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 intraterine 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-
NITROGEN DIOXIDE EFFECTS

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 Rich
ters (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 maintaining 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-fl levels within the chamber varied by no more than 5% of the mean. Continuous monitoring of the residual background levels (ppb) of NOx, ozone, particulate matter, and SO₂ 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 (Respirlog 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 subseg-
ments 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 subseg-
ments 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 passes of the brush were performed, distributed among the subsegments of the right lower lobe. With each subsequent bronchoscopy, brush-
ings 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 subse-
quent 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 $5 \times 10^4$ 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 fluoro-chrome-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 $-70\degree C$ 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$\degree$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 $-70\degree$C until use.

Aliquots ($2 \times 10^6$) 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$\degree$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$\degree$C in a 5% CO$_2$ atmosphere for 4 days; supernatant fluids were removed each 24 h and stored at $-70\degree$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 ($1 \times 10^6$) 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 $\mu$g/ml streptomycin, and 2.5 $\mu$g/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$\degree$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 agarase 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 $\mu$g/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$\degree$C in 5% CO$_2$ to allow the virus to absorb to the cells, 0.8 ml of medium was added to each well. Plates were then incubated at 37$\degree$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.
Table 1. Subject age and baseline pulmonary function

<table>
<thead>
<tr>
<th></th>
<th>Males (n = 12)</th>
<th>Females (n = 9)</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>26.9 ± 4.5</td>
<td>27.1 ± 4.1</td>
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<tr>
<td>FVC, liters</td>
<td>4.93 ± 0.85</td>
<td>3.88 ± 0.64</td>
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<tr>
<td>FVC, %predicted</td>
<td>97.7 ± 14.5</td>
<td>109.6 ± 13.7</td>
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<tr>
<td>FEV₁, liters</td>
<td>4.08 ± 0.71</td>
<td>3.39 ± 0.47</td>
</tr>
<tr>
<td>FEV₁, %predicted</td>
<td>96.5 ± 15.1</td>
<td>112.6 ± 13.1</td>
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<tr>
<td>FEV₁/FVC, %</td>
<td>83.0 ± 8.5</td>
<td>88.3 ± 7.8</td>
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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. Ve and estimated intake of NO₂

<table>
<thead>
<tr>
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<th>Rest</th>
<th>Exercise</th>
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<tr>
<td>0.60 ppm</td>
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</tr>
<tr>
<td>Males</td>
<td>7.93 ± 0.69</td>
<td>38.95 ± 2.63</td>
</tr>
<tr>
<td>Females</td>
<td>7.12 ± 0.69</td>
<td>34.52 ± 2.27</td>
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<tr>
<td></td>
<td>1,262 ± 102</td>
<td>2,363 ± 146</td>
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<tr>
<td>Intake Estimate, µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,070 ± 98</td>
<td>1,999 ± 131</td>
</tr>
<tr>
<td>Total</td>
<td>3,625 ± 239</td>
<td>3,068 ± 190</td>
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<table>
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<tr>
<th></th>
<th>Rest</th>
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<tr>
<td>1.5 ppm</td>
<td></td>
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<tr>
<td>Males</td>
<td>8.65 ± 0.53</td>
<td>40.70 ± 2.35</td>
</tr>
<tr>
<td>Females</td>
<td>7.39 ± 1.17</td>
<td>35.02 ± 2.48</td>
</tr>
<tr>
<td></td>
<td>3,117 ± 193</td>
<td>5,695 ± 343</td>
</tr>
<tr>
<td>Intake Estimate, µg</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>2,729 ± 428</td>
<td>4,975 ± 348</td>
</tr>
<tr>
<td>Total</td>
<td>7,812 ± 358</td>
<td>7,704 ± 598</td>
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Values are means ± SE. Ve, minute ventilation. Intake estimate is approximation of intake of NO₂ based on the product of concentration (µg/l) × time of exposure (min) × Ve (l/min).

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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. 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.

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.
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),
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-
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 cytoxicity 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 significant 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**

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

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.

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