure chamber was used for these experiments to maximize any potential problems that the mice might induce in NO_2 decomposition or regional gas concentrations. We checked the concentration of NO and NO_x both outside and inside animal huddles of both active and sleeping mice (additional sampling points). In later experiments, a custom-designed, solenoid-based, multipoint sampling system was installed. This system allowed for automatic sampling of NO/NO_x concentrations at seven points within the chamber, including the geometric center, one each at the top and

Table A.2 Analysis of Cylinders Containing Nitrogen Dioxide From Three Suppliers

	Analysis (ppm)				
	Certified[a]		University of Alabama[b]		
Supplier	NO_2	NO	NO_x	NO	Contamination (%)
Scott Specialty Gases	2,304	0	ND[c]	ND	–
	2,195	0	ND	ND	–
	2,070	0	17.3[d]	0.7	4
	19.9	0	19.9	0.7	4
	7.1	0	ND	ND	
	7.1	0	7.1	0.2	3
	7.2	0	ND	ND	
	6.4	0	6.0	0.3	5
	0.8	0	1.0	0.04	4
Tamm Medical	2,450	0	17.8[d]	0.7	4
	11.2	0	10.8[e]	0.4	4
	4.0	0	3.6	0.1	4
	2.3	0	2.6	0.1	4
	1.7	0	1.6	0.08	5
	0.7	0	0.7	0.03	4
Scott Marrin	1,909	0	17.0[d]	0.7	4

[a] From analytical report supplied by manufacturer.

[b] Analysis by Beckman analyzer.

[c] ND = Not done. Cylinders used prior to July 1989 were not checked at the university.

[d] The concentration in the cylinder was too high to be analyzed directly by the Beckman analyzer. Analysis was based on the reading from inside the exposure chamber using the highest concentration that could be obtained with the normal flow rate of 64 L/minute. Analysis by infrared spectrophotometry indicated that the upper limit of contamination was 6%.

[e] This mixture was also checked by infrared spectrophotometry, and contamination was too low to be detected by this experiment.

bottom of the chamber, and four points in diagonally opposite corners.

There were no apparent differences in the concentration of NO or NO_x in the different regions (Table A.3), indicating no appreciable breakdown of NO_2 within the chamber at the flow rates used. There were also no appreciable changes in either NO or NO_x concentrations between the beginning and end of the normal exposure time of four hours, indicating that NO_2 concentrations within the chamber were stable. In addition, there was no appreciable difference in the concentrations of NO or NO_x outside and inside animal huddles of either active or sleeping mice. After the chamber was fitted with the multiple probe system, no meaningful differences were observed in any position or at any time during any experiment.

Table A.3. Variation in Oxides of Nitrogen and Nitric Oxide Concentrations Inside the Chamber at Different Concentrations of Nitrogen Dioxide[a]

	Gas Concentration (ppm)					
	15.6		5		2	
Sampling Position	NO_x	NO	NO_x	NO	NO_x	NO
Chamber Empty						
1	15.6	0.7	5.0	0.2	ND[b]	ND
2	15.6	0.7	5.0	0.2	ND	ND
3	15.6	0.7	5.0	0.2	ND	ND
4	15.6	0.7	5.2	0.2	ND	ND
5	15.6	0.7	5.2	0.2	ND	ND
6	15.6	0.7	5.2	0.2	ND	ND
7	15.6	0.7	5.2	0.2	ND	ND
8	15.6	0.7	5.2	0.2	ND	ND
9	15.6	0.7	5.2	0.2	ND	ND
Chamber Full (80 mice)						
1	ND	ND	4.7	0.2	2.2	0.1
2	ND	ND	4.7	0.2	2.2	0.1
3	ND	ND	4.8	0.2	2.2	0.1
4	ND	ND	4.5	0.2	2.2	0.1
5	ND	ND	4.8	0.2	2.2	0.1
6	ND	ND	4.8	0.2	2.2	0.1
7	ND	ND	5.0	0.2	2.2	0.1
8	ND	ND	4.7	0.2	2.2	0.1
9	ND	ND	4.8	0.2	2.2	0.1
10	ND	ND	4.5	0.2	2.2	0.1
11	ND	ND	4.7	0.2	2.2	0.1

[a] Numbers represent either a single determination (empty) or the average of two determinations (full) of NO and NO_x done on different days. Positions 1 through 9 are the eight corners of the chamber and the geometric center. Positions 10 and 11, found only in the full chamber, are the averages of five readings per day taken from cages with active mice (position 10) or with sleeping mice (position 11).

[b] ND = Not done.

PRESENCE OF AMMONIA WITHIN THE EXPOSURE CHAMBER

A Drager Model 21 NH_3 detection system (Drägerwerk Aglübeck, Lübeck, Germany) was used to determine whether or not NH_3 was present in the chamber during an animal exposure. In initial tests, the chamber was loaded with 80 animals that were exposed to either 2 or 5 ppm of NO_2 for six hours. After the exposure, gas flow was stopped, the animals were removed, and the chamber was left closed with no gas exchange for an additional 30 minutes to provide the maximum opportunity for NH_3 buildup. The detector was used to measure NH_3 concentrations both at the level of the animals and at the bottom of the chamber, where the excreta was located. No detectable NH_3 was present within the chamber even when it was used three consecutive times without cleaning. The function of the detector was verified by using the same cartridges to check NH_3 concentrations in dirty cages that had been used to house conventional (i.e., not gnotobiotic) animals. The cartridges functioned correctly and gave readings of 10 to 25 ppm NH_3 in these cages. Even though the chambers were thoroughly cleaned and disinfected after each exposure, periodic NH_3 measurements were made after some experimental exposures to verify the complete absence of NH_3. No NH_3 was detected during any of the periodic checks.

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ABBREVIATIONS

AAALAC	American Association for the Accreditation of Laboratory Animal Care
CFUs	colony-forming units
ΔlogCFU	change in log colony-forming units
dpm	disintegrations per minute
Δdpm	change in disintegrations per minute
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
H_2O_2	hydrogen peroxide
HEPES	*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid
LD_{50}	lethal dose for 50% of animals
MMAD	mass median aerodynamic diameter
NaCl	sodium chloride
NaCl-G	sodium chloride containing dextrose
$NaHCO_3$	sodium bicarbonate
NH_3	ammonia
NO	nitric oxide
NO_2	nitrogen dioxide
NO_x	oxides of nitrogen
PBS	phosphate-buffered saline
PD_{50}	gross pneumonia dose for 50% of animals
ppm	parts per million
RITC	rhodamine isothiocyanate
RPMI-BA	RPMI-1640 medium containing bovine serum albumin
RPMI-S	RPMI-1640 medium containing fetal bovine serum
^{35}S	sulfur-35
σ_g	geometric standard deviation

INTRODUCTION

A Request for Preliminary Applications (RFPA 86–4), which solicited proposals for studies on "Health Effects of Automotive Emissions," was issued by the Health Effects Institute (HEI) in the summer of 1986. The purpose of preliminary applications is to invite investigators to submit short descriptions of research proposals on topics outside of those defined by other Requests for Applications. In response to RFPA 86–4, Dr. Jerry K. Davis of the University of Alabama at Birmingham submitted a preliminary application entitled "Mechanisms of Nitrogen Dioxide Enhancement of Murine Respiratory Mycoplasmosis." The HEI Health Research Committee considered this work as complementary to the HEI's ongoing human and animal studies of nitrogen dioxide and susceptibility to respiratory infections, and requested that Dr. Davis submit a full proposal. In April 1987, Dr. Davis submitted a proposal for a four-year study entitled "Murine Respiratory Mycoplasmosis: A Model to Study Effects of Oxidants on Host Susceptibility to Respiratory Infection." In December 1987, the HEI approved a two-year project that began in March 1988. Total expenditures were $305,682. The Investigators' Report was received at the HEI in June 1990 and was accepted by the Health Review Committee in January 1991. During the review of the Investigators' Report, the Review Committee and the investigator had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h), and Sections 207(c)(4), (5), and (6) impose specific requirements for reducing motor vehicle emissions of certain oxidants and other pollutants. Section 202(i) calls for the Administrator to study whether additional reductions in light-duty vehicle and light-duty truck emissions should be undertaken.

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

SCIENTIFIC BACKGROUND

A significant amount of the nitrogen dioxide in our environment is derived from motor vehicle emissions. The question of whether ambient levels of nitrogen dioxide produce detrimental effects in normal, healthy individuals has not been resolved; however, evidence from human and animal studies suggests that exposure to nitrogen dioxide may lead to an increased susceptibility to respiratory infections (U.S Environmental Protection Agency 1982; Morrow 1984; Samet et al. 1987).

ATMOSPHERIC CHEMISTRY

Nitrogen dioxide, a gas with an irritating odor, is derived primarily from nitric oxide, a product of the combustion of fossil fuel by industrial operations, the generation of electric power, and motor vehicles (Morrow 1984; Finlayson-Pitts and Pitts 1986). In the atmosphere, nitric oxide is oxidized rapidly by ozone and photochemical processes, and more slowly by oxygen, to form nitrogen dioxide (Urone 1976). A minor amount (between 5 and 10%) of the total oxides of nitrogen emitted by combustion processes is already in the form of nitrogen dioxide (U.S. Environmental Protection Agency 1982). Nitrogen dioxide (NO_2) also acts as an energy trap by absorbing energy from sunlight and forming nitric oxide (NO) plus a single atom of oxygen (O), which then reacts with an oxygen molecule (O_2) to form ozone (O_3), thus perpetuating the cycle by which ozone is formed during the daylight hours. The yellow-brown haze of photochemical smog often seen hovering over a city is due, in large part, to nitrogen dioxide and the aerosols it helps generate (Urone 1976).

EXPOSURE TO NITROGEN DIOXIDE

Urban residents are usually exposed to a diurnal cycle of nitrogen dioxide that consists of a low, basal concentration and higher concentration peaks, or spikes, of short duration (U.S. Environmental Protection Agency 1982). The current NAAQS for nitrogen dioxide is 0.053 ppm as an annual average; that means that the average of measurements taken over a 12-month period at any given measurement site should not exceed 0.053 ppm. In 1989, the annual mean concentrations of nitrogen dioxide ranged from slightly below 0.01 ppm at the "cleaner" measuring sites to approximately 0.045 ppm at the "higher" sites, with a median value slightly greater than 0.02 ppm at the "typical" sites. The 1989 composite average nitrogen dioxide level was 5% lower than the 1980 level, indicating a downward trend during this period. In 1989 only the Los Angeles–Long Beach California area, with a population of approximately 8.5 million people, exceeded the NAAQS for nitrogen dioxide (U.S. Environmental Protection Agency 1991).

Regulatory agencies have focused primarily on nitrogen dioxide in photochemical smog. Recently, however, it has been recognized that significant exposures occur in indoor atmospheres contaminated either by industrial processes or by burning gas in the home. Nitrogen dioxide generated indoors by gas stoves typically reaches levels of 0.025 ppm, although concentrations as high as 0.2 to 0.4 ppm have been recorded (Speizer et al. 1980; Samet et al. 1987), and concentrations as high as 2 ppm have been found in homes with portable kerosene heaters (Leaderer et al. 1984). A nitrogen dioxide level of 4 ppm was measured at an indoor sports arena after a malfunctioning engine of an ice resurfacer was tested (Hedberg et al. 1989).

Nitrogen dioxide is known to cause serious biological effects at high levels of exposure. Some evidence also suggests that exposure to nitrogen dioxide leads to increased susceptibility to respiratory infections (U.S. Environmental Protection Agency 1982; Morrow 1984; Samet et al. 1987).

EFFECTS OF NITROGEN DIOXIDE ON HUMANS

Nitrogen dioxide is relatively insoluble and penetrates beyond the upper airways to its major site of injury at the terminal bronchioles (Utell 1988). These are critical structures that serve as conductors of the air stream to the eventual sites of gas exchange. At concentrations of 5 ppm or higher, exposure of healthy human adults to nitrogen dioxide for as little as 15 minutes has been reported to increase resistance to air flow, impair the normal transport of gases between the blood and the lungs, and increase susceptibility to bronchoconstricting agents (U.S. Environmental Protection Agency 1982; Morrow 1984). A number of investigators have attempted to determine the effect of exposure to nitrogen dioxide in populations that might be more susceptible to decreases in pulmonary function. Studies carried out on asthmatic subjects have provided conflicting results, which have been summarized by Utell (1989).

Numerous epidemiologic studies have examined whether exposure to nitrogen dioxide in indoor or outdoor air is a risk factor for respiratory infection (Florey et al. 1979; Speizer et al. 1980; Ekwo et al. 1983; Pengelly et al. 1984; Honicky et al. 1985; Samet 1989); however, the results of these studies have not provided a causal link between nitrogen dioxide exposure and respiratory infection (Samet et al. 1987; Pennington 1988). Interpreting the epidemiologic data has been complicated by factors such as inadequate or inaccurate personal exposure data for study subjects, short duration of the exposure measurement period for the studies, and the impact of confounding effects that occur when the influence of one variable is not separated from the effects of other variables. Confounding variables for study subjects in epidemiologic studies on nitrogen dioxide effects include smoking, occupational exposure to nitrogen dioxide, socioeconomic status, and asthmatic conditions (Samet et al. 1987; Samet and Utell 1990).

Our understanding of the possible detrimental effects of nitrogen dioxide is complicated by the fact that, in addition to a lack of unequivocal epidemiologic evidence of a relationship between nitrogen dioxide exposure and increased acute respiratory tract infection (Jakab 1988), controlled studies in human subjects have rarely addressed this issue (Samet and Utell 1990). In a study designed to evaluate the role of nitrogen dioxide inhalation in susceptibility to viral infection in healthy nonsmoking adults, Goings and coworkers (1989) exposed subjects for two hours per day for three consecutive days to 1 to 3 ppm nitrogen dioxide, levels consistent with those reported in indoor exposures. After exposure on the second day, they administered a live, attenuated influenza virus to the subjects intranasally. The results showed that the rates of infection were comparable for the nitrogen dioxide–exposed and clean-air breathing control groups in the first two years of the study. In the third year of the study, each group exposed to 1 or 2 ppm nitrogen dioxide became infected more often than control subjects breathing clean air. These differences were not statistically significant; therefore, the findings suggest, but do not prove, that exposure to nitrogen dioxide alone may play a role in increasing the susceptibility of adults to respiratory virus infection. The restriction of the subjects to young volunteers without lung disease excluded potentially susceptible segments of the population, such as persons with chronic lung disease, infants, and the elderly (Samet 1989).

EFFECTS OF NITROGEN DIOXIDE ON LABORATORY ANIMALS

Laboratory animals have been exposed to high concentrations of nitrogen dioxide to study its effects on respiratory defense against infectious agents. The detrimental effects of inhaling nitrogen dioxide at levels as high as 14 to 17 ppm include both inflammation and damage to the ciliated cells that line the airways (Stephens et al. 1971, 1972; Evans et al. 1972).

The results of studies on animals generally support the hypothesis that exposure to nitrogen dioxide increases susceptibility to bacterial and viral infection (U.S. Environmental Protection Agency 1982). Gardner and associates (1977a,b) and Coffin and coworkers (1977) examined the relationship between the concentration of nitrogen dioxide and the duration of exposure, and their effect on the susceptibility of mice to bacterial respiratory infection. The concentrations of nitrogen dioxide varied from 1 to 14 ppm, and the duration of exposure ranged from 0.5 to 7 hours, so that the product of concentration and time was held constant at 7. When mice were exposed to high concentrations of nitrogen dioxide for brief periods of time, both the severity of infection and the degree of mortality were greater than those seen after prolonged exposure to lower concentrations. This indicated that susceptibility to infection was influenced more by concentration than by duration of exposure.

A number of investigators have examined the susceptibility of mice to a bacterial infection using an experimental protocol that simulated the typical urban exposure pattern of humans during a work week. This mode of exposure consists of a low background level of nitrogen dioxide with superimposed nitrogen dioxide peaks, or spikes. Miller and associates (1987) studied the effects of exposure to spikes of nitrogen dioxide on the resistance of mice to bacterial infection. These investigators compared the mortality rate of mice exposed for up to 52 weeks to 0.2 ppm nitrogen dioxide with that observed when two one-hour spikes of 0.8 ppm nitrogen dioxide were superimposed on the 0.2 ppm exposure 5 days per week. After being challenged with *Streptococcus zooepidemicus*, significantly more mice died that had been exposed to the spiked regimen than those exposed only to 0.2 ppm nitrogen dioxide. The same investigators also compared the mortality rate of mice challenged with the same bacterial pathogen after exposure to either a continuous level of 1.5 ppm nitrogen dioxide or to a spiked regimen. Two one-hour spikes of 4.5 ppm nitrogen dioxide were superimposed twice daily on the continuous exposure level (Graham et al. 1987). After two weeks of exposure to nitrogen dioxide, a suggestive trend was noticed toward increased mortality in those mice receiving the spiked regimen. These studies suggest that spike exposures may modify the lung's response to a background level of nitrogen dioxide by decreasing the activity of its defense mechanisms against bacterial infection.

The sequence of exposure to nitrogen dioxide and challenge with a bacterial pathogen has also been found to influence the ability of mice to resist infection. Jakab (1987, 1988) reported that the antibacterial defenses of the lung were decreased after exposure to 5 ppm nitrogen dioxide when the gas was administered after the lungs had been seeded with *Staphylococcus aureus*. In contrast, 10 ppm nitrogen dioxide was required to decrease bactericidal activity when mice were exposed to nitrogen dioxide before a bacterial challenge.

Rose and coworkers (1988) exposed mice to nitrogen dioxide for two consecutive days before intratracheal instillation of an endogenous murine virus and continued exposure for four days after viral inoculation. Their observation that exposure to 5 ppm nitrogen dioxide enhanced the susceptibility of mice to viral infection suggests that exposure to nitrogen dioxide may also predispose the lower respiratory tract to viral, as well as bacterial, infection.

The pulmonary defenses that are compromised in laboratory animals exposed to nitrogen dioxide are also present in humans (Green 1984). Similar deficits in defenses might also occur in humans exposed to elevated levels of nitrogen dioxide.

EFFECTS OF NITROGEN DIOXIDE ON THE FUNCTION OF PHAGOCYTIC CELLS

Two interdependent systems keep the lung sterile and remove particles (including bacteria and viruses) deposited in different locations of the respiratory tract. Mucociliary transport clears particles from the nasopharynx and tracheobronchial tree, and phagocytes primarily clear and kill microorganisms in the alveolar region.

The two classes of phagocytic cells consist of macrophages and polymorphonuclear leukocytes (or neutrophils). The alveolar macrophage resides in the alveolus and is the first line of defense within the gas exchange region of the lung. When the lung is exposed to bacteria or viruses, macrophages release factors that stimulate an influx of neutrophils. Microorganisms are eliminated by a three-step process. They are first bound to the surface of phagocytes and then ingested (phagocytosis). In this process, soluble components (or opsonins) in the alveolar space facilitate phagocytosis by coating the inhaled microorganisms and promoting their ingestion by phagocytes (Kaltreider 1976; Sibille and Reynolds 1990). Then, macrophages and other phagocytes kill the microorganisms with a variety of degradative enzymes and by the production of highly reactive

species of oxygen, such as superoxide anion radicals, that may damage bacterial cells (Fels and Cohn 1986). In addition to their ability to phagocytize and kill microorganisms, macrophages play a key role in the induction of an immune reaction by finding and processing antigens for subsequent presentation to immunocompetent lymphocytes (Unanue 1980).

Studies in mice indicate that exposure to nitrogen dioxide impairs the killing process of phagocytes, thereby increasing the infectivity of bacterial pathogens (Jakab 1980). Several investigators have exposed animals to nitrogen dioxide and isolated alveolar macrophages by bronchoalveolar lavage for subsequent study in vitro. Acton and Myrvik (1972) noted a decrease in the phagocytic capacity of alveolar macrophages isolated from rabbits exposed to 15 to 50 ppm nitrogen dioxide. Schlesinger (1987) observed alterations in both the mobility and phagocytic activity of alveolar macrophages isolated from rabbits exposed to either 0.3 or 1 ppm nitrogen dioxide. Nitrogen dioxide may also reduce phagocytosis by injuring cellular membranes containing receptors that are critical for ingesting microorganisms (Frampton et al. 1989). Amoruso and colleagues (1981) reported that alveolar macrophages isolated from rats exposed to nitrogen dioxide showed a decreased production of superoxide anion radicals. Macrophages have also been exposed to nitrogen dioxide in vitro in order to assess its effect on their biological activities. Voisin and associates (1977) and Voisin and Aerts (1984) reported that guinea pig macrophages exposed to 5 ppm nitrogen dioxide and murine macrophages exposed to 2 ppm nitrogen dioxide displayed reduced bactericidal activity and viability. Thus, both the phagocytic and killing activities of alveolar macrophages appear to be compromised after exposure to nitrogen dioxide. The impairment of these critical functions can result in extended residence times of inhaled microorganisms on the alveolar epithelium. If microorganisms are not rapidly removed they may proliferate and increase the severity of an infection (Green and Kass 1964).

Other factors that may play important roles in maintaining pulmonary microbicidal activity include alveolar lining material, natural killer cells, and extracellular substances. Therefore, it is clear that the search for the target of the effects of nitrogen dioxide on the lung's ability to kill inhaled microorganisms must examine possible effects on a variety of host defenses.

MODEL SYSTEMS FOR ASSESSING SUSCEPTIBILITY TO RESPIRATORY INFECTION

Ethical considerations limit studies of experimental infection of humans, and investigators often must rely on models of infection in laboratory animals to evaluate the likelihood that exposure to photochemical oxidants increases the risk of respiratory infection in humans (Goldstein et al. 1976).

In such models, animals are exposed to a test pollutant followed by a subsequent challenge with an aerosol containing a pathogen (Ehrlich 1980; Gardner 1982; Graham and Miller 1985; Van Loveren et al. 1988). The rate of mortality is then compared to that of control animals exposed only to the pathogen. This infectivity model has been employed to study the effects of nitrogen dioxide exposure in hamsters, mice, and squirrel monkeys (U.S. Environmental Protection Agency 1982). The concentrations of nitrogen dioxide required to induce a significant increase in the mortality of monkeys and hamsters, compared with control animals, were higher than that required to achieve the same increase in mice. The difference in mortality among the species was due to variations in their innate resistance to the infectious agent, rather than to differences in resistance to nitrogen dioxide (Ehrlich 1980); therefore, the choice of animal used in the experiment is of critical importance. The infectious agent and the sequence of exposures to pollutant and pathogen are also critical choices when using this model (Ehrlich 1980; Jakab 1988).

A variation of the infectivity model can be used to measure the functional integrity of the antibacterial defenses of the lung (Jakab 1980). Animals (usually mice) are exposed to a test pollutant and then challenged with aerosols of radiolabeled bacteria. The level of radioactivity and number of viable bacteria in the lung tissue are measured immediately after the bacterial challenge and at various time points thereafter. These values then are compared with those obtained from control animals exposed only to the pathogen. The rate of the radiolabel's disappearance measures mucociliary clearance. The change in the ratio between the radiolabel and the number of viable bacteria reflects the level of bactericidal activity (Green and Goldstein 1966).

The infectivity model can provide information on the overall damage to pulmonary defense systems caused by exposure to test pollutants. It cannot, however, discriminate between the various individual components that may have been compromised (Van Loveren et al. 1988). For these analyses, it is necessary to isolate lung components and study their activities in vitro. In the present study, Dr. Davis and colleagues used bronchoalveolar lavage to complement the radiolabeled bacteria infectivity model. Bronchoalveolar lavage washes constituents of the airways and alveolar spaces from the lung and allows them to be examined for changes resulting from exposure to nitrogen dioxide. These constituents include alveolar and airway macrophages, inflammatory cells, alveolar lining material, and extracellular

substances. These two techniques allow functional decrements in the overall pulmonary defense system to be correlated with cellular responses.

In humans, pulmonary infectious diseases are most often related to indigenous flora rather than to newly acquired external pathogens (Green 1984). Therefore, relevant experimental models for extrapolating findings in animals to humans must take this into consideration.

In view of the similarities between human and rodent lung defense systems, the infectivity model is a valid means of evaluating the propensity of an agent such as nitrogen dioxide to produce or amplify infections in humans and allows the mouse to act as a convenient surrogate for humans (Goldstein 1984; Green 1984). Identifying a pollutant-induced inhibition of the ability of alveolar macrophages to kill inhaled microorganisms, or identifying an impairment in mucocilary clearance, suggests that such derangements also may be expected after exposure of humans to nitrogen dioxide.

JUSTIFICATION FOR THE STUDY

The HEI initiated research to help resolve the issue of whether or not oxides of nitrogen, in or near the ambient exposure range, increase the severity of respiratory infections in humans or enhance the susceptibility to these infections. Five studies were supported by RFA 83-2, "Nitrogen Oxides and Susceptibility to Respiratory Infections." These studies included two epidemiological surveys of the effects of nitrogen dioxide on respiratory infections in coal miners and infants, and three laboratory investigations of the effects of nitrogen dioxide on bacterial and viral infectivity.

To expand the scope of the research in this area, the HEI later solicited preliminary applications under RFPA 86-4, "Health Effects of Automotive Emissions," dealing with studies of the health effects of substances found in or produced by automobile emissions, and effects that may occur at pollutant concentrations found in urban centers. The Institute considered as particularly valuable those studies that could help elucidate the mechanisms by which pollutants produce toxic effects. After reviewing the preliminary applications submitted under RFPA 86-4, the Institute requested a full proposal from Dr. Davis, because his planned investigations were directly relevant to the studies already funded under RFA 83-2. In the proposal by Dr. Davis and colleagues, the investigators planned to examine the effects of exposure to nitrogen dioxide on the lung's ability to eliminate mycoplasma, to determine the mechanisms by which the organism is killed in the lungs, and to identify the component of the lung's defense system that is affected by nitrogen dioxide.

Relatively few experimental studies have been directed toward understanding the effects of nitrogen dioxide exposure on respiratory disease induced by a lung pathogen in its natural host (Brownstein et al. 1977; Jakab 1988). Mycoplasma normally lead a parasitic existence in close association with animal or plant cells (Alberts et al. 1983). Certain strains of mycoplasma are also pathogenic and capable of producing severe respiratory disease in rodents and humans. Because mycoplasma are endemic to rodents, thereby providing a normal host-parasite relationship, this was a good model in which to study the effects of an oxidant on pulmonary mycoplasmacidal mechanisms.

Mycoplasma pneumoniae is one of the most common pathogens of the human respiratory tract. Infection by this microorganism is the leading cause of acute pneumonitus in high-contact environments, such as college campuses and military bases, and is a common cause of respiratory illness in children. Illnesses associated with *M. pneumoniae* infections range from mild upper respiratory infection to tracheobronchitis and to acute pneumonia (Hu et al. 1980; Pennington 1988). *Mycoplasma pneumoniae* attaches to the ciliated epithelium of the lower respiratory tract, and, although the epithelial cells are not invaded by the organism, they are eventually destroyed (Berkow 1982). Likewise, *M. pulmonis* attaches to ciliated epithelial cells in the airways of rats; however, lesions develop in both the upper and lower respiratory tracts. In mice, the lesions produced by this microorganism are similar to those seen in rats, with a few minor exceptions (Schoeb and Lindsey 1985a,b). Studies using *M. pulmonis* in an animal model can be expected to yield information that can be extrapolated to the human lung's antimycoplasmal defense systems (Pennington 1988) because the lesions produced by *M. pneumoniae* in humans are similar to those produced in animals by other respiratory mycoplasma (Schoeb and Lindsey 1985a).

An important feature of the proposed studies was the use of mice known to be free of endogenous *M. pulmonis* and other pathogens that might contribute to the severity of the experimentally induced respiratory disease. Dr. Davis has had extensive experience in using *M. pulmonis* to induce respiratory disease (Davis et al. 1982; Davis and Cassell 1982; Davis et al. 1985; Davidson et al. 1988) and in studying the interaction of mouse phagocytes with this microorganism (Davis et al. 1980). In an earlier study, Dr. Davis and coworkers demonstrated that exposure to either 10 or 15 ppm of nitrogen dioxide significantly increased the mortality of mice exposed to this pathogen, and exposure to both 5 and 10 ppm nitrogen dioxide decreased the ability of the

mouse lung to kill *M. pulmonis* (Parker et al. 1989). To examine more environmentally relevant levels of this pollutant, the HEI Research Committee requested that Dr. Davis and his colleagues also expose mice to nitrogen dioxide concentrations of less than 5 ppm before being challenged with *M. pulmonis*.

OBJECTIVES AND STUDY DESIGN

The primary objective of the research was to identify the mechanism by which exposure to nitrogen dioxide enhances the susceptibility of mice to subsequent respiratory infection by a pathogen such as *M. pulmonis*. The specific objectives of the study were:

1. To measure the effects of inhaling nitrogen dioxide on the ability of the mouse lung's antimicrobial defense systems to kill *M. pulmonis*;
2. To provide a direct experimental link between nitrogen dioxide exposure, decreased pulmonary clearance of *M. pulmonis* due to defects in intrapulmonary killing, and alveolar macrophage function;
3. To develop an in vitro assay capable of demonstrating the killing of *M. pulmonis*; and
4. To determine the components of the mouse lung's defense systems that are the targets for the effect of nitrogen dioxide on mycoplasmacidal activity.

To examine the effects of nitrogen dioxide exposure on the ability of the mouse lung's defense systems to kill *M. pulmonis* (specific objective 1), mice were exposed by inhalation first to either 0.5, 1, 2, or 5 ppm of nitrogen dioxide for four hours and then to aerosols of the mycoplasma that had been tagged with radioactive sulfur. The maximum amount of time between the end of nitrogen dioxide exposure and exposure to *M. pulmonis* aerosols was 30 minutes. The lungs of the mice were studied either immediately after mycoplasmal exposure or 24 hours thereafter. The ratio of living versus killed microorganisms in lung tissue samples was determined by comparing the number of *M. pulmonis* colonies that grew in cell culture with the amount of radioactivity present in the tissue samples. Because the level of radioactivity is a measure of both living microorganisms and debris remaining from killed microorganisms, an increase in the ratio of live cell colonies to radioactivity at the 24-hour time period indicates a decreased level of killing in the lung.

The investigators proposed to determine whether a decreased intrapulmonary clearance of mycoplasma, after exposure to nitrogen dioxide, was linked to changes in the function of alveolar macrophages (specific objective 2). Several approaches were used to attain this objective. First, mice were exposed in vivo to *M. pulmonis*, and bronchoalveolar lavage fluid was collected and separated into cellular and noncellular fractions. Cultures were then established in vitro with separated cells alone, with the noncellular supernatant fraction alone, and with recombined cells and supernatant. The number of viable mycoplasmas was determined in each culture immediately and four hours after the cultures were established. Second, the capacity of alveolar macrophages to kill *M. pulmonis* in the in vivo–in vitro system described above was also studied after first exposing mice to nitrogen dioxide. Third, the content of alveolar macrophages relative to other cell types was examined in bronchoalveolar lavage fluid taken immediately after mice were exposed to *M. pulmonis* and four hours after exposure. Individual cell types in the bronchoalveolar lavage fluid were identified by assays using specific antisera or purified antibodies. Fourth, after exposure to *M. pulmonis*, bronchoalveolar lavage fluid was treated with a specific antiserum to determine the predominant cell type with which the mycoplasma were associated.

Other investigators had shown that macrophages can kill *M. pulmonis* in vitro only in the presence of a specific antibody because in the absence of the antibody the organisms attach to phagocytes, but no killing occurs. To determine whether mouse alveolar macrophages alone, in vitro, are able to kill *M. pulmonis* (specific objective 3), macrophages were incubated in the presence of *M. pulmonis* and either normal rabbit serum or rabbit serum containing an antibody specific for *M. pulmonis*. The number of viable mycoplasmas was determined immediately after establishing the cultures and two and four hours later.

The inability to design successfully a completely in vitro assay for demonstrating alveolar macrophage killing of *M. pulmonis* (see Technical Evaluation section) hampered a precise determination of the intrapulmonary target for nitrogen dioxide (specific objective 4). In a further effort to implicate alveolar macrophages as one of the targets for nitrogen dioxide, several additional protocols were designed. First, the number of living bronchoalveolar lavage cells isolated from mice exposed to nitrogen dioxide was measured immediately after exposure and four and 24 hours later, and compared with the levels obtained from control mice. Cell viability was measured using the dyes trypan blue and fluorescein diacetate. (Living cells do not absorb trypan blue, because the dye cannot pass through the intact cell membrane. In contrast, fluorescein diacetate is absorbed by all cells, but can be degraded only by living cells, producing a compound with a characteristic green fluorescence.). Second, bronchoalveolar lavage cells were isolated from control mice exposed to air and from mice ex-

posed to a series of nitrogen dioxide concentrations. The dose of nitrogen dioxide that decreased the number of living alveolar macrophages was compared to the dose that decreased the level of mycoplasma killing obtained in the experiments performed under specific objective 1. Third, cell viability and intrapulmonary killing of *M. pulmonis* after nitrogen dioxide exposure was studied in two series of experiments. The viability of lavaged cells isolated from both control mice and mice exposed to nitrogen dioxide was compared immediately after pollutant exposure and one, three, and seven days after exposure. The intrapulmonary killing of *M. pulmonis* by mice exposed to nitrogen dioxide was also measured one, three, and seven days after exposure and compared to the levels observed with control mice.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

This study confirms earlier findings of the investigators (Parker et al. 1989) that exposure of mice to nitrogen dioxide concentrations of 5 ppm and higher diminishes the in vivo microbicidal activity of the mouse lung against *M. pulmonis* infection. Lower concentrations of nitrogen dioxide did not affect intrapulmonary killing of *M. pulmonis* (specific objective 1). Alveolar macrophages exposed to microorganisms in vivo and then lavaged and assayed in vitro did show mycoplasmacidal activity, which was decreased by exposing mice to nitrogen dioxide before exposing them to *M. pulmonis* (specific objective 2). Bronchoalveolar lavage studies also demonstrated that the alveolar macrophage was essentially the only phagocytic cell responding to mycoplasmal infection, and that by inference these cells were at least partially responsible for the mycoplasmacidal activity observed (specific objective 2). After exposure to *M. pulmonis*, most of the cell-associated mycoplasmas were associated with alveolar macrophages (specific objective 2). Attempts to identify directly the cellular target for nitrogen dioxide toxicity were unsuccessful, because the investigators were unable to demonstrate in vitro microbicidal activity of alveolar macrophages toward *M. pulmonis* in the absence of added immune serum (specific objective 3). The inability to develop the in vitro assay prevented attainment of this objective and objective 4, which was to determine the components of the mouse lung's defense system that are the target of the effect of nitrogen dioxide on mycoplasmacidal activity. However, the protocols that were substituted for objective 4 (effect of nitrogen dioxide on cell viability and recovery of mycoplasmacidal activity) did provide circumstantial evidence that damage to macrophages may be one mechanism by which nitrogen dioxide reduces mycoplasmacidal activity.

ASSESSMENT OF METHODS AND STUDY DESIGN

The investigators provided detailed descriptions of animal strain selection and breeding, microbiological procedures using *M. pulmonis*, microbial aerosol exposure methods, techniques of bronchoalveolar lavage, and in vitro microbicidal assays. An important feature of this study was the quality control and monitoring of breeding colonies with regard to intercurrent mycoplasmal, viral, and other infections. These methods have long been used successfully by the investigators, and they paid meticulous attention to avoid methodological pitfalls that could have resulted in flawed data. The health status of animals used in other studies of the effect of nitrogen dioxide on the respiratory tract has generally not been specified; therefore, it is possible that earlier results could have been confounded by the presence of *M. pulmonis* or other infectious agents. It has been estimated that over 60% of the "barrier-maintained" rodent colonies in the United States are infected with *Mycoplasma* species or other pathogens (Cassell et al. 1981).

The microbiological and in vivo assay systems were appropriately and skillfully carried out, but the in vitro work was hampered by the inability of the investigators to develop a completely in vitro assay system to demonstrate killing of *M. pulmonis* by alveolar macrophages. One aspect of the in vitro assay that might have accounted for the failure of macrophages to kill the mycoplasma in the absence of a specific antibody was that the assay was performed in vials, which may have prevented full aerobic metabolism by the macrophages. Although alveolar macrophages are very sensitive to being deprived of oxygen, the investigators have previously used this assay with other organisms and found that microbicidal activity did take place. In the current study, they were able to kill *M. pulmonis* in these cultures when anti-*M. pulmonis* serum was present.

Methods for chamber exposure of animals to nitrogen dioxide are concisely described. However, throughout the study there were significant uncertainties about quality control procedures for the nitrogen dioxide exposure system. During the early phases of the work, the analyzer used to determine nitrogen dioxide concentrations was not calibrated with cylinders of known concentrations of the gas. These problems were rectified during the course of the study via an improved protocol for the generation and measurement of nitrogen dioxide. Additional experiments conducted after the establishment of appropriate quality control procedures confirmed the experimental data obtained

earlier. Quality control procedures used to generate the nitrogen dioxide and distribute the gas in the exposure chamber are described in Appendix A of the Investigators' Report.

The nitrogen dioxide in the cylinders used for animal exposures can undergo chemical transformation to nitric oxide and other breakdown products during storage. Measuring the concentration of nitric oxide and nitrogen dioxide before an exposure period allows the investigator to determine the level of contamination of the test gas. The lack of nitric oxide standards for calibrating the analyzer throughout most of the study and the use of retroactively corrected, rather than actual, values of nitric oxide concentrations, raise additional concerns about the reliable monitoring of experimental exposure atmospheres. The retroactive corrections were necessitated when, shortly before the conclusion of the study, the investigators became aware of a modification of the analyzer used for their nitric oxide measurements that resulted in a constant error in their initial determination of the levels of nitric oxide.

In summary, the methodology the investigators employed, although not new, was generally sound. Problems with generating and measuring accurate levels of nitrogen dioxide were corrected, and the majority of the nitrogen dioxide exposures were carried out after the implementation of appropriate quality control procedures. The investigators were unable to compare mycoplasma killing in the in vivo-in vitro system using both control animals and animals exposed to nitrogen dioxide in the same experimental period. Therefore, directly parallel experiments comparing the effects of nitrogen dioxide on mycoplasmal killing were not carried out because it was not possible to process twice as many animals in the same time period.

STATISTICAL METHODS

The statistical methodology, which consisted of simple comparisons and analyses of variance, was appropriate in most cases. The authors made informed choices of analytical methodology for each experiment, taking into account elements of study design such as the number of groups of data, the mode of organization, the degree of conformity to Gaussian distribution, and the uniformity of variance. One might argue the merits of nonparametric testing versus inverse-variance weighting in cases of nonuniform variance (standard deviation changing over the range of the data), but the investigators' approach was generally appropriate. When the data had a three-factor structure, a three-factor ANOVA was performed (Figure 6), and the main effects and interactions were identified and followed up with a multiple-comparison procedure. This represented a high level of statistical effort, and the multiple-comparison strategy was chosen from among many respectable alternatives.

RESULTS AND INTERPRETATIONS

The principal results are shown in Table 1 of the Investigators' Report, which demonstrates that, assuming accurate measurements of nitrogen dioxide concentrations, acute exposure to 5 ppm nitrogen dioxide for four hours impairs the capability of the mouse lung mycoplasmacidal defense systems to kill *M. pulmonis*. Lower concentrations (0.5, 1, or 2 ppm) had no effect on mycoplasmacidal activity. In addition, exposure to concentrations of nitrogen dioxide at 0.5 ppm and 1 ppm (but not higher) stimulated the loss of ^{35}S-labeled *M. pulmonis* from lung tissue, thereby implying that nitrogen dioxide had a mild accelerating effect on mucociliary transport. This observation is compatible with the mild irritant effect of the pollutant at low concentrations and may be an indicator of the biological activity of nitrogen dioxide at 0.5 ppm.

The inability to demonstrate microbicidal activity in vitro by mouse alveolar macrophages in the absence of immune serum is shown in Figures 1 and 2 of the Investigators' Report. The investigators attribute this result to the loss of some molecular substance in the alveolar fluid that may have prevented the opsonization of *M. pulmonis* required for subsequent intracellular killing. These cells are highly sensitive to reductions in the oxygen content that take place when they are suspended in or overlaid by tissue culture fluid. The assay system used cells in vials, where the relative volume of fluid overlying the cells may have impeded the oxygenation required to manifest microbicidal activity. However, the investigators have previously obtained bactericidal activity using this method, and mycoplasmacidal activity was demonstrated in this study when anti-*M. pulmonis* was present.

In place of a completely in vitro assay for demonstrating mycoplasmal killing by alveolar macrophages, the investigators were able to demonstrate in vitro killing of *M. pulmonis* by murine lung cells obtained through lavage after the cells first had been exposed to the microorganisms in vivo. When lavage was carried out using 0.9% sodium chloride alone, the combination of cells plus the noncellular fraction of lavage fluid produced maximal killing of *M. pulmonis*. When cell culture medium containing bovine serum albumin was used for lavage, maximal killing was seen with lavaged cells alone. The lack of an increased microbicidal effect from the addition of the noncellular fluid fraction to lavaged cells may have been due to the binding and subsequent inactivation of an active component of the lavage fluid by albumin. This possibility was not discussed

by the investigators. They obtained evidence suggesting that exposure to nitrogen dioxide in vivo abrogated the mycoplasmacidal activity of alveolar macrophages in vitro; however they were unable to process both control mice and mice exposed to nitrogen dioxide on the same day to define more directly the action of nitrogen dioxide on microbicidal mechanisms.

The investigators noted that the viability of lavaged cells in the in vivo-in vitro experiments was lower in cultures prepared from the cells of mice exposed to 10 ppm nitrogen dioxide. Additional experiments demonstrated that the viability of alveolar macrophages was the same in cultures prepared from the cells of mice exposed to either clean air or 2 ppm nitrogen dioxide. Exposure of mice to 5 or 10 ppm nitrogen dioxide, however, decreased macrophage viability in vitro. The present study does not allow us to conclude that exposure to the higher levels of nitrogen dioxide affected the viability of alveolar macrophages in vivo. The reduced viability in vitro may have been due to cells having been injured after exposure to nitrogen dioxide, thus becoming more susceptible to the stresses involved in bronchoalveolar lavage. The cold lavage fluid used in the in vivo–in vitro experiments, may have presented an additional stress for the alveolar macrophages (Senior et al. 1981).

The putative role of alveolar macrophages in the microbicidal activity of the rodent lung against *M. pulmonis* is supported by the data in Table 6 of the Investigators' Report. This table shows that 95% of the recovered cells in both control animals and animals exposed to nitrogen dioxide were alveolar macrophages, and almost all of the cell-associated microorganisms were associated with alveolar macrophages. Although the role of fixed tissue cells in the production of factors necessary for mycoplasmacidal activity cannot be ruled out, alveolar macrophages were essentially the only cell type overtly involved in the response to mycoplasmal infection.

The demonstration that exposure to 10 ppm nitrogen dioxide caused a decreased viability of lavaged cells, which are almost exclusively (greater than 95%) alveolar macrophages, supports the proposal that one of the targets of nitrogen dioxide in decreasing the intrapulmonary killing of *M. pulmonis* may be the alveolar macrophage. Additional circumstantial evidence for this interpretation may be adduced from the observations that, after mice were exposed to nitrogen dioxide, the nitrogen dioxide dose level that reduced cell viability was comparable to that which reduced intrapulmonary mycoplasmacidal activity, and the time course for the recovery of cell viability matched the recovery of mycoplasmacidal activity. The observation that seven days were required for full recovery of mycoplasmacidal activity suggests that exposure to nitrogen dioxide may prolong the disease state, in addition to increasing its severity.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

Additional investigations are required to determine more precisely how exposure to nitrogen dioxide enhances the susceptibility of mice to infection with *M. pulmonis*. As pointed out by the investigators, although the results of their studies suggest that damage to alveolar macrophages may be at least one mechanism by which exposure to nitrogen dioxide alters intrapulmonary killing of *M. pulmonis*, they may not be the only target. The experiment that showed an increase in killing when alveolar macrophage cultures were supplemented with the noncellular fraction of lavage fluid suggests that soluble factors, such as a nonspecific opsonin, may also mediate the microbicidal activity of the mouse lung. An earlier study by this group (Parker et al. 1989) showed that significant killing of *M. pulmonis* takes place in normal animals within eight hours of infection and before adaptive immunity can develop. This latter finding, together with the effect of the noncellular fraction of lavage fluid on mycoplasmacidal activity, suggests that macrophages are not the only defense mechanism responsible for killing *M. pulmonis*, and that certain opsonins can replace an antibody. Miller and Cottrell (1987) reported that exposure to nitrogen dioxide alters surfactant concentrations in the lungs, and surfactant is thought to be a nonspecific opsonin for some bacteria (LaForce et al. 1978). Multiple targets for nitrogen dioxide exposure have previously been suggested by Jakab (1987).

In order to remain consistent with their earlier study (Parker et al. 1989), the investigators chose to use only a single four-hour exposure of mice to nitrogen dioxide before infection with aerosols of *M. pulmonis*. Either more prolonged or multiple exposures might have induced decrements in mycoplasmacidal activity at lower concentrations of nitrogen dioxide than the 5 ppm reported here. Indeed, Miller and associates (1987) have demonstrated an increased susceptibility of mice to infectious respiratory disease following long-term (52-week) exposure to 0.2 ppm nitrogen dioxide, with superimposed spikes of 0.8 ppm.

The demonstration that alveolar macrophages show mycoplasmacidal activity in vitro in the presence of specific immune serum suggests that additional studies of the action of nitrogen dioxide as a possible immune system suppressant may also be an important area for additional research.

CONCLUSIONS

The investigators used a mouse infectivity model to study the effect of exposure to nitrogen dioxide on mycoplasmacidal activity. Mice were exposed to aerosols of *M. pulmonis* after inhaling various concentrations of nitrogen dioxide. Using cell culture techniques, the investigators compared the content of living mycoplasma in lung tissue from mice exposed to nitrogen dioxide to the number of surviving mycoplasma present in lung tissue from control animals exposed to air. Dr. Davis and colleagues isolated both cellular and noncellular constituents of the airways and alveolar spaces by bronchoalveolar lavage to determine which component was responsible for killing *M. pulmonis*, and whether exposure to nitrogen dioxide compromised the activity of these constituents.

No new information was discovered concerning the mechanisms by which exposure to nitrogen dioxide impairs mycoplasmal killing. The viability of alveolar macrophages appeared to decrease after exposure to nitrogen dioxide; however, the study provided no clarification of the mechanism responsible for this effect.

The investigators found that:

1. Exposure to nitrogen dioxide levels of 5 ppm or higher for four hours increases the incidence and severity of respiratory disease in mice subsequently exposed to aerosols of *M. pulmonis*.
2. Exposure to nitrogen dioxide levels of 0.5, 1, and 2 ppm for four hours had no effect on the susceptibility of mice to mycoplasmal infection.
3. *Mycoplasma pulmonis* is killed in the lungs of naive mice in the absence of a specific antibody. Although killing is presumed to be carried out by the alveolar macrophages, additional soluble factors may also play a role in the microbicidal activity.
4. The predominant cell type present in the early hours after *M. pulmonis* infection is the alveolar macrophage, and those mycoplasmas that are associated with cells are attached to alveolar macrophages. These are apparently the only mobile cells overtly active soon after mycoplasmal infection.
5. The viability of alveolar macrophages declines at the same concentrations of nitrogen dioxide that produce a reduction in intrapulmonary mycoplasmacidal activity.
6. The time course of recovery of alveolar macrophage viability, after exposure to nitrogen dioxide for four hours, matches the recovery of mycoplasmacidal activity.

The investigators were unable to develop a completely in vitro assay system for demonstrating the killing of *M. pulmonis* by alveolar macrophages in the absence of immune serum. Therefore, they were unable to determine whether a reduction in the phagocytic or cytocidal activities of these cells represents a mechanism by which mycoplasmacidal activity is reduced after exposure of mice to nitrogen dioxide.

The study reported here by Dr. Davis and colleagues provides additional evidence that decreased pulmonary clearance or killing may enhance the infectivity of an inhaled pathogen after exposure of animals to nitrogen dioxide. The investigators also developed a model that may be useful for future investigations of the interactions between exposure to nitrogen dioxide, lung defense mechanisms, and respiratory tract mycoplasma infection.

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LIST OF HEI PUBLICATIONS

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Special Reports

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

Research Reports

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

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Research Reports

Report No.	Title	Principal Investigator	Publication Date
17	Studies on the Metabolism and Biological Effects of Nitropyrene and Related Nitro-polycyclic Aromatic Compounds in Diploid Human Fibroblasts	V.M. Maher	March 1988
18	Respiratory Infections in Coal Miners Exposed to Nitrogen Oxides	M. Jacobsen	July 1988
19	Factors Affecting Possible Carcinogenicity of Inhaled Nitropyrene Aerosols	R.K. Wolff	August 1988
20	Modulation of Pulmonary Defense Mechanisms Against Viral and Bacterial Infections by Acute Exposures to Nitrogen Dioxide	G.J. Jakab	October 1988
21	Maximal Aerobic Capacity at Several Ambient Concentrations of Carbon Monoxide at Several Altitudes	S.M. Horvath	December 1988
22	Detection of Paracrine Factors in Oxidant Lung Injury	A.K. Tanswell	February 1989
23	Responses of Susceptible Subpopulations to Nitrogen Dioxide	P.E. Morrow	February 1989
24	Altered Susceptibility to Viral Respiratory Infection During Short-Term Exposure to Nitrogen Dioxide	R.M. Rose	March 1989
25	Acute Effects of Carbon Monoxide Exposure on Individuals with Coronary Artery Disease	HEI Multicenter CO Study Team	November 1989
26	Investigation of a Potential Cotumorigenic Effect of the Dioxides of Nitrogen and Sulfur, and of Diesel-Engine Exhaust, on the Respiratory Tract of Syrian Golden Hamsters	U. Heinrich (U. Mohr)	May 1989
27	Cardiovascular Effects of Chronic Carbon Monoxide and High-Altitude Exposure	J.J. McGrath	July 1989
28	Nitrogen Dioxide and Respiratory Infection: Pilot Investigations	J.M. Samet	September 1989
29	Early Markers of Lung Injury	J.N. Evans	September 1989
30	Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust	J.L. Mauderly	October 1989
31	DNA Binding by 1-Nitropyrene and Dinitropyrenes in Vitro and in Vivo: Effects of Nitroreductase Induction	F.A. Beland	November 1989
32	Respiratory Carcinogenesis of Nitroaromatics	R.C. Moon	April 1990
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42	Effects of Methanol Vapor on Human Neurobehavioral Measures	M.R. Cook	August 1991
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44	Leukocyte-Mediated Epithelial Injury in Ozone-Exposed Rat Lung	K. Donaldson (J.M.G. Davis)	October 1991
45	The Effects of Exercise on Dose and Dose Distribution of Inhaled Automotive Pollutants	M.T. Kleinman	November 1991
46	Role of Ring Oxidation in the Metabolic Activation of 1-Nitropyrene	F.A. Beland	November 1991

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

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