

Background Materials for **Frampton (2002)**

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Nitrogen dioxide exposure: effects on airway and blood cells

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air pollution; influenza virus; respiratory syncytial virus; blood; epithelial cells

NITROGEN DIOXIDE (NO₂), a byproduct of oxidation and combustion, is a primary outdoor and indoor air pollutant (14). Because of its oxidative potential and limited solubility, NO₂ is a deep lung irritant, and accidental exposures to high concentrations can cause acute lung injury and death (13, 49). NO₂ interacts with the lung epithelial lining fluid and epithelial cell membranes, with local production of reactive oxygen

and nitrogen species (47). A National Ambient Air Quality Standard has been established for NO₂ as an annual mean of 0.053 ppm (100 μg/m³), and the State of California has established a short-term NO₂ standard at 0.25 ppm for 1 h (6). Indoor NO₂ concentrations are often greater than those found outdoors, with peak levels exceeding 2.0 ppm (29) in homes with unvented sources of combustion.

Epidemiological studies have linked NO₂ exposure with increased respiratory illness in children (20, 28, 30, 33). A meta-analysis found that a 16-ppb increase in indoor NO₂ levels was associated with a 20% increased risk of respiratory illness in children (21). Exposure to NO₂ inside ice hockey arenas, from operation of natural gas-fueled ice resurfacing machines in the presence of inadequate ventilation, has been associated with “epidemics” of acute respiratory illness in exposed players and fans. Concentrations as high as 4–5 ppm have been measured in ice arenas (22). Recent studies have linked ambient NO₂ exposure with increased mortality (16), increases in cardiac arrhythmias in patients with implantable defibrillators (35), and with increased intrauterine mortality (34). However, it is difficult in epidemiology studies to separate effects of NO₂ exposure from other combustion-related pollutants.

Human clinical studies have generally found no effects of NO₂ exposure on pulmonary function at concentrations <2.0 ppm. However, exposures to 1.5–2.0 ppm for 1–3 h increased nonspecific airway responsiveness (11, 32), and recent studies suggest that exposures as low as 0.26 ppm NO₂ for 30 min at rest induce increased responsiveness to specific allergen challenge in patients with asthma (25, 46). Exposures to 2.0 ppm for 6 h with intermittent exercise caused a very mild airway inflammatory response in healthy subjects, with no changes in lung function (1) or alveolar macrophage (AM) phenotype (17). These data suggest that there are effects on airway epithelium at concentra-

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tions below those associated with pulmonary function changes or inflammation.

Animal exposure studies have indicated that NO₂ may increase susceptibility to infection. For example, rodents exposed to NO₂ at levels only 5- to 10-fold higher than peak indoor levels showed impaired responses to infectious challenges, in part through impairment of AM function (19, 24, 41). Damji and Richers (9) found alterations in circulating and splenic lymphocyte subsets after exposure to NO₂ for 8 h at levels as low as 4 ppm. These findings suggest that NO₂ exposure may alter both local and systemic host defenses. However, clinical studies have been inconclusive. Goings et al. (18) exposed healthy volunteers to 1–3 ppm NO₂ or air for 2 h/day for three consecutive days. A live, attenuated cold-adapted influenza A vaccine virus was administered nasally to all subjects after exposure on *day 2*. Volunteers exposed in the third year of the three-year study became infected more frequently in association with NO₂, but the effect was not statistically significant.

The health effects of NO₂ exposure may therefore result both from the direct oxidant effects of the pollutant and from increasing airway susceptibility to other challenges, including respiratory virus infection. We hypothesized that NO₂ causes a cascade of events, beginning with injury and inflammation of the distal airway epithelium, recruitment of T lymphocytes from blood to the airways, and increased susceptibility of the injured epithelial cells to viral infection.

METHODS AND STUDY DESIGN

Subjects. Subjects were 18–40 yr of age, of both genders, lifetime nonsmokers with normal spirometry, free of cardiac or respiratory disease, and without symptoms of respiratory infection within 6 wk of study. Informed consent was obtained, and the study was approved by the Research Subjects Review Board of the University of Rochester.

Study design. The overall protocol required 4 days for completion. The first day was devoted to subject screening, informed consent, and baseline measurements. The 2nd, 3rd, and 4th days were exposure days, all separated by at least 3 wk. All subjects were exposed in double-blind fashion to air and two concentrations of NO₂ (0.6 and 1.5 ppm) in randomized order for 3 h in an environmental chamber with exercise for 10 of each 30 min at an intensity sufficient to increase the minute ventilation to 40 l/min. Symptoms were assessed by questionnaire after each exposure; subjects ranked the severity of each symptom on a scale from zero (“not present”) to five (“incapacitating”). Pulmonary function was measured before and immediately after exposure. Phlebotomy and fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) and brush biopsies were performed 3.5 h after exposure.

Exposure facilities. All exposures were undertaken in a 45-m³ environmental chamber with an independently controlled ventilation system. The capabilities for generating and maintaining pollutant levels and constant temperature and humidity have been described previously (48). For comfort, temperature and relative humidity were maintained at 37.1 ± 3.0°C and 21.2 ± 0.92% (mean ± SD), respectively.

NO₂ concentrations were generated by introducing NO₂ gas in air (5,000 ppm compressed gas; Air Products, Allentown, PA) in a Venturi mixer with purified intake air from

the hospital ventilation system and was discharged in the exposure room via five ceiling diffusers. A comparable number of exhausts near the floor removed air from the exposure chamber, resulting in ~0.3 atmospheric changes/min. This enabled NO₂ levels to reach >90% of target levels within 4 min. The concentrations of NO₂ at the 3- and 6-ft levels within the chamber varied by no more than 5% of the mean. Continuous monitoring of the residual background levels (ppb) of NO_x, ozone, particulate matter, and SO_x in the purified intake air was performed.

Physiological testing. Spirometry was performed using a pneumotachograph interfaced with a computer (model CPS-F; Medical Graphics, St. Paul, MN). Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV₁), and forced expiratory flow between 25 and 75% of FVC were recorded from the best of three determinations, based on FEV₁. Airway resistance and thoracic gas volume were measured during panting using an integrated-flow, pressure-corrected body plethysmograph. Specific airway conductance (sGaw) was determined as the reciprocal of airway resistance, corrected for thoracic gas volume. Minute ventilation was measured initially at rest and during exercise using inductive plethysmography (Respigraph model PN SY01; NonInvasive Monitoring Systems, Miami Beach, FL), calibrated with a rolling seal spirometer (model 840; Ohio Medical Products, Houston, TX).

Fiberoptic bronchoscopy. We performed fiberoptic bronchoscopy with BAL and airway brush biopsy on each subject 3.5 h after exposures. Subjects were premedicated with 0.75–1.0 mg intravenous atropine, and topical anesthesia of the upper airway was established using lidocaine spray. Oxygen was administered by a nasal cannula, and cardiac rhythm was monitored throughout the procedure. The fiberoptic bronchoscope (FB-19H, outer diameter 6.3 mm; Pentax, Orangeburg, NY) was passed orally, and topical lidocaine was administered through the bronchoscope to suppress cough. The bronchoscope was gently wedged in a subsegmental airway of the inferior segment of the lingula. Four 50-ml aliquots of sterile normal saline were instilled sequentially and immediately withdrawn under gentle suction. The return of the first 50-ml aliquot was collected as the “bronchial lavage” (BL) sample, and the return from the subsequent three aliquots were pooled as the “alveolar lavage” (AL) sample. The bronchoscope was then gently wedged in a subsegmental airway of the right middle lobe, and the lavage was repeated. The same lingular and middle lobe subsegments were entered during each subject’s three procedures. All lavage fluids were collected on ice and processed immediately. The BL and AL samples from the right middle lobe were combined with the respective samples from the lingula before cell counting.

Brush biopsies were obtained from the lower lobe subsegments by passing a 3-mm bronchial brush (model 149; Mill Rose Laboratories, Mentor, OH) through the suction channel of the bronchoscope and gently rubbing it against the bronchial mucosa. The brush was withdrawn and agitated in 1.5 ml sterile normal saline on ice. Up to 30 passages of the brush were performed, distributed among the subsegments of the right lower lobe. With each subsequent bronchoscopy, brushings were obtained from the alternate lung to avoid the possibility of carry-over effects from local inflammation at the brushed site. Inspection of the airways during subsequent procedures showed no evidence of residual effects from the previous bronchoscopy. Cells recovered were consistently >95% epithelial cells.

Cell quantitation and characterization. Total cell counts were performed separately on the BL and AL samples and on

cells recovered by airway brush biopsy, using a hemocytometer. Viability was assessed using trypan blue dye exclusion. Cytospin slides (Shandon, Pittsburgh, PA) were prepared from aliquots of BL, AL, and epithelial cells of sufficient volume to contain 5×10^4 cells. Slides were stained with Diff-Quick (American Scientific Products, McGraw Park, IL) for differential counts; at least 500 cells from each slide were counted. A separate slide of cells from AL was stained with Mayer's hematoxylin and toluidine blue for enumeration of mast cells.

Venous blood was analyzed for hematocrit, hemoglobin, red blood cell indexes, and total and differential leukocyte counts in the clinical hematology laboratories of Strong Memorial Hospital (Rochester, NY). Differential cell counts as percentages were multiplied by the total white blood cell count and expressed as concentrations of cells.

Immunofluorescence analysis. Flow cytometry was used as a sensitive method for evaluating cell differential counts, and for assessing changes in phenotype and expression of activation markers, for both blood and lung lymphocytes. Fresh heparinized whole blood or cells from AL fluid that had been washed one time in cold PBS were stained with fluorochrome-labeled monoclonal antibodies (Becton-Dickinson, Mountain View, CA) with appropriate isotype control antibodies. Red blood cells were lysed, and cells were analyzed on a FACScan flow cytometer (Becton-Dickinson) equipped with a 15-mW argon ion laser at 488 nm. Data (forward scatter, linear scale; wide angle light scatter, log scale; and fluorescence emission at 500–560 nm and 543–627 nm, log scale) were collected in list mode for subsequent analysis. Irrelevant antibodies of the appropriate subclass showed no non-specific binding. The lymphocyte gate was selected based on light scattering properties, and lymphocyte subsets were determined as a percentage of gated cells. These percentages were then multiplied by the concentration of lymphocytes to express lymphocyte subsets as concentrations of cells.

Infection with influenza and respiratory syncytial virus *in vitro*. Influenza A/AA/Marton/43 H1N1 virus was grown in allantoic cavities of 10-day-old embryonated hens' eggs (8) and was stored at -70°C until use. The long strain of respiratory syncytial virus (RSV) was grown in HEp-2 cell monolayers cultured in Eagle's MEM (Biowhittaker, Walkersville, MD) with 2% heat-inactivated FBS in 5% CO_2 at 37°C . The virus was harvested after cytopathological changes involved 90% of the HEp-2 monolayer. The cells were scraped and sonicated to release cell-associated virus. The cell-free supernatant containing the virus was stored at -70°C until use.

Aliquots (2×10^5) of BL, AL, and epithelial cells were resuspended in serum-free growth medium (LHC-8; Biowhittaker), plated in 24-well culture dishes, and exposed to influenza virus or RSV for 1 h at 37°C at a virus-to-cell ratio of 1:1. Control wells contained virus without cells and cells without virus. After infection, virus was removed by washing two times with PBS. Cells were then incubated in LHC-8 medium at 37°C in a 5% CO_2 atmosphere for 4 days; supernatant fluids were removed each 24 h and stored at -70°C until assay for infectious influenza and RSV. Viability of BL, AL, and epithelial cells, with and without virus infection, was assessed by measuring lactate dehydrogenase (LDH) in culture supernatants on *day 1* and *day 4* of culture and by trypan blue dye exclusion after 4 days in culture. To provide direct comparison with our previous studies of the effects of NO_2 exposure on AM inactivation of influenza virus (16), and because we expected to see less infectious RSV released by AM than by epithelial cells, separate samples of AL cells (1×10^6) were infected with influenza and RSV at a virus-to-cell

ratio of 10:1. Supernatant fluids were removed each 24 h and stored for subsequent analysis of infectious virus.

Assay of infectious virus. All samples from a given subject were analyzed simultaneously without knowledge of the exposure. For influenza virus, confluent monolayers of Madin-Darby canine kidney cells were grown in 24-well cluster dishes in MEM containing 10% FBS, 100 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 2.5 $\mu\text{g}/\text{ml}$ amphotericin B. Aliquots of lavage cell culture fluids to be assayed for virus were serially diluted 10-fold in Earle's balanced salt solution, inoculated in quadruplicate (0.2 ml/well), and incubated at 37°C for 1 h with agitation every 15 min to ensure an even distribution of inoculum and to maintain moisture on cell surfaces. The cell monolayers were then washed with PBS and were overlaid with agarose containing MEM, antibiotics, and trypsin (2.5%; Biowhittaker), without FBS. After 2 days of further incubation, the cells were fixed with 10% formalin, the agarose layer was removed, and the wells were washed with water. Methylene blue stain (0.3%) was added for 15 min, the cultures were washed and air-dried, and the viral plaques were counted. Results were recorded as the mean values of quadruplicate determinations for each assay.

For RSV assay, HEp-2 cells were grown in 24-well tissue culture plates in MEM plus 5% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin in a 5% CO_2 atmosphere. When the HEp-2 cell monolayers were between 30 and 50% confluent, quadruplicate wells were inoculated with virus. Wells were first washed with warm, serum-free medium and then inoculated with 0.2 ml of diluted cell-free culture supernatants plus 1.8 ml MEM containing 1% FBS. After 2 h of incubation at 37°C in 5% CO_2 to allow the virus to adsorb to the cells, 0.8 ml of medium was added to each well. Plates were then incubated at 37°C and examined daily for the cytopathic effect from *day 3* to *day 7*. Plates showing no cytopathic effect by *day 7* were recorded as negative for the presence of virus. The virus titer was calculated by end-point dilution, using the method originally described by Reed and Muench (37).

Data handling and statistical methods. The study was designed as a standard, three-period cross-over design with three different treatments. The statistical analysis was a standard ANOVA (26) that included both period and carry-over effects in addition to an effect of treatments and gender (a between-subjects effect). Each ANOVA included an examination of residuals as a check on the assumptions of normally distributed errors with constant variance. If the required assumptions were not satisfied then data transformations such as the logarithm were considered. A P value <0.05 was required for significance. Data means shown in RESULTS include all study subjects, even though statistical outliers were excluded for the ANOVA.

RESULTS

Subject characteristics. Twenty-one subjects were studied (9 females, 12 males). Table 1 shows their age and baseline pulmonary function. All subjects completed all exposures and procedures without significant adverse effects.

Exposure data. Actual achieved NO_2 concentrations were 0.61 ± 0.02 and 1.50 ± 0.02 (SD) ppm. Minute ventilation during rest and exercise, and estimated total NO_2 intake, are shown in Table 2. Total NO_2 intake did not differ significantly between males and females at either exposure level using the Student's t -test.

Table 1. Subject age and baseline pulmonary function

	Males (n = 12)	Females (n = 9)
Age, yr	26.9 ± 4.5	27.1 ± 4.1
FVC, liters	4.93 ± 0.85	3.88 ± 0.64
FVC, %predicted	97.7 ± 14.5	109.6 ± 13.7
FEV ₁ , liters	4.08 ± 0.71	3.39 ± 0.47
FEV ₁ , %predicted	96.5 ± 15.1	112.6 ± 13.1
FEV ₁ /FVC, %	83.0 ± 8.5	88.3 ± 7.8

Values are means ± SD; n, no. of subjects. FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s.

Pulmonary function. There were no significant effects of NO₂ exposure on FVC, FEV₁, their ratio, or sGaw in either males or females.

Symptoms. Most subjects did not experience symptoms during any of the three exposures. Occasionally subjects reported mild respiratory symptoms during NO₂ exposure; respiratory symptom scores were generally highest after exposure to 1.5 ppm NO₂, but differences between NO₂ and air exposure were not significant for any single symptom, or for total symptom scores, by Wilcoxon analysis.

Blood cells. Analysis of data from complete blood counts performed 3.5 h after each exposure showed significant dose-related decreases in hematocrit, hemoglobin (Fig. 1), and red blood cell count (data not shown) in association with NO₂ exposure for both males and females. For all subjects, the decrease in hematocrit was ~1.7 percentage points or an overall reduction of 4.1%. There were no significant changes in red blood cell indexes, including mean corpuscular hemoglobin concentration, mean corpuscular volume (Fig. 1), and mean corpuscular hemoglobin (data not shown). These findings were therefore consistent with a decrease in red blood cell number, but not size or content of hemoglobin.

Figure 2 shows changes in blood leukocyte differential counts. All classes of leukocytes decreased in association with NO₂ exposure, but the change was significant by ANOVA only for lymphocytes, with no gender differences.

Blood lymphocyte phenotype. When lymphocyte subsets were expressed as percentages of gated cells, there were no significant NO₂ effects for the group as a whole

(Fig. 3). However, ANOVA suggested gender differences in the response to NO₂ for the ratio CD4⁺/CD8⁺, which increased in association with increasing concentrations of NO₂ in males but decreased in females ($P < 0.001$). Effects of NO₂ also differed by gender for CD16⁺ lymphocytes (NK cells; $P = 0.047$). The percentage of gated cells that were CD4⁺ was increased ($P = 0.014$) and CD16⁺ cells were decreased ($P = 0.015$) in females relative to males, independent of NO₂ exposure.

When data were expressed as concentration of cells, analysis showed gender differences in the concentration of T lymphocytes, including both CD4⁺ and CD8⁺ T cells, but no evidence for effects of NO₂ exposure.

Airway cells. There were no significant effects of NO₂ on the volume of fluid recovered, or on the total concentration of cells recovered, in BL or AL. ANOVA indicated significant NO₂ effects on the recovery of polymorphonuclear leukocytes (PMN) and lymphocytes in BL (Fig. 4). BL PMN increased in association with NO₂ in a dose-response fashion ($P = 0.003$). Lymphocytes increased after exposure to 0.6 ppm NO₂ ($P = 0.045$) but were closer to air exposure levels after 1.5 ppm NO₂. The increase in BL lymphocytes after 0.6 ppm NO₂ exposure was slightly greater in males than females ($P = 0.039$; Fig. 4). A significant association was observed between symptom scores and PMN in BL after exposure to 1.5 ppm NO₂ ($r = 0.56$, $P = 0.008$). Differential recovery of cells in AL was not altered by NO₂.

Airway lymphocyte phenotype. Because of limited cell numbers in BL, lymphocyte phenotype was determined only for cells in the AL fraction. The percentage of CD4⁺ lymphocytes increased after NO₂ exposure in a dose-related fashion, with no gender difference ($P = 0.034$; Fig. 5). There were no significant NO₂ effects on lymphocyte expression of CD16, CD25 (activation marker), or HLA-DR. AL CD25⁺ lymphocytes, as a concentration of cells, were increased in males relative to females ($P = 0.007$, data not shown).

Respiratory cell viability. Viability of cells recovered in BL and AL generally exceeded 90% and did not change with NO₂ exposure. Viability of airway epithelial cells recovered by bronchial brushing was generally <50% and decreased slightly but nonsignificantly in association with NO₂ exposure (data not shown).

Table 2. \dot{V}_E and estimated intake of NO₂

	\dot{V}_E , l/min		Intake Estimate, μg		
	Rest	Exercise	Rest	Exercise	Total
0.60 ppm					
Males	7.93 ± 0.69	38.95 ± 2.63	1,262 ± 102	2,363 ± 146	3,625 ± 239
Females	7.12 ± 0.69	34.52 ± 2.27	1,070 ± 98	1,999 ± 131	3,068 ± 190
1.5 ppm					
Males	8.65 ± 0.53	40.70 ± 2.35	3,117 ± 193	5,695 ± 343	8,812 ± 358
Females	7.39 ± 1.17	35.02 ± 2.48	2,729 ± 428	4,975 ± 348	7,704 ± 598

Values are means ± SE. \dot{V}_E , minute ventilation. Intake estimate is approximation of intake of NO₂ based on the product of concentration ($\mu\text{g/l}$) × time of exposure (min) × \dot{V}_E (l/min).

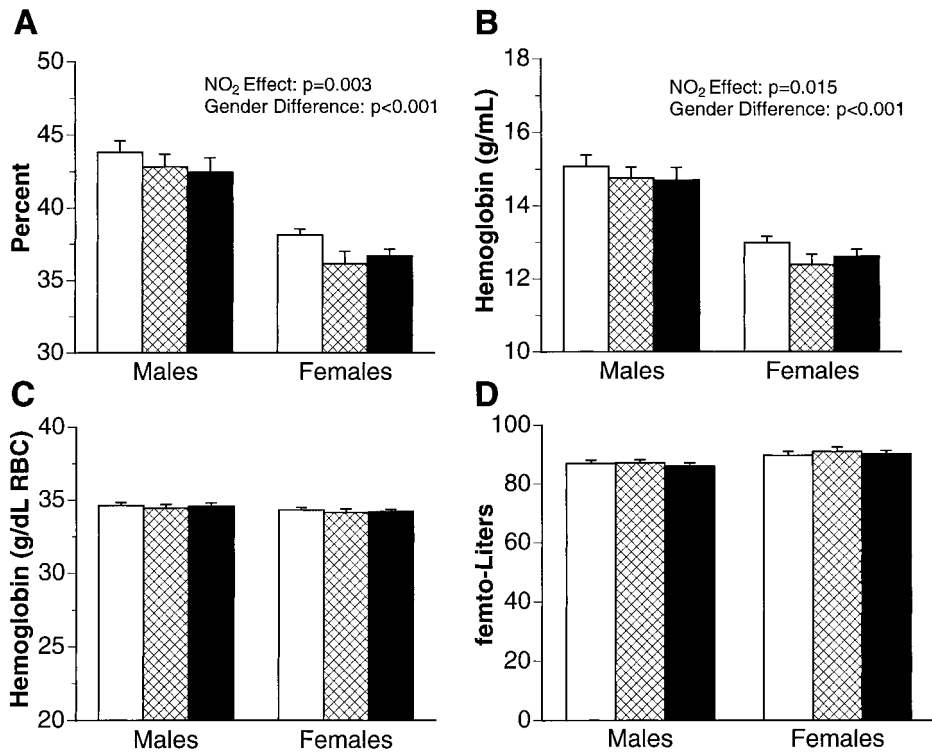


Fig. 1. Hematocrit (A), hemoglobin (B), mean corpuscular hemoglobin concentration (MCHC; C), and mean corpuscular volume (MCV; D) in blood after exposure to air or nitrogen dioxide (NO₂). Open bars, air exposure; crosshatched bars, 0.6 ppm NO₂ exposure; filled bars, 1.5 ppm NO₂ exposure. Data are means ± SE. RBC, red blood cells.

Combined effects on epithelial cell viability of prior NO₂ exposure and in vitro infection with influenza or RSV were assessed by measuring release of LDH in the culture supernatant on the 1st and 4th days of culture. As shown in Fig. 6, an NO₂ dose-related 40% increase in LDH release was seen on *day 1* of culture in the presence of RSV ($P = 0.024$), with a similar trend seen with influenza ($P = 0.076$). Similar findings were seen

after *day 4* of culture. Overall, these findings suggested a significant combined effect of NO₂ and infection with RSV on epithelial cell viability, with a possible similar effect for influenza virus.

Susceptibility to infection with influenza and RSV. No NO₂ effects were found on the susceptibility of AM or bronchial epithelial cells to infection in vitro with influenza or RSV. Influenza virus titers in the super-

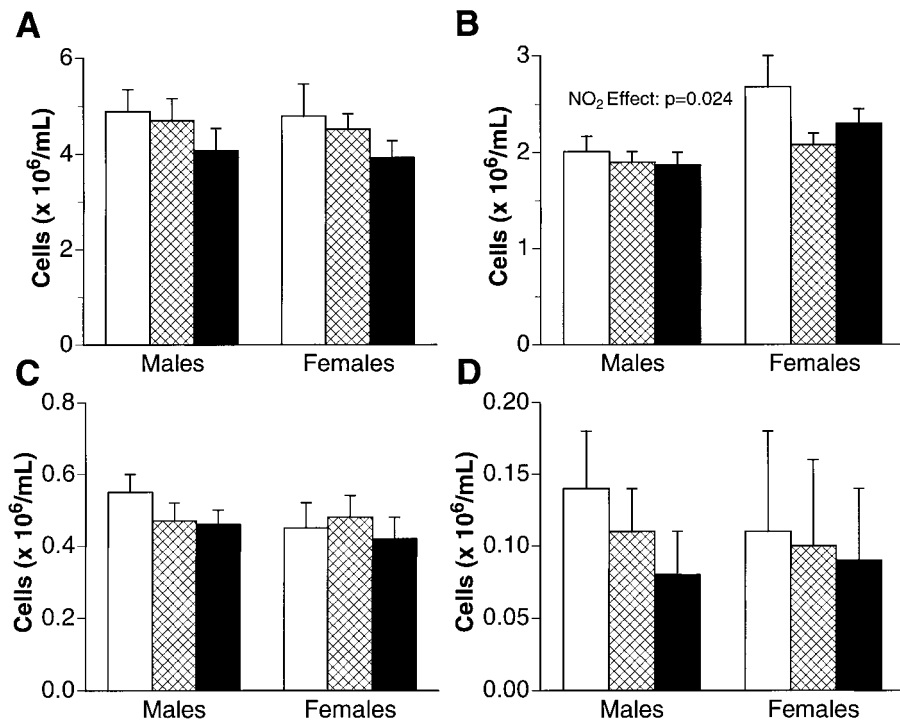
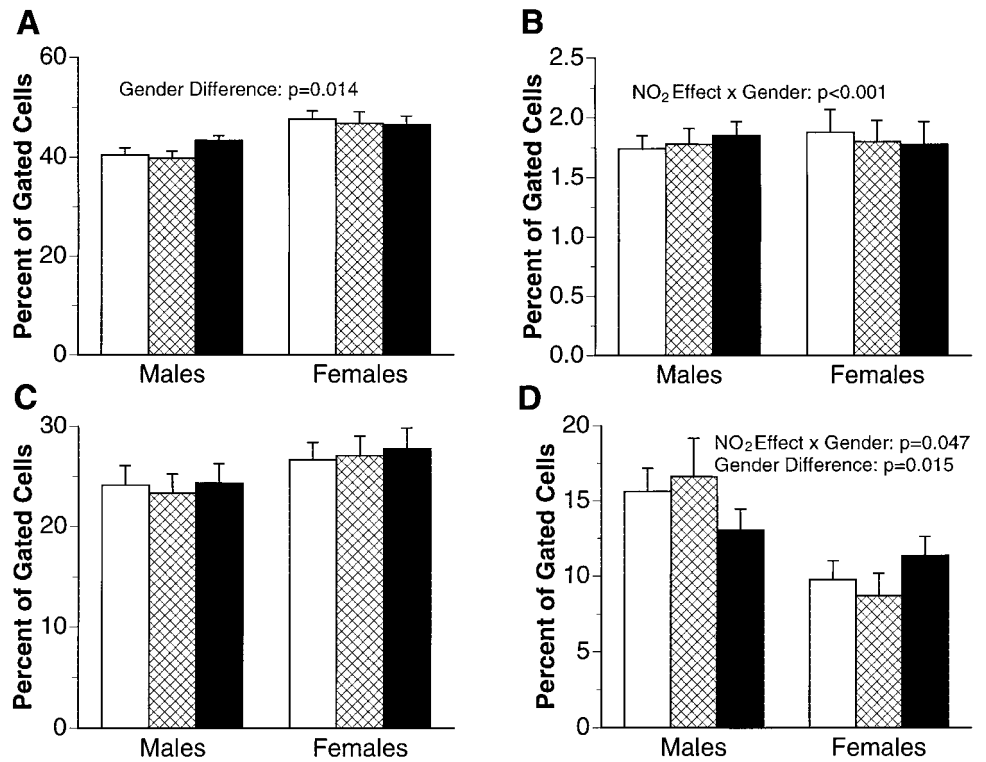


Fig. 2. Blood leukocytes after exposure to air or NO₂. A: polymorphonuclear leukocytes (PMN); B: lymphocytes; C: monocytes; D: eosinophils. Open bars, air exposure; crosshatched bars, 0.6 ppm NO₂ exposure; filled bars, 1.5 ppm NO₂ exposure. Data are means ± SE.

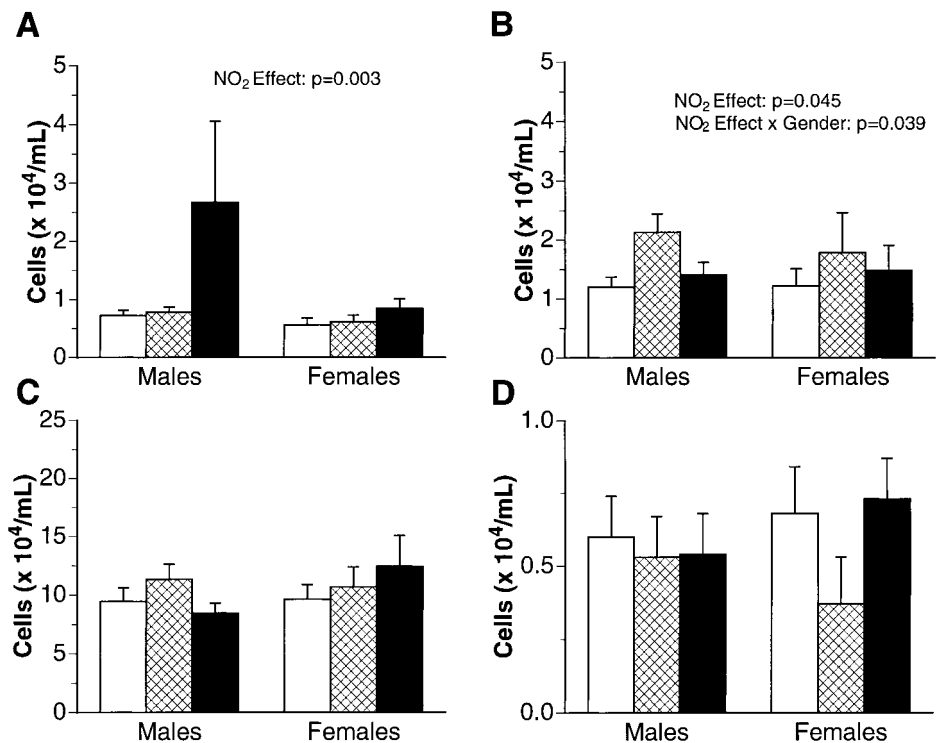
Fig. 3. Blood lymphocyte subsets, determined using immunofluorescence techniques, as a percentage of gated cells. A: CD4⁺; B: CD4⁺/CD8⁺; C: CD8⁺; D: CD16⁺. Open bars, air exposure; cross-hatched bars, 0.6 ppm NO₂ exposure; filled bars 1.5 ppm NO₂ exposure. Data are means ± SE.



natant fluids of both AM and epithelial cells declined with time, similar to the heat inactivation curve, indicating that infections were not productive. Infection with RSV resulted in relatively stable virus titers over 4 days of culture, suggesting that infection was minimally productive. There were no NO₂ effects on virus titers for any cell type.

Proteins in BL and AL fluid. Total protein and albumin were measured in both BL and AL as indicators of changes in epithelial permeability. There were no NO₂ effects on the concentration of total protein or albumin in either BL fluid or AL fluid. The concentration of albumin was lower in females compared with males for both BL fluid ($P = 0.045$) and AL fluid ($P = 0.019$),

Fig. 4. Cells recovered in bronchial lavage fluid. A: PMN; B: lymphocytes; C: alveolar macrophages; D: eosinophils. Open bars, air exposure; crosshatched bars, 0.6 ppm NO₂ exposure; filled bars, 1.5 ppm NO₂ exposure. Data are means ± SE.



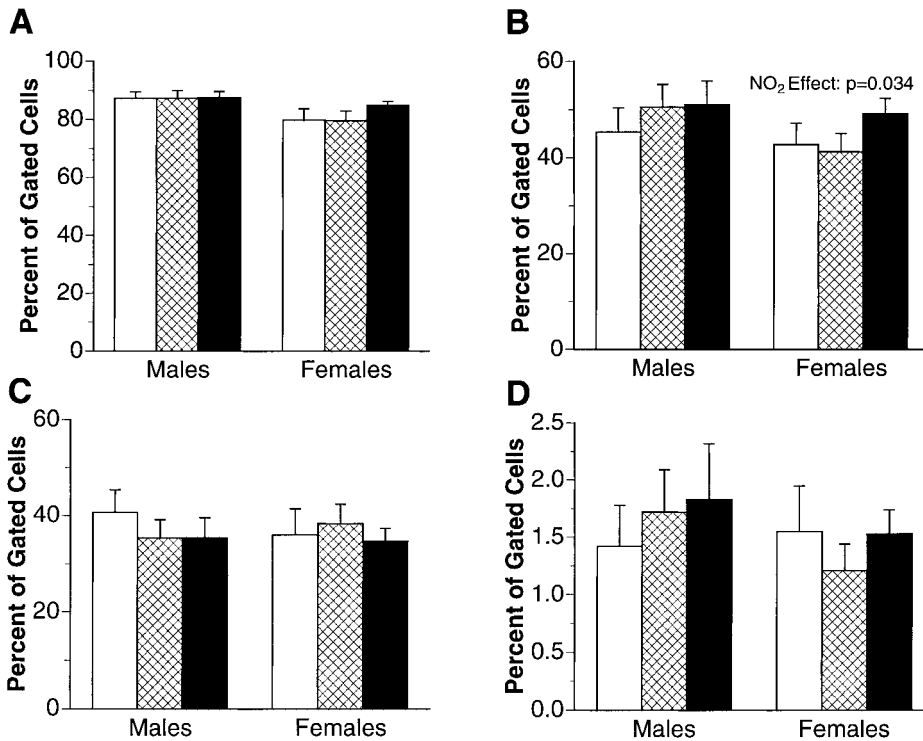


Fig. 5. Lymphocyte subsets recovered in alveolar lavage fluid, determined using immunofluorescence techniques. A: CD3⁺; B: CD4⁺; C: CD8⁺; D: CD4⁺/CD8⁺. Open bars, air exposure; crosshatched bars, 0.6 ppm NO₂ exposure; filled bars, 1.5 ppm NO₂ exposure. Data are means ± SE.

possibly related to differences in epithelial lining fluid volume related to lung size.

DISCUSSION

The objectives of this study were to determine effects of NO₂ exposure on airway inflammation, blood lymphocyte recruitment to the lung, and the susceptibility of airway cells to infection with influenza virus and RSV. Each subject was exposed to air and two concentrations of NO₂ to assess exposure-response relationships, with both concentrations below the threshold for

induction of airway inflammation suggested by previous studies.

Susceptibility to respiratory viruses. We found no effect of NO₂ exposure on infection with either influenza virus or RSV for cells obtained by BL, AL, or bronchial brush biopsy. Our laboratory found previously that AM obtained from subjects by BAL 3.5 h after exposure to 0.60 ppm NO₂ for 3 h were less effective at inactivating influenza virus in vitro compared with AM obtained after air exposure (12), although the difference did not reach statistical significance (1.96 vs. 1.25 log₁₀ plaque-forming units on day 2 of incubation, *P* < 0.07). Effects were seen in four of nine subjects, suggesting differences in susceptibility. No effects on virus inactivation were found when BAL was performed 18 h after exposure (47).

The present study did not show evidence for susceptible or nonsusceptible groups of subjects. We conclude that single exposures to 0.6 and 1.5 ppm NO₂ in vivo do not alter the susceptibility of lower airway cells to in vitro infection with influenza virus or RSV when tested under the conditions used in this study.

Influenza virus infection of bronchial epithelial cells or of AL cells (mostly AM) was not productive of infectious virus under the conditions of our study, and RSV was at best minimally productive. Previous studies have found that human AM become abortively infected with influenza virus in vitro (40); viral protein synthesis occurs, but infectious virus is not released, and no cytopathic effect is observed. However, AM do appear to support productive infection with RSV (3). Both influenza and RSV have the capability of infecting respiratory epithelial cells cultured from airway ex-

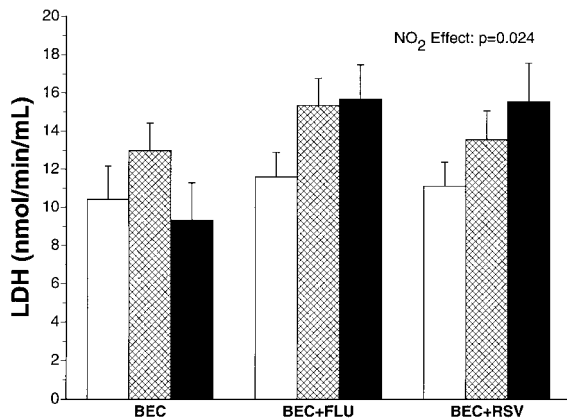


Fig. 6. Release of lactate dehydrogenase (LDH) in the culture supernatant fluids by bronchial epithelial cells (BEC) after 1 day in culture, with or without previous 1-h exposures to influenza virus (FLU) or respiratory syncytial virus (RSV). Differences were significant for BEC+RSV. Open bars, air exposure; crosshatched bars, 0.6 ppm NO₂ exposure; filled bars, 1.5 ppm NO₂ exposure. Data are means ± SE.

plants (2, 7, 39). It is possible that cells obtained by bronchial brushing did not yield productive infections because of disruption of membrane viral receptors during processing or because the cells obtained were superficial cells that were terminally differentiated. These findings do not rule out the possibility of NO₂ effects on viral infectivity in vivo.

Although NO₂ exposure did not alter susceptibility to infection with viruses in this study, there was a significant effect of prior NO₂ exposure on LDH release by airway epithelial cells exposed to RSV, with a similar trend for influenza virus (Fig. 6). This was seen in the absence of any direct effect of NO₂ on the viability of epithelial cells. The magnitude of the effect varied with NO₂ concentration and was seen after both 1 and 4 days of culture. This finding may not be specific for viral challenge but may represent enhanced cytotoxicity from a variety of infectious or noninfectious challenges. Our data suggest that NO₂ exposure enhances the cytotoxic effects of respiratory viruses or other challenges on epithelial cells and could thereby increase the severity of epithelial injury after such challenges.

The mechanism responsible for this effect is unknown but may involve injury to the epithelial cell membrane by reactive oxygen and nitrogen species generated from NO₂. Alternatively, epithelial susceptibility may have been enhanced by the small increase in airway PMN observed after exposure to 1.5 ppm NO₂.

Leukocyte subsets and activation. NO₂ exposure appeared to have an effect on lymphocyte recovery in both blood and BL fluid. In blood, NO₂ exposure resulted in a decrease in lymphocytes in both males and females (Fig. 2). In BL fluid, lymphocytes increased after exposure to 0.60 ppm NO₂ (Fig. 4). There were no signifi-

cant effects on lymphocyte recovery in AL. These findings suggest that NO₂ exposure may induce the recruitment of lymphocytes from the blood to the conducting airways.

There were gender differences in blood lymphocyte responses to NO₂. The ratio of CD4⁺ to CD8⁺ lymphocytes increased slightly in males in response to NO₂ but decreased in females. This may be in part because males and females differed in the concentration of blood T cells at baseline in our study, consistent with published findings (38). There was also a marginal gender difference in the response to NO₂ with regard to the percentage of CD16⁺ cells (NK cells) in the blood. There were no NO₂-related changes in the expression of the activation markers CD25 or HLA-DR.

Analysis of lymphocyte subsets in AL fluid revealed significant increases in the percentage of CD4⁺ T cells after NO₂ exposure (Fig. 5). There were no NO₂ effects on other lymphocyte subsets or activation markers in AL fluid. There were insufficient numbers of cells for lymphocyte subset characterization in BL fluid, where significant effects on total lymphocytes were seen.

Table 3 summarizes the findings from the current and previous studies examining NO₂ effects on lymphocyte subsets. Three previous studies have examined changes in airway lymphocyte subsets after single exposures to NO₂. Helleday et al. (23) observed increases in CD3⁺ lymphocytes and NK cells 24 h after 20-min exposures to 3.5 ppm NO₂ in nonsmokers. Blomberg et al. (4) found an increase in B lymphocytes, CD69⁺ lymphocytes, and NK cells 6 h after, but not 1.5 h after, 4-h exposures to 2.0 ppm NO₂. Circulating leukocytes increased 6 h after exposure, but blood lymphocyte subsets were not reported. In a previous study in our laboratory, 6-h exposures to 2.0 ppm NO₂ were associated with a small but significant decrease

Table 3. NO₂ effects on BAL lymphocyte subsets and activation

Reference	Exposure	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD8 ⁺	NK Cells	Activation	B Cells
<i>Blood</i>								
Current study	0.6, 1.5 ppm, 3 h	↓	NC	NC	↑ Males ↓ Females	NC	NC	NT
Azadniv et al. (1)	2.0 ppm, 6 h	NC	NC	↓	NC	NC	NC	NT
Rubinstein et al. (42)	0.60 ppm, 2 h × 4 days	NC	NC	NC	NC	NC	NT	NC
Solomon et al. (45)	2.0 ppm, 4 h × 3 days	NC	NC	NC	NC	NC	NC	NC
<i>BAL</i>								
Current study	0.6, 1.5 ppm, 3 h	NC	↑	NC	NC	NC	NC	NT
Azadniv et al. (1)	2.0 ppm, 6 h	NC	NC	NC	NC	NC	NC	NT
Blomberg et al. (4)	2.0 ppm, 4 h	NC	NC	NC	NC	↑	(CD69)	↑
Blomberg et al. (5)	2.0 ppm, 4 h × 4 days	NC	NC	NC	NC	NC	(CD25)	NC
Helleday et al. (23)	3.5 ppm, 20 min	↑	NC	NC	NC	↑	NT	NC
Rubinstein et al. (42)	0.60 ppm, 2 h × 4 days	NC	NC	NC	NC	↑	NT	NC
Sandstrom et al. (44)	1.5 ppm, 20 min every 2nd day × 6	NC	NC	↓	↑	↓	NT	NC
Sandstrom et al. (43)	4.0 ppm, 20 min every 2nd day × 6	NC	NC	↓	↑	↓	NT	↓
Solomon et al. (45)	2.0 ppm, 4 h × 3 days	NC	↓	NC	NC	NC	NC	NC

BAL, bronchoalveolar lavage; NC, no change; NT, not tested; ↑, increased; ↓, decreased.

in the percentage of CD8⁺ T lymphocytes in blood, with no changes in BAL fluid, 18 h after exposure (1).

Thus effects of NO₂ exposure on lymphocytes in BAL fluid are small and not consistent among studies. This may reflect differing exposure protocols, subject selection, or differences in sampling times among the studies. It is also possible that NO₂ exposure alters lymphocyte populations predominantly in the conducting airways rather than in the alveoli; lymphocyte subsets in the bronchial fraction of lavage were not assessed in any of these studies. In the current study, the data appear consistent with an NO₂-induced reduction in circulating T lymphocytes. In addition, the current findings suggest the possibility of gender differences in the lymphocyte responses to NO₂, and this possibility needs to be considered in the design of future studies.

Airway inflammation. Exposure to NO₂ was followed by an exposure-related increase in PMN recovered in BL 3.5 h after exposure in this study. Azadniv et al. (1) observed a small increase in PMN in unfractionated BAL fluid both immediately and 18 h after exposure to 2.0 ppm for 6 h. Blomberg et al. (4) found a 2.5-fold increase in PMN in bronchial wash 6 h, but not 1.5 h, after 4-h exposures to 2.0 ppm NO₂, with an increase in interleukin-8 levels 1.5 h after exposure. Thus single exposures to NO₂ at concentrations as low as 2.0 ppm induce a mild airway inflammatory response in healthy subjects, which may persist at least 18 h. In the current study, a weak association was observed between respiratory symptoms and PMN in BL after exposure to 1.5 ppm NO₂, suggesting that these low concentrations of NO₂ may be associated with clinical effects in some subjects.

Red blood cell effects. Analysis of data from complete blood counts revealed a small but highly significant decrease in red blood cell number and hemoglobin concentration in association with NO₂ exposure (Fig. 1). The mean reduction in hematocrit was ~4.1% and was similar for males and females despite the expected difference at baseline, with no change in red blood cell size or hemoglobin content. There was also an overall trend toward a decrease in the white blood count (Fig. 2), although this change was not statistically significant.

The decrease in hematocrit and hemoglobin in association with NO₂ exposure was an unexpected finding. However, this effect has been observed previously in human studies of NO₂ exposure. Posin et al. (36) observed small but significant decreases in hemoglobin and hematocrit immediately after 2.5-h exposures to 1 or 2 ppm NO₂. In addition, mice exposed to 5 ppm NO₂ for 1 h demonstrated reductions in hemoglobin and erythrocyte counts along with increases in bilirubin and methemoglobin concentrations, suggesting a mild hemolytic anemia (10). Other studies in animals provide evidence supporting increased red blood cell turnover after exposure to low concentrations of NO₂ (27, 31). Considered in light of these previous findings, it appears possible that NO₂ exposure, even at the low concentrations used in this study, leads to small reductions in circulating red blood cells. The duration of this

effect is unknown. Mechanisms may involve red blood cell membrane changes, methemoglobin formation, or cellular redistribution within the circulation. We believe hemodilution effects are an unlikely explanation because subjects performed a similar intensity of exercise on each exposure day.

The magnitude of these changes is small and unlikely to be of clinical significance for most individuals. However, in a 70-kg male, this drop in hematocrit would be equivalent to removal of ~200 ml of blood. Therefore, clinical consequences are possible for individuals with cardiovascular compromise or for competing athletes. Future studies of NO₂ exposure should consider assessment of red blood cell membranes, reticulocyte counts, and methemoglobin levels.

We conclude that, in healthy subjects, single exposures to NO₂ with exercise, at levels found indoors in homes with unvented combustion sources, induce the following effects: 1) mild airway inflammation; 2) mild respiratory symptoms in some subjects; 3) small reductions in hematocrit and hemoglobin; 4) possible small reductions in circulating T lymphocytes; and 5) possible increased susceptibility of airway epithelial cells to injury from exposure to respiratory viruses.

In addition, there may be gender differences in the effects of NO₂ on blood and/or airway lymphocytes. We found no effects of NO₂ on pulmonary function or on the susceptibility of airway epithelial cells or AM to productive infection by influenza virus or RSV in vitro.

With the relatively large number of statistical tests in this study, some significant *P* values could have occurred by chance. Our a priori approach was to evaluate findings based on the level of significance, consistency with the primary hypotheses and other study findings, and biological plausibility. The reductions in hematocrit and hemoglobin were the only findings not consistent with the primary hypotheses; here the highly significant *P* value (*P* = 0.003) from the ANOVA, and the similar effect in males and females despite baseline differences, makes the finding unlikely to be a chance occurrence.

All NO₂ effects observed in this study were small and unlikely to be of clinical significance for healthy subjects. However, young children, the elderly, and individuals with underlying respiratory or cardiovascular disease may be more susceptible to such effects. Indeed, time-series epidemiology studies indicate that these groups are at risk for adverse health effects from even modest increases in ambient air pollution (15). It is possible that a combination of airway and blood effects of exposure to NO₂ could exacerbate underlying airway disease, particularly after infections with respiratory viruses or other respiratory challenges.

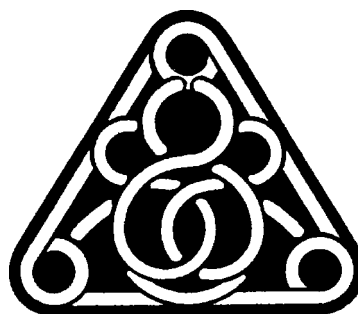
This work was supported by Contract 93-07 from the Center for Indoor Air Research and National Institutes of Health Grants RR-00044 and ES-01247.

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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

October 7th, 2014

MEMORANDUM

Subject: Nitrogen Dioxide: Evaluation of inhalation toxicity study, Frampton et al 2002

PC Code: 000596	DP Barcode: 421314
Decision No.: N/A	Registration No.: N/A
Petition No.: N/A	Regulatory Action: Data Evaluation Record (DER), Toxicology Review for Product Registration, Section 3
Risk Assess Type: Single chemical, non aggregate	Case No.: 4045
TXR No.: 1003341	CAS No.: 10102-44-0
MRID No.: 49420001	40 CFR: N/A

From: Jonathan Leshin, PhD, Toxicologist
Risk Assessment and Science Support Branch (RASSB)
Antimicrobials Division (7510P)

Thru: Tim McMahon, PhD, Senior Scientist
Steven H. Weiss, Branch Chief *Jaune Parone for Steve Weiss*
Risk Assessment and Science Support Branch (RASSB)
Antimicrobials Division (7510P)

To: Jaqueline Hardy, Product Manager
Regulatory Management Branch II (RMB2)
Antimicrobials Division (7510P)

Agency Conclusion: The Agency has reviewed the following journal article reporting about a study conducted with nitrogen dioxide. The study was not conducted according to the 870.3465 guidelines, but is acceptable for regulatory purposes.

Frampton, M.W., Boscia, J., Roberts, J.R., et al (2002) Nitrogen dioxide exposure: effects on airway and blood cells Department of Medicine, Environmental medicine and Biostatistics (University of Rochester, School of Medicine) MRID 49420001 American Journal of Physiology – Lung, Cellular and Molecular Physiology, 282: L155-165

EXECUTIVE SUMMARY: In an inhalation toxicity study (MRID 49420001) nitrogen dioxide was administered to 21 people aged 18-40 years (9 females, 12 males) in an environmental chamber at concentrations of 0, (room air), 0.6 ppm or 1.5 ppm nitrogen dioxide for a total of three hours. Exercise was performed for 10 minutes out of each thirty minutes at a sufficient level to raise minute ventilation to 40 l/min. Each exposure period was separated by at least three weeks from the previous exposure period. The subjects were assessed for pulmonary function before and after each exposure. Phlebotomy and bronchoscopy with bronchoalveolar lavage and

brush biopsies were performed 3.5 hours post exposure. Recovered cells were challenged with respiratory viruses *in vitro*.

There was no effect of nitrogen dioxide exposure on infection with influenza or respiratory syncytial virus (RSV) from cells found in the bronchial lavage (BL), alveolar lavage (AL) or bronchial brush biopsy. It is likely that single exposures to 0.6 and 1.5 ppm nitrogen dioxide do not alter susceptibility to cells in the airway to common respiratory viruses. Cells from the alveolar macrophage (AM) fraction do not support infection by influenza but do support infection from RSV, albeit minimally.

Although there was no change in susceptibility to infection, there was a significant effect on lactate dehydrogenase (LDH) release following RSV release with a trend towards LDH release in influenza due to prior exposure to nitrogen dioxide. This was seen even in the absence of a direct effect on the viability of the cells. The magnitude of effect varied with nitrogen dioxide concentration.

Nitrogen dioxide exposure had an effect on lymphocyte recovery from both blood and BL fluid. In blood, nitrogen dioxide exposure resulted in a decrease of lymphocytes, while in BL fluid, there was an increase in lymphocytes following exposure to .6 ppm nitrogen dioxide. There was no effect on lymphocytes in AL.

There are differences between genders of the blood lymphocyte response to nitrogen dioxide. The ratio of CD4+ to CD8+ increased slightly in males in response to nitrogen dioxide but decreased in females. This may be due to differences in initial blood t cell concentration, which is consistent with previously published work. There was a marginal gender difference in response to nitrogen dioxide in terms of percentage of CD16+ cells in blood. CD25 and HLA-DR exhibited no nitrogen dioxide dependent changes. Lymphocytes in AL fluid revealed increases in the percentage of CD4+ cells post nitrogen dioxide exposure. No other changes in lymphocyte subsets were seen in AL fluid. BL fluid could not be measured for lymphocyte subsets due to insufficient fluid volume. There was an increase in polymorphonuclear leukocytes after exposure to nitrogen dioxide in the BL. This indicates a mild inflammatory response to nitrogen dioxide. Overall, there appear to be some gender differences in lymphocyte subsets expression due to nitrogen dioxide exposure and a decrease in lymphocytes in the blood due to nitrogen dioxide exposure.

There was a small but statistically significant decrease in red blood cell number and hemoglobin concentration in association with nitrogen dioxide. This change was similar for both males and females and occurred despite no change in hemoglobin content or red blood cell size. It is unclear the reason for this. This change would unlikely be clinically significant in a healthy individual but indicates there may be some effect in persons with cardiovascular compromise, children or the elderly.

This study is listed as quantitative and acceptable/nonguideline. The LOAEL is 600 ppb. There is no NOAEL for this study.

ATTACHMENT

Data Evaluation Record (DER)



Inhalation Toxicity

For

Nitrogen Dioxide

PC Code: 000596

EPA Reviewer: Jonathan Leshin, Ph.D.
RASSB, Antimicrobials Division (7510P)
Secondary Review: Tim McMahon, Toxicologist
RASSB, Antimicrobials Division (7510P)

Signature: 
Date: 10/7/14
Signature: 
Date: 10/7/14
Template version 02/06

Data Evaluation Record

STUDY TYPE: Inhalation Toxicity – [human]

PC CODE: 000596

DP BARCODE: 421314

TXR#: 1003341

TEST MATERIAL (PURITY): Nitrogen Dioxide

SYNONYMS: None

CITATION: Frampton, M.W., Boscia, J., Roberts, J.R., et al (2002) Nitrogen dioxide exposure: effects on airway and blood cells Department of Medicine, Environmental medicine and Biostatistics (University of Rochester, School of Medicine) MRID 49420001 American Journal of Physiology – Lung, Cellular and Molecular Physiology, 282: L155-165

SPONSOR: Center for Indoor Air Research and National Institutes of Health

INVESTIGATORS' EXECUTIVE SUMMARY:

In an inhalation toxicity study (MRID 49420001) nitrogen dioxide was administered to 21 people aged 18-40 years (9 females, 12 males) in an environmental chamber at concentrations of 0, (room air), 0.6 ppm or 1.5 ppm nitrogen dioxide for a total of three hours. Exercise was performed for 10 minutes out of each thirty minutes at a sufficient level to raise minute ventilation to 40 l/min. Each exposure period was separated by at least three weeks from the previous exposure period. The subjects were assessed for pulmonary function before and after each exposure. Phlebotomy and bronchoscopy with bronchoalveolar lavage and brush biopsies were performed 3.5 hours post exposure. Recovered cells were challenged with respiratory viruses *in vitro*.

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infection from RSV, albeit minimally.

Although there was no change in susceptibility to infection, there was a significant effect on lactate dehydrogenase (LDH) release following RSV release with a trend towards LDH release in influenza due to prior exposure to nitrogen dioxide. This was seen even in the absence of a direct effect on the viability of the cells. The magnitude of effect varied with nitrogen dioxide concentration.

Nitrogen dioxide exposure had an effect on lymphocyte recovery from both blood and BL fluid. In blood, nitrogen dioxide exposure resulted in a decrease of lymphocytes, while in BL fluid, there was an increase in lymphocytes following exposure to .6 ppm nitrogen dioxide. There was no effect on lymphocytes in AL.

There are differences between genders of the blood lymphocyte response to nitrogen dioxide. The ratio of CD4+ to CD8+ increased slightly in males in response to nitrogen dioxide but decreased in females. This may be due to differences in initial blood t cell concentration, which is consistent with previously published work. There was a marginal gender difference in response to nitrogen dioxide in terms of percentage of CD16+ cells in blood. CD25 and HLA-DR exhibited no nitrogen dioxide dependent changes. Lymphocytes in AL fluid revealed increases in the percentage of CD4+ cells post nitrogen dioxide exposure. No other changes in lymphocyte subsets were seen in AL fluid. BL fluid could not be measured for lymphocyte subsets due to insufficient fluid volume. There was an increase in polymorphonuclear leukocytes after exposure to nitrogen dioxide in the BL. This indicates a mild inflammatory response to nitrogen dioxide. Overall, there appear to be some gender differences in lymphocyte subsets expression due to nitrogen dioxide exposure and a decrease in lymphocytes in the blood due to nitrogen dioxide exposure.

There was a small but statistically significant decrease in red blood cell number and hemoglobin concentration in association with nitrogen dioxide. This change was similar for both males and females and occurred despite no change in hemoglobin content or red blood cell size. It is unclear the reason for this. This change would unlikely be clinically significant in a healthy individual but indicates there may be some effect in persons with cardiovascular compromise, children or the elderly.

This study is listed as quantitative and acceptable/nonguideline. The LOAEL is 600 ppb. There is no NOAEL for this study.

I. MATERIALS AND METHODS

A. MATERIALS:

1. <u>Test Material:</u>	Nitrogen dioxide
Description:	Gas
Lot/Batch #:	Unknown
Purity:	Unknown

Compound Stability: Unknown
 CAS # of TGAI: 10102-44-0

2. Vehicle and/or positive control: Room air

3. Baseline characteristics of study:

Twenty one individuals (9 females, 12 males), ages 18-40, lifetime non smokers, with normal spirometry, free of cardiac or respiratory disease and without symptoms of respiratory infection within six weeks of the study gave informed consent to participate in this study as approved by the research subjects review board of the University of Rochester. Baseline measurements were taken the day of study selection. The baseline measurements are shown in table 1. These included spirometry to determine forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1) and forced expiratory flow between 25% and 75% of FVC.

Table 1: Subject age and baseline pulmonary function

	Males (n=12)	Females (n=9)
Age, yr	26.9 ± 4.5	27.1 ± 4.1
FVC, liters	4.93 ± 0.85	3.88 ± 0.64
FVC, %predicted	97.7 ± 14.5	109.6 ± 13.7
FEV1, liters	4.08 ± 0.71	3.39 ± 0.47
FEV1, % predicted	96.5 ± 15.1	112.6 ± 13.1
FEV1/FVC, 5	83.0 ± 8.5	88.3 ± 7.8

Vales are measn ± SD

B. STUDY DESIGN:

1. Dose assignment:

Subjects underwent exposure to all three of the potential conditions. These exposures were separated by at least three weeks. Exposure was either to air, 0.6 ppm or 1.5 ppm nitrogen dioxide for three hours. The order of exposure was randomized. Pulmonary function was measured before and immediately after exposure. During the exposure, subjects exercised for at least ten minutes every thirty minutes at a level that would increase their minute volume to 40 l/min. Subjects rated their symptoms after each exposure via questionnaire. The severity of any symptoms were assessed on a scale of zero (“not present”) to five (“incapacitating”). Temperature and humidity were controlled during exposure.

2. Generation of the test atmosphere / chamber description:

Exposures were performed in a 45 m³ environmental exposure chamber. Nitrogen dioxide gas concentrations were generated by introducing nitrogen dioxide gas in a Venturi mixer with purified air from the hospital ventilation system. Gases were discharged from the room via 5

ceiling based diffusers. There were approximately 0.3 atmosphere changes per minute. Target gas levels reached 90% target within 4 minutes and concentrations at 3 and 6 feet varied by no more than 5%. Continuous measurement of background levels of nitrogen oxides, ozone, particulate matter and sulfur oxides of the intake air was performed.

3. Physiological testing:

Spirometry was performed using a pneumotachograph connected to a computer. Forced vital capacity, forced expiratory volume in 1 s and forced expiratory flow between 25 and 75% of FVC were recorded from the best of three determinations based on FEV1. Airway resistance and thoracic gas volume were measured during panting using a plethysmograph. Specific airway conductance (sGAW) was determined as the reciprocal of airway resistance corrected for gas volume. Minute ventilation was measured at rest and during exercise using an inductive plethysmograph.

4. Bronchial lavage and blood draw:

Bronchoalveolar lavage and brush biopsy was performed on each subjects 3.5 hours after exposure. Prior to the procedure, subjects were premedicated with 0.75 to 1.0 mg atropine and topical anesthesia via lidocaine spray was applied. Oxygen was administered via nasal cannula and cardiac rhythm was monitored. The lavage and biopsy were performed using a fiberoptic bronchoscope and four 50 ml aliquots of sterile normal saline were applied sequentially and immediately suctioned in the subsegmental airway of the inferior segment of lingula. The first aliquot was collected as bronchial lavage (BL) and the three subsequent aliquots were pooled as an alveolar lavage (AL). The lavage was repeated in the subsegmental airway of the right middle lobe. These samples were combined before assessment. Brush biopsies were obtained from the lower lobe subsegments using a 3 mm bronchial brush gently rubbed against the bronchial mucosa. This brush was withdrawn and agitated in 1.5 ml of sterile normal saline on ice. Up to 30 passages of the brush were performed, distributed among the subsegments of the right lower lobe. With each subsequent bronchoscopy, the brushing were obtained from the alternate lung, to minimize carryover from local inflammation of the brushed sites. Inspection of the airways indicated no residual effects from previous bronchoscopy. Cells recovered were consistently 95% epithelial cells. No mention was provided on how alveolar macrophages (AM) were isolated.

4. Cell and viral assays:

Total cell counts were performed on the BL, AL and brush biopsy samples. Viability was assessed using trypan blue. Differential counts were performed using Diff-Quick solution. A separate slide of cells from AL was stained with Mayer's hematoxylin and toluidine blue to check for mast cells. Venous blood was checked for hematocrit, hemoglobin, red blood cell indexes and total and differential leukocyte counts at Strong Memorial Hospital.

Flow cytometry was used as another method of evaluating cell differential counts and assessing changes in phenotype and expression of activation markers, for both blood and lung

lymphocytes. Lymphocytes were stained with monoclonal antibodies for CD4+, CD8+, CD16+, CD25 and HLA-DR. The lymphocyte gate was selected based on light scattering properties and lymphocyte subsets were determine as a percentage of gated cells.

Influenza A/AA/Marton/43 H1N1 and respiratory syncytial virus (RSV) were used to examine the potential for increased susceptibility to infection after exposure to nitrogen dioxide. Cells from the BL, AL, and brush biopsies were plated and assessed for viability by measuring lactate dehydrogenase in culture supernatants and by trypan blue dye exclusion. Viral propagation was measured by using methylene blue staining followed by counting of viral plaques for H1N1. For RSV virus titer was determined by using the endpoint dilution method.

6. Statistics: The study was designed as a standard, three period, cross over design with three different treatments. Statistical analysis was standard ANOVA that included both period and carryover effects in addition to an effect of treatments and gender. Each ANOVA included an examination of residuals as a check on the assumptions of normally distributed errors with constant variance. If required assumptions were not satisfied then data transformation such as the logarithm were considered. A $p < 0.05$ was required for significance. Data means shown in results include all study subjects, even though statistical outliers were excluded for the anova.

II. RESULTS

A. OBSERVATIONS :

1. Physiological Testing:

There was no statistically significant changes in minute ventilation or intake of nitrogen dioxide. This is shown in table 2. There was no change in FVC, FEV1, their ratio or sGAW in either males or females during exposure. This data was not reported. Most subjects did not experience any symptoms during any of the three exposures. Occasionally, a subjects would report mild respiratory symptoms. These symptoms were generally scored highest after exposure to 1.5 ppm nitrogen dioxide, but these differences between nitrogen dioxide and air exposure were not considered significant for any single symptom or for total symptom when analyzed by Wilcoxon analysis.

Table 2: Minute ventilation and estimated intake of nitrogen dioxide.

	Minute ventilation, l/min		Nitrogen Dioxide intake, µg		
	Rest	Exercise	Rest	Exercise	Total
0.6 ppm					
Males	7.93 ± 0.69	38.95 ± 2.63	1,262 ± 102	2,363 ± 146	3,625 ± 239
Females	7.12 ± 0.69	34.52 ± 2.27	1,070 ± 98	1,999 ± 131	3,068 ± 190
1.5 ppm					
Males	8.65 ± 0.53	40.70 ± 2.35	3,117 ± 193	5,695 ± 343	8,812 ± 358
Females	7.39 ± 1.17	35.02 ± 2.48	2,729 ± 428	4,975 ± 348	7,704 ± 598

Values are mean ± S, Intake estimate is approximation of intake of nitrogen dioxide based on the

product of concentration ($\mu\text{g/l}$) X time of exposure (min) X minute ventilation

2. Bronchial lavage and blood draw:

Figure 1 shows the effects of nitrogen dioxide on blood factors with significant decreases in hematocrit and hemoglobin in association with nitrogen dioxide exposure for both males and females. Red blood cell counts also decreased (data not shown). Hematocrit decreased 4.1% across all subjects. There were no significant changes in red blood cell indexes, including mean corpuscular volume and mean corpuscular hemoglobin. These findings were consistent with a decrease in red blood cell number but not size or content of hemoglobin.

Figure 2 shows how the differential count of blood leukocytes changes. All types of leukocytes decreased after exposure to nitrogen dioxide but only lymphocytes had a statistically significant change, with no difference based on gender.

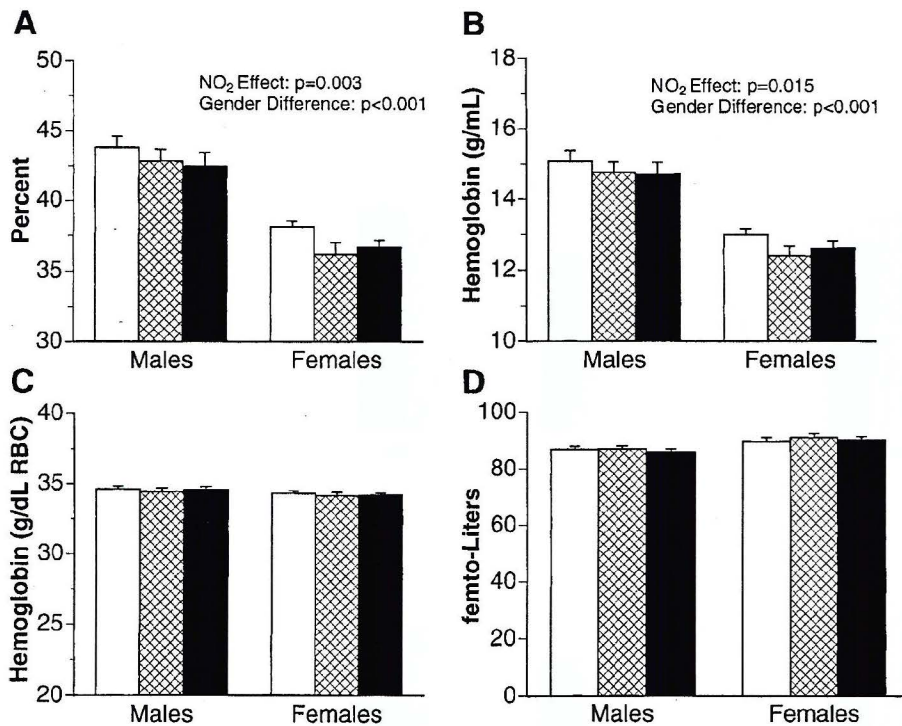
Figure 3 describes the blood lymphocyte subsets as a percentage of gated cells. As a group as a whole, there were no significant effects of nitrogen dioxide. ANOVA suggests that there are potential gender differences in the response to nitrogen dioxide for the ratio of CD4+/CD8+. This increased in relation to increasing concentration of nitrogen dioxide in males, but decreased in females ($p < 0.001$). Effects of nitrogen dioxide also differed by gender for CD16+ lymphocytes (also known as NK cells, $p = 0.047$). The percentage of CD4+ cells was increased and CD16+ cells were decreased in females relative to males, but this was independent of exposure to nitrogen dioxide. Expressed as a concentration of cells, there were gender differences in the concentration of T lymphocytes, but no evidence of effects due to nitrogen dioxide exposure.

In the lungs, there was no effect of nitrogen dioxide on the volume of the fluid recovered or the total concentration of cells in the BL or AL. ANOVA indicated significant nitrogen dioxide effects on the recovery of polymorphonuclear leukocytes (PMN) and lymphocytes in BL. These results are seen in figure 4. BL PMN showed a dose dependent increase ($p = 0.003$). Lymphocytes increased after exposure to 0.6 ppm nitrogen dioxide ($p = 0.045$) but returned closer to normal levels after exposure to 1.5 ppm nitrogen dioxide. The increase in BL lymphocytes after 0.6 ppm exposure was slightly greater in males than females ($p = 0.039$). A significant association was observed between symptom scores and PMN in BL after exposure to 1.5 ppm nitrogen dioxide ($r = 0.56$, $p = 0.008$).

Phenotypic differentiation for the lymphocytes recovered for the lung was only possible on the AL fraction due to the limited number of cells recovered from the BL. The percentage of CD4+ lymphocytes increased after nitrogen dioxide. This increase was dose related ($p = 0.034$) but not gender related. This is shown in figure 5. There were no nitrogen dioxide effects on lymphocyte differentiation of CD16, CD 25, or HLDA-DR expressors. AL CD 25+ lymphocytes were increased as a concentration of cells in males relative to females ($p = 0.007$, data not shown).

Figure 1: Hematocrit (A), hemoglobin (B), mean corpuscular hemoglobin concentration (C) and

mean corpuscular volume (D) in blood after exposure to air (open bars), 0.6 ppm nitrogen dioxide (crosshatched bars) or 1.5 ppm nitrogen dioxide (filled bars). Data are means \pm SE.



[Nitrogen Dioxide/000596]

Figure 2: Blood leukocytes after exposure to air or nitrogen dioxide. A: polymorphonuclear leukocytes, B: lymphocytes, C: monocytes, D: eosinophils. Open bars, air exposure, crosshatched bars, 0.6 ppm nitrogen dioxide, filled bars 1.5 ppm nitrogen dioxide exposure. Data are means \pm SE.

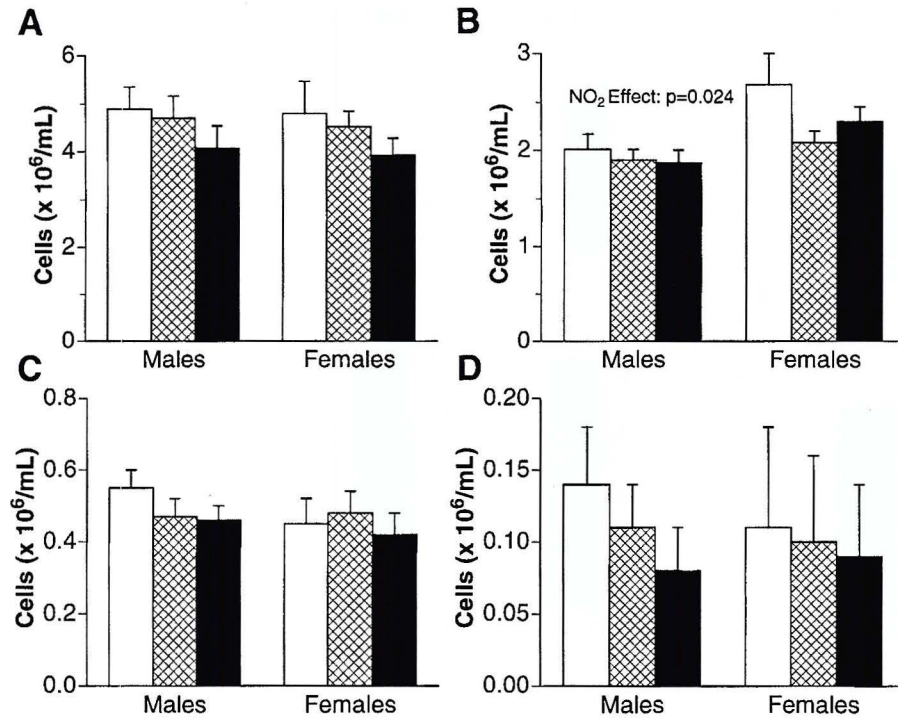
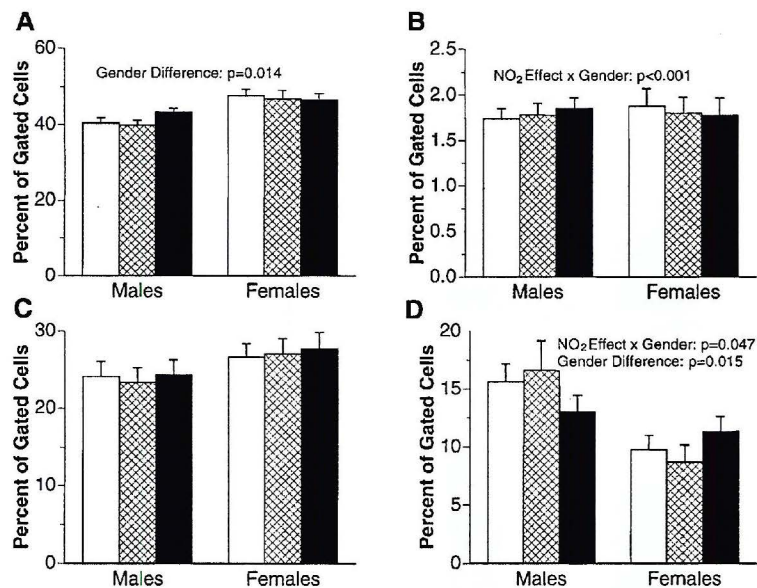


Figure 3: Blood lymphocyte subsets, determined using immunofluorescence techniques as a percentage of gated cells. A: CD4+, B: CD4+/CD8+, C: CD8+, D: CD16+. Open bars, air exposure, crosshatched bars, 0.6 ppm nitrogen dioxide, filled bars 1.5 ppm nitrogen dioxide exposure. Data are means \pm SE.



[Nitrogen Dioxide/000596]

Figure 4: Cells recovered in bronchial lavage fluid. A: Polymorphonuclear leukocytes, B: lymphocytes, C: alveolar macrophages, D: eosinophiles. Open bars, air exposure, crosshatched bars, 0.6 ppm nitrogen dioxide, filled bars 1.5 ppm nitrogen dioxide exposure. Data are means \pm SE.

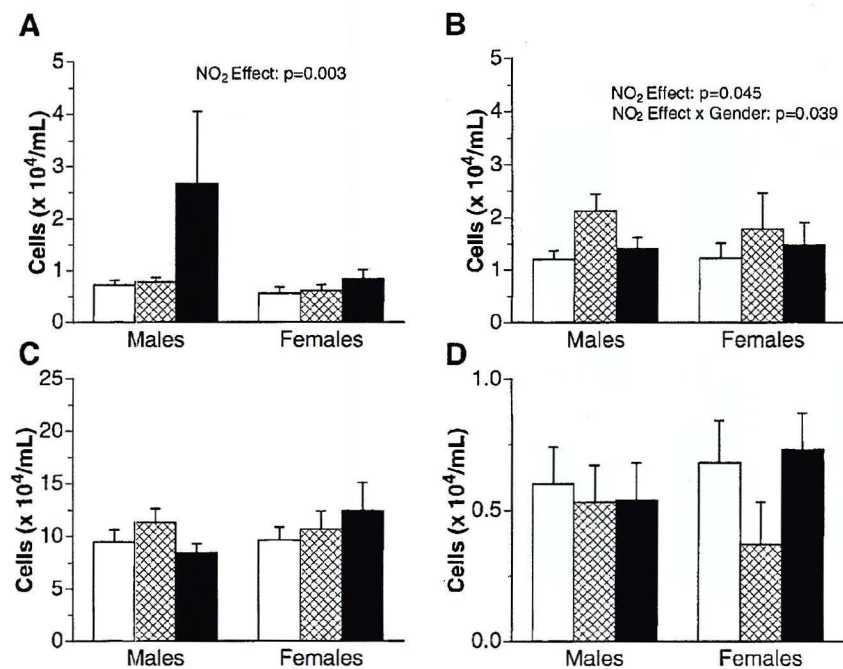
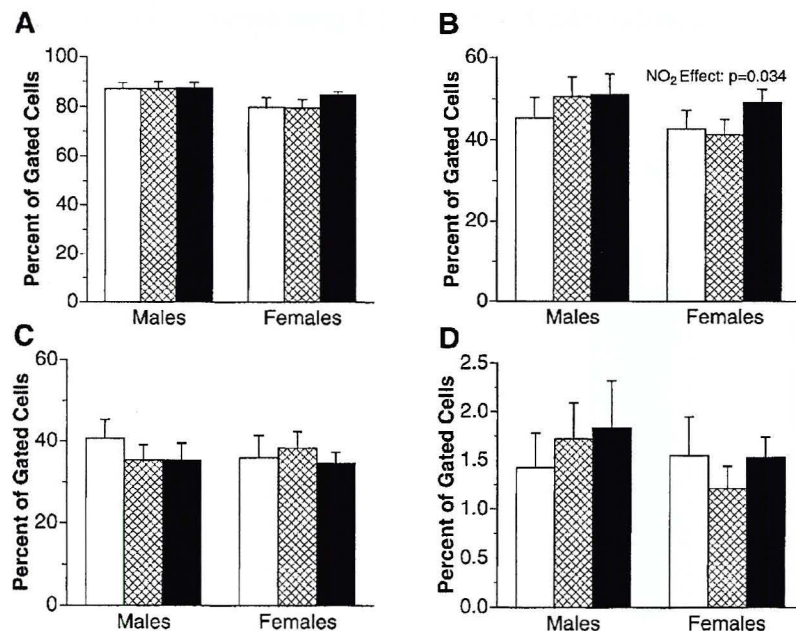


Figure 5: Lymphocyte subsets recovered in alveolar lavage fluid, determined using immunofluorescence techniques. A: CD3+, B: CD4+, C: CD8+, D: CD4+/CD8+ Open bars, air exposure, crosshatched bars, 0.6 ppm nitrogen dioxide, filled bars 1.5 ppm nitrogen dioxide exposure. Data are means \pm SE.



3. Cell and viral assays:

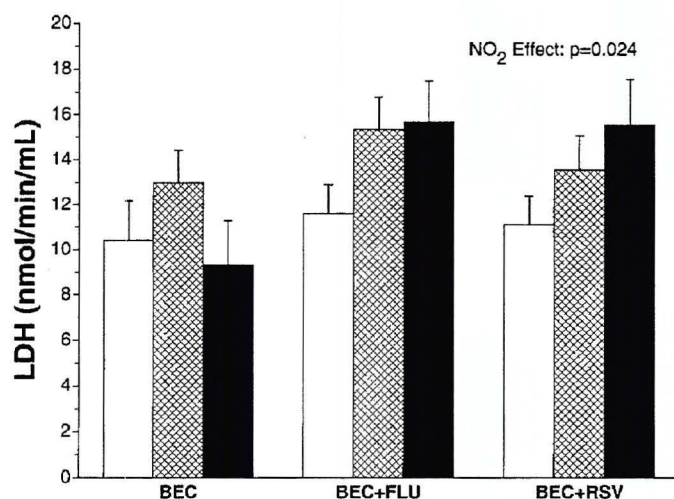
Viability of cells from the BL and AL fractions was generally greater than 90% and there was no effect due to nitrogen dioxide exposure. Brushed cells from the bronchial airway had lower viability, often <50%. This was decreased slightly, but non-significantly, in response to nitrogen dioxide exposure. Effects on viability due to prior nitrogen dioxide exposure and infection with influenza or RSV were measured on days 1 and 4 post culture. There was a nitrogen dioxide dose related 40% increase in LDH release on day 1 of culture in the presence of RSV and a similar, but nonstatistically significant, trend in cells treated with influenza. This was similar to findings on day 4, which suggests that RSV has a greater effect on cell viability post nitrogen dioxide exposure than influenza and that nitrogen dioxide does decrease cell viability post nitrogen dioxide exposure.

No nitrogen dioxide effects were found on AM or bronchial epithelial cells after infection with influenza or RSV. Influenza titers in the supernatants of both AM and epithelial cells both declines, indicating a lack of productive culture. RSV produced stable viral titers, indicating the infections was minimally productive. No nitrogen dioxide dependence was found.

Total protein and albumin were measured in both BL and AL as a way to measure epithelial

permeability. No nitrogen dioxide effects on the concentration of either total protein or albumin were found, though albumin was found to generally be lower in females compared to males in both fluids. There was no indication on how these results were determined in the methods section of the paper.

Figure 6: Release of lactate dehydrogenase in the culture supernatant fluids by bronchial epithelial cells after 1 day in culture, with or without previous 1 hour exposure to influenza or RSV. Differences were significant for bronchial epithelial cells and RSV. Open bars, air exposure, crosshatched bars, 0.6 ppm nitrogen dioxide, filled bars, 1.5 ppm nitrogen dioxide exposure. Data are means \pm SE.



III. INVESTIGATORS' DISCUSSION AND CONCLUSIONS:

The goal of the paper was to study the effects of nitrogen dioxide on airway inflammation, blood lymphocyte recruitment to the lung and the susceptibility of airways cells to infection with influenza and RSV. There was no effect of nitrogen dioxide exposure on infection with influenza or RSV from cells found in the BL, AL or bronchial brush biopsy. It is likely that single exposures to 0.6 and 1.5 ppm nitrogen dioxide do not alter susceptibility to cells in the airway to common respiratory viruses. Cells from the AM fraction do not support infection by influenza but do support infection from RSV, albeit minimally. Due to the potential for mechanical disruption of cells recovered from bronchial brushing, it is not possible to rule out the effect of nitrogen dioxide on infectability of respiratory viruses. Although there was no change in susceptibility to infection, there was a significant effect on LDH release following RSV release with a trend towards LDH release in influenza due to prior exposure to nitrogen dioxide. This was seen even in the absence of direct effect on the viability of the cells. The magnitude of effect varied with nitrogen dioxide concentration. The authors suspect that this might represent enhanced cytotoxicity post nitrogen dioxide exposure to any potential challenge. This may indicate that nitrogen dioxide exposure could increase the severity of lung tissue damage following other challenges.

Nitrogen dioxide exposure had an effect on lymphocyte recovery from both blood and BL fluid. In blood, nitrogen dioxide exposure resulted in a decrease of lymphocytes, while in BL fluid, there was an increase in lymphocytes following exposure to .6 ppm nitrogen dioxide. There was no effect on lymphocytes in AL. This suggests that nitrogen dioxide induces the recruitment of lymphocytes from blood to the airways.

There are differences between genders of the blood lymphocyte response to nitrogen dioxide. The ratio of CD4+ to CD8+ increased slightly in males in response to nitrogen dioxide but decreased in females. This may be due to differences in initial blood T cell concentration, which is consistent with previously published work. There was a marginal gender difference in response to nitrogen dioxide in terms of percentage of CD16+ cells in blood. CD25 and HLA-DR exhibited no nitrogen dioxide dependent changes. Lymphocytes in AL fluid revealed increases in the percentage of CD4+ cells post nitrogen dioxide exposure. No other changes in lymphocyte subsets were seen in AL fluid. BL fluid could not be measured for lymphocyte subsets due to insufficient fluid volume. There was an increase in polymorphonuclear leukocytes after exposure to nitrogen dioxide in the BL. This indicates a mild inflammatory response to nitrogen dioxide. Overall, there appear to be some gender differences in lymphocyte subsets expression due to nitrogen dioxide exposure and a decrease in lymphocytes in the blood due to nitrogen dioxide exposure.

There was a small but statistically significant decrease in red blood cell number and hemoglobin concentration in association with nitrogen dioxide. This change was similar for both males and females and occurred despite no change in hemoglobin content or red blood cell size. It is unclear the reason for this. This change would unlikely be clinically significant in a healthy individual but indicates there may be some effect in persons with cardiovascular compromise, children or the elderly.

In general, these effects are small and unlikely to be of clinical significance to healthy individuals. These effects occur in a largely dose dependent fashion, with more significant effects present at the 1.5 ppm dose level. However, individuals with underlying respiratory or heart conditions, the young or old or persons who are immunocompromised may be at risk at these concentrations.

This study is listed as quantitative and acceptable/nonguideline. The LOAEL is 600 ppb. There is no NOAEL for this study.



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON D.C., 20460

OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

October 9, 2014

MEMORANDUM

SUBJECT: Ethics Review of Human Toxicity Study with Nitrogen Dioxide

FROM: Kelly Sherman, Human Studies Ethics Review Officer
Office of the Director
Office of Pesticide Programs

TO: Steven Weiss, Chief
Risk Assessment Science Support Branch
Antimicrobials Division
Office of Pesticide Programs

REF: Frampton, M.W. et al. Nitrogen Dioxide Exposure: Effects on Airway and Blood Cells. *Am J Physiol Lung Cell Mol Physiol*. 282: L155-L165, 2002. (MRID 49420001)

I have reviewed the referenced human toxicity study with nitrogen dioxide. I conclude that if the study is determined to be scientifically valid and relevant, there is no regulatory barrier to EPA relying on this research in actions taken under FIFRA or §408 of FFDCA.

Summary Characteristics of the Research

In this study, nitrogen dioxide was administered to 21 people aged 18-40 years (9 females, 12 males) in an environmental chamber at concentrations of 0 (room air), 0.6 ppm or 1.5 ppm nitrogen dioxide for a total of three hours. Exercise was performed for 10 minutes out of every thirty minute period at a sufficient level to raise minute ventilation to 40 l/min. Each exposure period was separated from the previous exposure period by at least three weeks. The subjects were assessed for pulmonary function before and after each exposure. Phlebotomy and bronchoscopy with bronchoalveolar lavage and brush biopsies were performed 3.5 hours after exposure. Recovered cells were challenged with respiratory viruses *in vitro*.

To supplement the ethics information provided in the journal article, Jonathan Leshin and I (both of EPA) spoke to Dr. Mark Frampton by telephone on August 20, 2014. After that conversation, I emailed Dr. Frampton with some specific questions about the ethics of the study.

Dr. Frampton replied via email on September 11, 2014. A copy of the questions and Dr. Frampton's responses are provided as Attachment 1.

In addition, I contacted the reviewing institutional review board, the Research Subjects Review Board of the University of Rochester, and requested copies of the available records about this study. On September 25, 2014, I received copies of the protocol, consent form, and approval letter for this study via email. These documents are provided as Attachment 2.

1. Value of the Research to Society:

The objective of this study was to examine the effects of nitrogen dioxide (NO₂) exposure on airway inflammation, blood cells, and antiviral respiratory defense. The study was conducted at the University of Rochester School of Medicine. It was funded in part by the Center for Indoor Air Research and National Institutes of Health grants. The results were published in the *American Journal of Physiology, Lung Cellular and Molecular Physiology* in 2002. EPA is proposing to use the study in its risk assessment for nitrogen dioxide as medical equipment sterilant.

2. Subject Selection:

- a. **Demographics.** Twenty one subjects aged 18-40 years (9 females, 12 males) participated in the study. The subjects were lifetime non-smokers with normal spirometry, no cardiac or respiratory disease, and without symptoms of respiratory infection within 6 weeks of the study. (Frampton et al., p. L156)
- b. **Pregnancy and Nursing Status.** Dr. Frampton stated that none of the subjects were pregnant and that subjects were tested for pregnancy prior to each exposure. (Attachment 1)
- c. **Recruitment.** The subjects were recruited from the University of Rochester community. As part of the recruitment and consent process, the study coordinator described the study and initially screened interested individuals by telephone. Next, each potential subject met with one of the researchers and received a copy of the consent form to take home and review prior to deciding whether to sign the consent form and enroll. (Attachments 1, 2). No vulnerable subjects were recruited or participated in the study (Attachment 1).

3. Risks and Benefits:

- a. **Risks.** The risks to subjects are discussed briefly in the consent form. Risks were minimized by enrolling only healthy subjects and closely monitoring the subjects during and after the procedure. (Attachment 1) Medical oversight and assistance from nursing staff at the hospital also minimized potential risks to the subjects. (Attachment 2)
- b. **Benefits.** There are no benefits to the subjects. The consent form states "personal therapeutic benefit may not result from participation in this study, although knowledge gained may be beneficial to others." (Attachment 2)

- c. **Risk-Benefit Balance.** The consent form and article are silent regarding the risk-benefit balance. Dr. Frampton stated that “the potential societal benefits of increased understanding of NO₂ toxicity and effects outweighed the small risks associated with the study.” (Attachment 1)
4. **Independent Ethics Review:** The study was reviewed and approved by the University of Rochester Research Subjects Review Board. (Attachment 2)
5. **Informed Consent:** Dr. Frampton stated: “The study coordinator described the study and screened interested subjects by telephone. The study coordinator explained all aspects of the study during a visit to the medical center and provided the consent form, with a copy to take home. Consent was obtained by a study physician.” (Attachment 1) Each subject provided written informed consent before participating. (Attachment 1) A copy of the consent form is included in Attachment 2.
6. **Respect for Subjects.** Subjects were paid \$50 for completing the first exposure and bronchoscopy, another \$50 for completing the second exposure and bronchoscopy, and \$450 for the third exposure and bronchoscopy, for a total of \$550. (Attachment 2) The consent form states that subjects were free to withdraw from the study at any time. However, it appears that subjects were only partially compensated if they withdrew early. The subjects’ identities are not revealed in the study report.

Applicable Standards

Standards Applicable to the Conduct of the Research

This research was conducted in 1995-1996, before EPA’s Rule for Protection of Human Subjects of Research became effective in 2006. Thus, 40 CFR part 26 did not apply when this research was conducted.

The prevailing ethical standards for medical research conducted in 1995-1996 are articulated in the 1989 and 1996 revisions of the Declaration of Helsinki. Key elements are:

1. Research must be scientifically sound and conducted by qualified personnel
2. There must be a clear purpose and protocol, reviewed and approved by an independent ethics committee
3. The interests of science and society should never take precedence over considerations related to the well-being of the subject
4. Participants should give prior, informed, voluntary consent

Standards Applicable to EPA’s Reliance on the Research

The Agency’s rule (40 CFR part 26 subpart Q) defines standards for EPA to apply in deciding whether to rely on research—like this study—involving intentional exposure of human subjects. The applicable acceptance standards from 40 CFR part 26 subpart Q are these:

§26.1703. Except as provided in §26.1706, EPA must not rely on data from any research subject to this subpart involving intentional exposure of any human subject who is a pregnant woman (and therefore her fetus), a nursing woman, or a child.

§26.1704 EPA must not rely on data from any research subject to this section if there is clear and convincing evidence that: (1) The conduct of the research was fundamentally unethical (e.g., the research was intended to seriously harm participants or failed to obtain informed consent); or (2) The conduct of the research was deficient relative to the ethical standards prevailing at the time the research was conducted **in a way that placed participants at increased risk of harm (based on knowledge available at the time the study was conducted) or impaired their informed consent.**

EPA has submitted this study for review by the Human Studies Review Board (HSRB) because 40 CFR §26.1602 requires HSRB review for pre-2006 studies intended for EPA reliance that were conducted for the purpose of identifying or measuring a toxic effect. This study meets those criteria.

Compliance with Applicable Standards

All of the subjects in this study were adults. The nine female subjects were not pregnant. Therefore, this research did not involve intentional exposure of any pregnant or nursing female subjects or any children. EPA's reliance on the research is not prohibited by 40 CFR §26.1703.

The subjects provided written informed consent and the protocol underwent independent ethics review and approval. Based on these facts, and the absence of any information suggesting that the research was fundamentally unethical or intended to harm participants, I conclude that reliance on the research is not prohibited by 40 CFR §26.1704(1).

With regard to the study's compliance with prevailing ethical standards, I considered all available information in the published article, the information provided by Dr. Frampton, and the documents provided by the reviewing institutional review board. The potential risks of the study were explained to subjects and detailed in the consent form. All of the subjects provided written informed consent. Some of the subjects may have been employees or students at the University, so it is possible that some of the subjects may have had a subordinate relationship with one or more of the researchers. However, recruiting among employees and students was common practice at the time of this study, and there is no clear and convincing evidence to suggest undue influence or lack of fully informed, fully voluntary consent. The research was reviewed and approved by the University of Rochester Research Subjects Review Board.

Based on these facts, I conclude that the study was not deficient relative to the prevailing ethical standards in a way that placed participants at increased risk of harm or impaired their informed consent. Therefore, reliance on this study is not prohibited by 40 CFR §26.1704(2).

Conclusion

I find no barrier in law or regulation to reliance on MRID 49420001 in EPA actions taken under FIFRA or §408 of FFDCA. I defer to others for a full review of the scientific validity of this study. If it were determined not to have scientific validity, it would also not be ethically acceptable.

ATTACHMENT 1

Sherman, Kelly

From: Frampton, Mark <Mark_Frampton@URMC.Rochester.edu>
Sent: Thursday, September 11, 2014 2:37 PM
To: Sherman, Kelly
Cc: Little, Erika
Subject: RE: Questions re Frampton et al. (2002)

Kelly,

Below are the answers to most of your questions. We are pulling the study records out of archive to get you a copy of the protocol and consent form, but can't say how long that will take.

1. Were any of the 9 female subjects pregnant or nursing during the study? **No.** Were the female subjects tested for pregnancy at the beginning of the study? **Yes.** Were they tested for pregnancy during the study, which lasted 12+ weeks? **Subjects were tested prior to each exposure session.**
2. How and from what population were the subjects recruited? If subjects were recruited from a potentially vulnerable population, were there procedures in place to minimize coercion or undue influence? **Subjects were recruited from the University community and local population. No vulnerable populations were included.**
3. Did all subjects complete the informed consent form before the research began? **Yes.**
4. What were subject told about the research prior to signing the consent form? **The study was described in detail and all questions were answered.**
5. Could you describe the informed consent process? **The study coordinator described the study and screened interested subjects by telephone. The study coordinator explained all aspects of the study during a visit to the medical center and provided the consent form, with a copy to take home. Consent was obtained by a study physician.**
6. What risks to subjects were identified by the researchers and how were those risks minimized? **This was all part of the study protocol. The primary risks were related to fiberoptic bronchoscopy, and included adverse effects of medications and the procedure itself. Risks were minimized by studying only healthy subjects, minimizing the amount of medication used, and closely monitoring the subjects during and after the procedure.**
7. How were risks to subjects weighed against the benefits to the subjects and society? **The potential societal benefits of increased understanding of NO2 toxicity and effects outweighed the small risks associated with the study.**
8. Were there procedures in place to protect the privacy of the subjects? **Yes. Identifying information was kept separate from study data, under lock and key.**
9. Were subjects compensated for their participation? If so, how much were they paid? **Yes. I don't remember the exact amount, but it was based on the time spent in the study. It was likely in the range of \$1,000-\$1,200.**

Mark W. Frampton MD
Professor of Medicine & Environmental Medicine
Pulmonary & Critical Care
University of Rochester Medical Center
601 Elmwood Ave., Box 692
Rochester, NY 14642-8692
585-275-4861
mark_frampton@urmc.rochester.edu

From: Sherman, Kelly [mailto:Sherman, Kelly@urmc.rochester.edu]

ATTACHMENT 2

APPROVAL OF RESEARCH

Date: 09/07/95
RSRB# 1905

Principal Investigator: Utell, Mark, M.D.

Project Title: Effects of Air Pollution on Breathing Assessed by Lung Lavage

This board has reviewed the information you have submitted regarding the above application and has given REAPPROVAL*.

Additional Remarks:

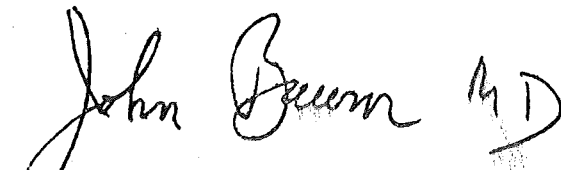
Modifications in the above proposal as approved by this Board should be submitted for additional evaluation. This includes changes in the subjects of study, the means of obtaining consent, or in the risk to the subjects.

Consent forms with patient/subject signatures must be kept in the principal investigator's study file at all times. (Records must be retained for a minimum of (3) years after a project is completed.)

If a drug is to be used in the study, the drug should be stored in the Pharmacy for dispensing and inventory control. Any adverse reaction to biologicals, drugs, radioisotopes or medical devices must be reported to the Research Subjects Review Board for evaluation. (This is in addition to any other agency to whom you would report adverse reactions.) Adverse reactions involving any substance used in this study as reported by other investigators should also be reported to this Board.

A PROGRESS REPORT WILL BE REQUIRED AT LEAST ANNUALLY.

The Department of Health and Human Services has approved the University of Rochester for five (5) years thru 11/30/98 (Multiple Project Assurance #M1357-01) in accordance with the Federal Regulations of March 8, 1983 and its subparts B, C & D for the Protection of Human Subjects.



John Baum, M.D.
Chairman

Valerie Bartlett
Executive Director

Complete Board listing on reverse side.

* (The approval will be withdrawn if at any time the conditions and requirements of the RSRB are not met.)
November 9, 1994



THE UNIVERSITY OF ROCHESTER

MEDICAL CENTER

SCHOOL OF MEDICINE AND DENTISTRY • SCHOOL OF NURSING
STRONG MEMORIAL HOSPITAL

RSRB # 1905h

601 ELMWOOD AVENUE
ROCHESTER, NEW YORK 14642
AREA CODE 716

CONSENT FORM

Title: **Effects of Air Pollution on Breathing Assessed by Lung Lavage in Healthy Humans: Nitrogen Dioxide**

Investigators: Mark W. Frampton, M.D.
Assistant Professor of Medicine

Mark J. Utell, M.D.
Professor of Medicine and Toxicology

Paul E. Morrow, Ph.D.
Emeritus Professor of Toxicology

Norbert J. Roberts, Jr., M.D.
Associate Professor of Medicine

Paul C. Levy, M.D.
Assistant Professor of Medicine



Introduction:

You are being asked to participate in a study to evaluate the effects of various common forms of air pollution on the lung.

It is important that you read and understand the following general statements that apply to all participants in this study: (a) participation is entirely voluntary, (b) personal therapeutic benefit may not result from participation in this study, although knowledge gained may be beneficial to others, and (c) withdrawal from this study may be accomplished at any time without prejudice or jeopardy to your health care.

The nature of the study, the risks, inconveniences, discomforts, and other important information about the study are discussed below. Please feel free to ask any questions that may arise as you review this material or at any point in the study, should you decide to participate.

Title: Effects of Air Pollution on Breathing Assessed by Lung Lavage in Healthy Humans: Nitrogen Dioxide

This study is designed to investigate the effects of exposure to the pollutant gas nitrogen dioxide (NO₂) on the cellular and biochemical make-up of the lung. The highest concentration of the pollutant that will be used for the exposures is 1.5 ppm, a concentration that has been observed in homes using gas cooking. These investigations are needed to help define any potential health consequences of exposure, to determine if some individuals are more susceptible to the effects of NO₂ than others, and to help establish guidelines for acceptable limits of exposure.

For this study, participants will participate in three separate exposures, at least 3 weeks apart, to clean air, NO₂ at 0.60 ppm, and NO₂ at 1.5 ppm. Bronchoalveolar lavage and bronchial brush biopsy will be performed approximately 3 hours after each exposure. Cells and fluid obtained from the procedures will be analyzed in the laboratory.

Consent:

Upon admission to this study, I will receive a physical examination and review of my medical history, and breathing tests will be performed. I will exercise on a stationary bicycle to determine an appropriate level of exercise.

On a separate day, I will undergo a 3-hour exposure to either clean, filtered air or one of two low concentrations of the pollutant gas nitrogen dioxide. Exposures to similar levels of this pollutant have been performed safely in normal volunteers many times in the past. There is always the possibility that the exposure could be irritating to the airways and cause coughing or shortness of breath. These possibilities are very unlikely. The exposure will be done in an environmental chamber in the Clinical Research Center at Strong Memorial Hospital. This is a room-sized facility and I will be free to be active within the confines of the chamber. I will have a 3-hour exposure to air or one of the pollutant gases, and for 10 minutes of every half hour (two 10-minute periods each hour) I will be required to exercise on the stationary bicycle. The investigators will monitor my breathing during these exercise periods. Breathing tests will be performed before and after the exposure.

Approximately 3 hours following the exposure, a procedure called bronchoscopy with bronchoalveolar lavage and bronchial brush biopsy will be performed to collect fluid and cells from my lung. This procedure has been explained to me in detail by one of the investigators, and I understand that this same procedure has been performed on many normal volunteers from this and other institutions without serious side effects. It has also been explained to me that this is an established procedure used by pulmonary physicians to evaluate certain lung diseases. It is important that on the day of the procedure I will have refrained from eating or drinking for at least 6 hours before the procedure. The bronchoscopy will be performed in the Pulmonary Unit at Strong Memorial Hospital by Dr. Frampton or by a senior Pulmonary Fellow and Dr. Frampton, or one of the other full-time faculty members of the Pulmonary Unit.

RS
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8/19/94

Title: Effects of Air Pollution on Breathing Assessed by Lung Lavage in Healthy Humans: Nitrogen Dioxide

I understand that the procedure requires the following: (a) having a needle placed into a vein in my arm and a blood sample withdrawn, (b) receiving 1 mg atropine through the intravenous line which will cause my heart rate to speed up and my mouth to become dry. I understand that the atropine will counteract reflexes that may tend to lower my heart rate during bronchoscopy and make my lungs temporarily (1 hour) produce less mucus. It also involves (c) being attached to an EKG monitor that will monitor my heart rate and rhythm during the procedure, (d) being given supplemental oxygen, and (e) having my nose, throat, vocal cords, trachea, and bronchi anesthetized (numbed up) with a mist of Lidocaine, a widely used local anesthetic. The application of this spray may make me cough and have a gagging sensation until the medicine takes affect. Also, I will have the bronchoscope, a small flexible tube with a light on it, passed through my mouth and into my lung where, after being properly positioned, segments of my lungs will be washed repeatedly with 50 ml (approximately 1 1/2 ounces) of a solution of salt and water that will be immediately suctioned back to collect the lung fluid and cells. Following the lavage, cells lining the airway will be sampled through the bronchoscope using a small brush. The procedure itself generally takes less than one hour to complete and after approximately another 30 minutes of observation following the procedure I will be free to leave the hospital until the next study day.

Fiberoptic bronchoscopy with lavage and bronchial brush biopsy is a routine, widely-used procedure that is safely performed on a daily basis to evaluate lung problems in patients. There are, however, risks attendant to its performance. These have been explained to me by one of the investigators and are: (1) possible spasm of my voice box making it difficult for me to breathe; (2) possible spasm of the breathing tubes in my lungs causing me to wheeze and be short of breath; (3) lowered amounts of oxygen in my blood for a short time that is usually not noticeable; (4) irregular heart beat; (5) very infrequent side effects from the local anesthetic, Lidocaine, which has been reported to cause sleepiness, nervousness, twitching, and even seizures in high doses; (6) possible cough for a short time following the procedure which may be productive of small amounts of blood, and (7) a small blood clot or bruise with some pain may be left at the site of the intravenous line. The procedure will be terminated at any sign of problems or at my own request at any time, for any reason.

At intervals of at least 3 weeks, I will return for the other two exposures to either filtered air or nitrogen dioxide. All exposures will be 3 hours in duration with the same degree of exercise. Two of the exposures will be to nitrogen dioxide and one to filtered air; I will not know which exposure I will be getting.

Bronchoscopy and bronchoalveolar lavage will be performed after each of the other two exposures. The procedure will be identical to the first one previously described. As before, I will have nothing by mouth for at least 6 hours before the procedure, and no food or liquid intake will be permitted until after the lavage.

I understand that although this form enumerates several potential though unlikely risks of this study, it is not comprehensive in that it does not include every possible complication both foreseen and unforeseen. I also understand that standard

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Title: Effects of Air Pollution on Breathing Assessed by Lung Lavage in Healthy Humans: Nitrogen Dioxide

methods of medical care will be administered if I have any problem. Strong Memorial Hospital will provide medical care for essential acute, emergency medical treatment for physical injuries incurred that the University determines to be a result of my direct participation in the research. Compensation for injury is not available from Strong Memorial Hospital.

An honorarium will be paid to me after completion of each phase of the study, according to the following schedule:

First exposure and bronchoscopy:	\$50
Second exposure and bronchoscopy:	\$50
Third exposure and bronchoscopy:	\$450
Total:	\$550

All medical records and personal study data are available to each study participant or to any physician they choose. No information identified by name will be released from the study without specific consent from the participant.

By signing this consent form, I agree that I have had sufficient opportunity to discuss the research study, to ask questions about risks and benefits of the study, and that I feel competent to decide whether to participate in it. Furthermore, I understand my consent may be withdrawn at any time, for any reason, and I may gain release from the study without prejudice and without jeopardy to my future medical care. If I feel I have an injury, I should contact the investigator.

ROSE
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1/2/93
8/19/94

Title: Effects of Air Pollution on Breathing Assessed by Lung Lavage in Healthy Humans: Nitrogen Dioxide

Volunteer's Signature

Date

Ann Bauman
Auditor Witness's Signature

2/10/95
Date

I have fully explained possible complications to the volunteer and the nature of the benefits and risks involved.

Mark W. Franz
Investigator's Signature

2/10/95
Date

Ann Bauman
Auditor Witness's Signature

2/10/95
Date

(This witness's signature attests that the above information was provided to the volunteer.)

5/5/92 B
R
2/21/93 J
8/19/94 J

CRC Protocol Report

Effects of Air Pollution on Breathing Assessed by Lung Lavage in Healthy Humans (CRC #240)

Investigators: Mark W. Frampton, M.D. (Principal Investigator)
Norbert J. Roberts, Jr., M.D.
Mark J. Utell, M.D.
Paul Morrow, Ph.D.

SPECIFIC AIMS

Nitrogen dioxide (NO₂), an important indoor air pollutant, has been linked epidemiologically with increased frequency of respiratory illness and infection, particularly in children. Since respiratory infections in humans are predominantly viral, there is a need to examine effects of NO₂ exposure on host defense against viral infections in humans.

Following are the aims of this project:

1. Quantitate the effects of *in vivo* NO₂ exposure on the susceptibility of human bronchial epithelial cells and alveolar cells to infection with respiratory viruses.
2. Determine the mechanism(s) by which *in vivo* NO₂ exposure enhances the retention of infectious virus by alveolar lavage cells in humans.
3. Determine the effects of NO₂ exposure on lymphocyte subset distribution and activation in bronchoalveolar lavage fluid and peripheral blood.

BACKGROUND AND SIGNIFICANCE

Nitrogen dioxide is a product of combustion and is classified as a criteria pollutant by the Environmental Protection Agency. Although NO₂ was originally considered exclusively an outdoor pollutant, it is now recognized that NO₂ levels indoors often exceed those outdoors (Samet and Spengler, 1991). Unvented combustion sources in the home, including natural gas cooking and heating and kerosene heaters, have been shown to produce indoor NO₂ levels in excess of 0.60 ppm (Speizer et al., 1980) with peaks as high as 2.0 ppm (Leaderer et al., 1984). The most important potential health effect of NO₂ exposure is increased susceptibility to respiratory infection, and it is this issue that is being explored in the current protocol.

PROGRESS REPORT

We have utilized bronchoalveolar lavage (BAL) to explore the effects of NO₂ exposure on host defense in humans since this gas penetrates to the distal airways and may exert its most important effects on the terminal airways and alveolar region of the lung. The technique of BAL allows the enumeration of inflammatory cells in the distal airways and alveolar space, measurement of protein and enzyme levels in the alveolar lining fluid, and study of alveolar macrophages after exposure to air or pollutants. We are also able to sample airway epithelial cells using the technique of bronchial brushing. A tiny nylon brush is passed through the suction channel of the bronchoscope and gently rubbed against the bronchial mucosa. Using this technique, a sufficient number of superficial bronchial epithelial cells can be obtained for further study.

In our initial studies, exposure to 0.60 ppm NO₂ for 3 hours impaired the ability of alveolar macrophages, obtained by bronchoalveolar lavage 3 1/2 hours after exposure, to inactivate influenza virus *in vitro* (Frampton et al., 1989). We then examined the effect of exposure to continuous 1.5 ppm NO₂ for 3 hours, with BAL performed 3 1/2 hours after exposure. A similar trend towards decreased inactivation of virus by alveolar macrophages was observed, with the greatest difference between air and NO₂ exposure again seen on cells harvested on the second day (36 hours) after exposure (Utell et al., 1991). We have recently completed two series of exposure studies using a higher level of NO₂, 2.0 ppm for 6 hours with intermittent exercise. In the first series, BAL was performed 18 hours after exposure, and in a separate series BAL was performed 1 hour after exposure. In contrast to our observations at lower levels of exposure, in which there was no evidence of an inflammatory response, in these latest studies we observed a small increase in PMN in association with NO₂ at both time points (Frampton et al., 1992b). We again saw a slight trend towards decreased inactivation of influenza virus by alveolar macrophages in these latest studies. Based on these data, we have hypothesized that exposure to NO₂, at levels below those which induce an airway inflammatory response, alters the interaction between BAL cells and influenza virus, resulting in greater retention of infectious virus in association with cells retrieved by BAL.

We also sought to determine whether NO₂ exposure altered the proportion of lymphocyte subsets in the lung or peripheral blood. Although no effect on BAL lymphocytes was found, we observed a small but significant decrease in the proportion of CD8+ T-lymphocytes in peripheral blood 18 hours after exposure to NO₂ (air, 29.0±1.7%; NO₂, 27.3±1.4%, p=0.01). Similarly, a small but significant decrease in T-lymphocytes bearing neither CD4 nor CD8 antigens was observed in association with NO₂ exposure. Since CD8+ lymphocytes include "cytotoxic" lymphocytes important in controlling viral infections, these observations may have implications in understanding mechanisms of NO₂ effects on host defense.

Our future studies will determine the mechanisms by which NO₂ exposure enhances the retention of infectious virus by alveolar lavage cells, will further examine the effects of NO₂ exposure on lymphocyte subset distributions, and will examine exposure-response relationships.

EXPERIMENTAL DESIGN AND METHODS

To provide sufficient statistical power and to examine exposure-response relationships, 24 healthy subjects (12 males and 12 females) will be exposed to filtered air and to two levels of NO₂, 0.60 and 1.50 ppm, with each exposure separated by at least three weeks. Studies will be conducted in a randomized, double-blind fashion. Bronchoalveolar lavage and bronchial brush biopsies will be performed 3 1/2 hours after each exposure. Bronchial epithelial cells obtained by bronchial brushing, and alveolar cells obtained by bronchoalveolar lavage, will be challenged with influenza virus and respiratory syncytial virus *in vitro*. Flow cytometry and two-color immunofluorescence will be used to examine lymphocyte phenotype and activation in both alveolar lavage fluid and peripheral blood. In addition, BAL fluid and conditioned medium from alveolar cells maintained in culture will be examined for effects on virus infectivity and content of key pro-inflammatory cytokines (interleukin-1, interleukin-6, and tumor necrosis factor). The principal statistical approach will be analysis of variance. The study of 24 subjects provides a reasonable margin to assure adequate statistical power, and allows a balanced design (4 subjects in each of 6 possible orderings).

SUBJECTS

Subjects will be 12 males and 12 females, all healthy life-time nonsmokers between the ages of 18 and 40, enrolled from the University environment, with absence of symptoms of upper respiratory infection for at least six weeks prior to the study. Approval has been obtained from the Research Subjects Review Board, and a copy of the consent form is attached. Minority subjects will be included in the study; at least four subjects will be African-American.

JUSTIFICATION FOR USE OF GCRC RESOURCES

The environmental chamber used for the pollutant exposure in these studies is located in the Clinical Research Center. These exposures cannot be done elsewhere. In addition, nursing support from the CRC assists in monitoring subjects during exposure and further ensures subject safety.

ESTIMATED NUMBER OF OUTPATIENT VISITS

Twenty-four patients each will undergo 4 outpatient visits: a screening visit, two exposures to NO₂ and one exposure to air (control). Allowing for the small number of subjects who may not qualify based on screening criteria, or who withdraw from the study, we expect approximately 100 to 110 outpatient visits will occur over a period of three years.

OTHER GCRC RESOURCES

Computer support.

PROTOCOL

Attached is the protocol to be followed on each exposure day. During the first year of the study we expect to complete the study of 6 subjects, which will entail at least 18 exposures followed by bronchoscopy.

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CRC Protocol #240

Screening Day: Questionnaire and informed consent, history and physical exam, baseline PFTs

Criteria: 18-40 years, nonsmoker (no tobacco use x 3 years, life-time less than 1 pack year), no respiratory infection within 6 weeks of study

Exposure: Air, 0.6 ppm NO₂ or 1.5 ppm NO₂

07:30 - 0:800 Baseline pulmonary function tests, resting minute ventilation

08:00 Begin exposure

08:20 - 08:30 Exercise 1 with minute ventilation

08:50 - 09:00 Exercise 2 with minute ventilation

09:30 - 09:40 Exercise 3 with minute ventilation

10:10 - 10:20 Exercise 4 with minute ventilation

10:40 - 10:50 Exercise 5 with minute ventilation

11:00 End exposure
Pulmonary function tests
Questionnaire

14:00 Prepare for bronchoscopy
phlebotomy
intravenous line
premedication with atropine and lidocaine

14:30 Bronchoscopy with BAL, bronchial brushing

15:00 Observation period

15:30 End of study for subject

**GCRC Annual Report
January 3, 1996**

1. Effects of Air Pollution on Breathing Assessed by Lung Lavage in Healthy Humans (CRC #240)

Nitrogen dioxide (NO₂), an outdoor and indoor air pollutant, has been linked epidemiologically with increased frequency of respiratory illness and infection. Human clinical studies from our laboratory and others suggest that exposure to NO₂ may alter the interaction between the alveolar macrophages and influenza viruses, and may alter lymphocyte subsets in the lung and/or blood. Our study is quantitating the effects of NO₂ exposure *in vivo* on the susceptibility of bronchial epithelial cells and alveolar cells to infection with influenza virus and respiratory syncytial virus (RSV). In addition, mechanisms are being explored by which NO₂ exposure enhances the retention of infectious virus by lavage cells in humans. Finally, NO₂ effects on lymphocyte subset distribution and activation in bronchoalveolar lavage and peripheral blood are being examined.

To provide sufficient statistical power and to examine exposure-response relationships, 24 healthy subjects (12 males and 12 females) will be exposed for 3 hours to filtered air and to two levels of NO₂, 0.60 and 1.50 ppm, each separated by least three weeks. Bronchoalveolar lavage and bronchial brush biopsies are being performed 3 1/2 hours after each exposure. Airway and alveolar cells are being challenged with influenza virus and respiratory syncytial virus *in vitro*, and lymphocyte phenotype is being determined by using two-color immunofluorescence and flow cytometry.

A total of 29 subjects have been recruited for these studies thus far. Six subjects dropped out of the study for reasons unrelated to NO₂ exposure. Eighteen subjects have completed all three exposures of the study. An additional five subjects are in various stages of completion; an outbreak of a respiratory virus in the community necessitated a delay for several subjects. We are in the process of recruiting the additional subject needed to complete a total of 24. We expect to complete exposures by the end of March, 1996. Remaining virus, cytokine, and biochemical determinations will then be completed so that the exposure codes can be broken and data analysis initiated.

2. Effects of Repeated Exposures to Ozone in Healthy and Asthmatic Subjects (CRC #385)

Data from our laboratory suggest that underlying airway inflammation associated with cigarette smoking alters cellular responses to ozone exposure. For example, although smokers are less responsive to ozone than non-smokers in terms of changes in lung function, cells obtained by bronchoalveolar lavage following exposure to 0.22 ppm ozone release increased quantities of superoxide anion, compared with non-smokers. Individuals with asthma also have airway inflammation, and therefore may be at increased risk from repeated exposures to ozone. We have investigated the effects of single 4-hour exposures to 0.22 ppm ozone on the airways of asthmatic subjects. Bronchoalveolar lavage was performed 18 hours after exposure to ozone and to filtered air. FEV₁ decreased $13.4 \pm 4.3\%$ after ozone exposure, and increased $6.4 \pm 2.5\%$ after air exposure. PMN recovered in the bronchial fraction of BAL after ozone exposure increased nearly 9-fold ($p < 0.01$), eosinophils increased more than 2-fold ($p = 0.08$), and lymphocytes increased 1.4-fold ($p = 0.03$) compared with air exposure. Lymphocyte expression of activation markers increased following ozone exposure (CD25⁺: $20.2 \pm 3.1\%$ after air, $25.0 \pm 4.1\%$ after ozone, $p = 0.03$; HLADR⁺: $49.3 \pm 2.8\%$ after air, $54.9 \pm 3.3\%$ after ozone, $p = 0.02$). The majority of T cells were CD45RO⁺, and the number further increased after ozone exposure ($80.9 \pm 2.9\%$ after air, $86.1 \pm 1.7\%$ after ozone, $p = 0.06$). The percentage of CD4⁺ or CD8⁺ T cells did not change following ozone exposure.

We plan to extend these studies to determine the effects of four sequential daily ozone exposures on the airways of subjects with mild asthma. Data obtained from these studies will help to determine whether repeated exposures to ozone enhance airway inflammation and injury in healthy and asthmatic subjects.

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