CONTRACT NO. 93-317
FINAL REPORT
AUGUST 1997

The Effects of Serial-Day Exposure to Nitrogen Dioxide on Airway and Blood Leukocytes and Lymphocyte Sub-Sets

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY
AIR RESOURCES BOARD
Research Division

The Effects of Serial-Day Exposure to Nitrogen Dioxide on Airway and Blood Leukocytes and Lymphocyte Sub-Sets

Final Report
Contract No. 93-317

Prepared for:

California Air Resources Board
Research Division
2020 L Street
Sacramento, CA 95814

Prepared by:

The Lung Biology Center and Department of Medicine
University of California
San Francisco, CA 94143

John R. Balmes, MD, Principal Investigator
David J. Erle MD, Co-Investigator
Colin Solomon, Ph.D.
Dorothy L. Christian
Lisa L. Chen MD
Barbara S. Welch
Michael T. Kleinman, Ph.D.
Elizabeth Dunham

August 1997

For more information about the ARB's Research Division,
its research and activities, please visit our Web site:

http://www.arb.ca.gov/rd/rd.htm

ABSTRACT

Nitrogen dioxide (NO_2) is a free radical and oxidant gas present in both indoor and outdoor air. Inhalation of NO_2 could cause airway inflammation, and also decrease local or systemic immune function by changing the distribution of lymphocyte subsets, or inhibiting lymphocyte activation. This experiment was designed to test the hypothesis that exposure to NO_2 would: 1) increase leukocytes in bronchoalveolar lavage (BAL); 2) change the distribution of lymphocyte sub-sets and inhibit lymphocyte activation in BAL and peripheral blood (PB); and 3) increase proteins in BAL. Within a counter-balanced, repeated-measures design, fifteen healthy volunteers ($\bar{x} \pm$ SD: age = 29.2 ± 4.8 yr; FEV_1 = 4.38 ± 0.8 l) were exposed to filtered air (FA) or 2.0 ppm NO_2 for 4 h per day, alternating 30 min periods of rest and exercise, for 3 consecutive days . Bronchoscopy was performed 18 h following each exposure set, and PB was drawn immediately pre-exposure, and pre-bronchoscopy. Flow cytometry was used to enumerate B cells (CD19+), T-helper cells (CD3+CD4+), T-suppressor cells (CD3+CD8+), and NK cells (CD3-CD16+CD56+), and to examine the expression of the activation markers CD25, and CD69, on T and NK cells in both BAL and PB. In the Bronchial Fraction [(BFx), defined as the first 15 ml of BAL collected], there was an increase in percent neutrophils following NO_2 exposure compared to FA [median (range): 10.6 (2.2-43.6)% vs 5.3 (1.0-14.3)%; P=0.005]. There were no between-condition differences for BAL or BFx in total leukocyte count, or percent of macrophages, eosinophils, lymphocytes, or neutrophils. In BAL, there was a significant decrease in the percent of T-helper cells following NO_2 exposure compared to FA [median (range): 55.9 (29.2-73.9)% vs 61.6 (42.0-85.9)%; P=0.022]. For BAL and PB, there were no other significant, between- or within-exposure differences in any lymphocyte subsets, or in lymphocyte sub-set activation. In the BFx, there was a decrease in total protein following NO_2 exposure compared to FA [median (range): 0.117 (0.054-0.225) μg ml vs 0.171 (0.068-0.538) μg ml; P=0.004]. There were no between condition differences in the BAL total protein, or the BFx or BAL lactate dehydrogenase. We conclude that serial-day exposure to 2.0 ppm NO_2 results in bronchial inflammation and a minimal change in a BAL lymphocyte sub-set, with no changes in PB lymphocyte subsets or activation.

ACKNOWLEDGEMENTS

The investigators gratefully acknowledge Mr. Ronald E. Ferrando for assistance in conducting the exposure and bronchoscopy procedures, and Mr. Robert Montanti for assistance in the bronchoscopy procedures.

DISCLAIMER

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

TABLE OF CONTENTS

Abstract	1
Acknowledgements	2
Disclaimer	2
Summary	4
Conclusions and Significance	4
Recommendations	4
Body of Report	5
References	14
Publications/Presentations	18
Tables and Figures	19

SUMMARY

The results of this experiment demonstrate that exposure to 2.0 ppm NO_2 for 4 h per day on 3 consecutive days produces an increase in neutrophils in the BFx and a decrease in CD3+CD4+ cells in the BAL.

CONCLUSIONS AND SIGNIFICANCE

The results of this experiment indicate that serial-day ambient level NO_2 exposure can induce mild bronchial airway inflammation and a small change in cellular immune function. Taken in conjunction with other data, the NO_2-induced increase in bronchial neutrophils appears to be a function of the total dose of NO_2, as opposed to the exposure format. The NO_2-induced increase in neutrophils in the BFx was not found in the BAL, suggesting a regional difference in NO_2 exposure (delivery), absorbance, clearance, or antioxidant defenses. The single-day-exposure-induced inflammatory response found in other experiments appears to be attenuated following multiple-day exposures. This difference may be due to an increase in the clearance of NO_2 from the airway epithelium or upregulation of antioxidant defenses. The decrease in CD3+CD4+ cells in the BAL has not previously been reported. However, taken together, the results of controlled human NO_2 exposure experiments show no consistent effect on a specific lymphocyte sub-set or group of subsets over a range of NO_2 exposure conditions. The finding of no change in any of the leukocyte differential cell counts or lymphocyte sub-sets in the peripheral blood suggests that it is unlikely that this level of NO_2 exposure has any systemic effect on immune function. Although chronic exposure (multiple years) or higher concentrations (> 2.0 ppm), of NO_2 could conceivably produce larger changes in airway inflammation and cellular immune function, the minor changes observed in this experiment are unlikely to produce the increase in NO_2-associated respiratory tract illness observed in epidemiological studies.

RECOMMENDATIONS

The results of this experiment, taken in conjunction with other data, suggest that exposure to NO_2 at even a relatively high ambient concentration results in minimal changes in cellular immune function in the lungs and has no effect on systemic cellular immunity. However, as the NO_2-induced cellular changes we observed are not specific and have not been consistently observed in other experiments, further research is required to elucidate the source of this variability. Also, further research is required to determine if the minimal changes produced in this experiment are of any functional significance.

INTRODUCTION

Nitrogen dioxide (NO_2) is a free-radical and oxidant gas. Atmospheric NO_2 is derived directly from combustion, but primarily indirectly from the partial oxidation of nitric oxide in the presence of oxygen (1). The primary sources of NO_2 in indoor air are gas-burning equipment and tobacco smoke (2, 3), and in outdoor air are combustion engine and fossil-fuel burning emissions (4). The concentration of NO_2 in indoor and outdoor air can have ranges of 0.5 - 0.6 ppm (45 min period), and 0.007 - 0.065 ppm (48 h mean), respectively (2, 5). In healthy humans, the fraction of inhaled NO_2 retained during normal resting respiration is 81-90%, and during maximal ventilation is 91-92% (6). Nitrogen dioxide has low water solubility (0.037 ml ml H_2O @ 35°C; 7), therefore a large fraction of inhaled NO_2 is deposited in the peripheral airways. Inhaled NO_2 will be delivered initially and locally to the airway lining fluid and epithelium. As inhaled NO_2 can subsequently be delivered via tissue absorption and transfer across the blood:gas interface to the blood, systemic effects are possible.

Some epidemiological data suggest that exposure to ambient NO_2 is associated with an increased incidence of respiratory symptoms and illness (8-12). However, other studies have indicated no association between NO_2 exposure and respiratory symptoms or illness (13-15). A meta-analysis of the data from studies, involving children (age = 5 - 12 yr), indicated that a long-term increase in NO_2 exposure of 0.015 ppm was associated with a 20% increase in the odds of respiratory illness (16). No such analysis is available for data on adults. The methodological constraints inherent in epidemiological studies of health effects of air pollution (17) do not allow direct association of NO_2 exposure and respiratory illness.

Due to its cytotoxicity inhalation of NO_2 could produce airway inflammation and change the leukocyte distribution in the airway lining fluid and in the blood. In controlled human exposures, involving healthy individuals, single exposures to NO_2 change the leukocyte distribution in BAL (18-20). Exposure to 4.0 ppm NO_2 for 20 min increased the number of lymphocytes and decreased the number of macrophages at 4, 8, and 24 h post-exposure, and increased the number of mast cells at 4 and 24 h post-exposure, compared to pre-exposure (18). Similarly, exposure for 20 min to 4.0, and 5.5 ppm NO_2 increased the number of lymphocytes and decreased the number of macrophages, and exposure to 2.25, 4.0, and 5.5 ppm increased the number of mast cells all measured at 24 post-exposure compared to pre-exposure (19). In both of these experiments, there was no change in the total cell count or the number of neutrophils, eosinophils, or epithelial cells. Using the BFx, in addition to the BAL, exposure to 3.5 ppm NO_2 for 20 min increased the number, but not the differential percent, of neutrophils in the BFx and lymphocytes in the BAL 24 h post-exposure, compared to pre-exposure (20). There was no change in the total cell count, or in the number of macrophages or mast cells in the BFx and BAL (20). Also, in peripheral blood, there was no change in the total cell count, or in the number of lymphocytes, neutrophils, or monocytes (20). Contrary to these changes in leukocyte distribution following NO_2 exposure, exposure to 3.0 or 4.0 ppm NO_2 for 3 h did no change the percent of lymphocytes, macrophages, or neutrophils in BAL, 3.5-4.0 h post-exposure (21). However, this experiment used a cross sectional, as opposed to a repeated-measures (18-20) design.

Multiple-day exposures (22-24) to NO_2 do not appear to change the distribution of leukocytes to the same degree as single-day exposures (18-20). Exposure to 0.60 ppm NO_2 for 2 h on 4 out of 6 days did not change the percent of macrophages, neutrophils, or lymphocytes in the BAL 2 h post-exposure, compared to pre-exposure (22). Similarly, exposure to 1.5 ppm NO_2 for 20 min every alternate day for 6 exposures did not change the percent of macrophages, neutrophils, lymphocytes, or mast cells in either the BFx, or BAL 24 h post-exposure compared to pre-exposure (23). However, exposure to 4.0 ppm for 20 min every alternate day for 6 exposures decreased the percent of mast cells in the BFx 24 h post-exposure as compared to pre-exposure, but there was no change in macrophages, neutrophils, or lymphocytes in the BFx or BAL (24). The relative absence of changes in leukocyte distribution following multiple-day exposure, as compared to single-day, suggest an adaptation response to NO_2.

A NO_2-induced decrease in lymphocyte function or viability could decrease cellular immune function. Cellular immunity involves subpopulations of lymphocytes; T cells, B cells, and natural killer (NK) cells. The subpopulations of lymphocytes are differentiated on the basis of surface-antigen cluster designations (CD). T cells express CD3, and can be defined as inducer, which express CD4, or suppressor, which express CD8. B cells express CD19 and CD20, and natural killer cells express CD16 and/or CD56. T cells become activated when the cell contacts the specific antigen recognized by the T-cell receptor. When activated, T cells express specific surface proteins at higher levels. CD25, an intermediate time-frame activation maker, is expressed on a substantial proportion of T cells within 24-48 h following activation (25), and is also expressed in several pulmonary immunological disorders including asthma (26). CD69, an early (within hours) time-frame activation marker, is expressed following *in vitro* T cell activation (25), decreased on BAL fluid lymphocytes from tobacco smokers (27), and induced by NK cell activation (25). Quantification of the expression of these activation markers allows assessment of the effects of NO_2 on specific lymphocyte immune function.

The effect of exposure to NO_2 on immune function has been assessed via determination of host defense to induced respiratory tract infection. Following exposure to 0.6 ppm NO_2 for 3 h, there was no significant change in the *in vitro* inactivation of the influenza A virus by macrophages collected from BAL (28). Similarly, following exposure to 1.0-3.0 ppm NO_2, for 2 h per day for 3 days, there was no significant increase in the rate of infection from influenza A virus in the subjects exposed to NO_2 (29). These results suggest that susceptibility to influenza A infection may not be increased following NO_2 exposure. However, given the variety of pathogens capable of infecting the respiratory tract, it is important to determine the effect of NO_2 on cellular immune function in general, as relevant to a spectrum of inhaled pathogens.

Single- and multiple-day exposures to NO_2 have changed the distribution of lymphocyte sub-sets in BAL and peripheral blood (20, 22-24). A single exposure to 3.5 ppm NO_2 for 20 min increased the number of CD3+ and CD15+CD56+ in BAL 24 h post-exposure. There was no change in the number of CD19+, or the percent of CD4+, CD8+ cells, or the CD4+/CD8+ ratio (20). For multiple-day exposures, exposure to 0.60 ppm NO_2 for 2 h on 4 of 6 days increased the percent of CD16+ cells in BAL 2 h post-exposure, but there was no change in total lymphocytes or the percent of

CD3+, CD4+, CD8+, or CD20+ cells (22). There was no change in any of the blood lymphocyte sub-sets (22). Exposure to 1.5 ppm NO_2 on alternate days for 6 exposures decreased the percent of CD8+ cells and increased the CD4+/CD8+ ratio, but there was no change in the number of total T-cells or CD20+ cells, or in the percent of CD4+ cells, in BAL 24 h post-exposure (23). Similarly, exposure to 4.0 ppm NO_2 for 20 min on alternative days for 6 exposures, decreased the percent of CD8+ cells and the number of CD20+ and CD16+CD56+ cells, and increased the CD84+/CD8+ ratio, but there was no change in the number of total T-cells, or the percent of CD4+ cells (24).

Due to free-radical activity and oxidation, NO_2 can affect cell function and viability by damaging lipids, proteins, and other biomolecules (30). In combination with water, NO_2 can form nitric and nitrous acids, which are also potentially harmful to cell function. Exposure to 3.0 or 4.0 ppm NO_2 for 3 h decreased the functional capacity of alpha-1-protease inhibitor by 45% in bronchoalveolar lavage (BAL), suggesting increased susceptibility to elastase-induced protolytic damage (21). However, at lower concentrations of 1.5-2.0 ppm, for 3 h, the function of alpha-1-protease inhibitor was not changed (31). Exposure to 0.06 ppm NO_2 decreased alpha-2-macroglobulin in BAL 3.5 h post-exposure, but concentrations of 0.60 - 2.0 ppm NO_2 do not change albumin, total protein or lactate dehydrogenase (LDH), in BAL 3.5 - 18 h post-exposure (32). In another experiment, exposure to 3.5 ppm NO_2 for 20 min did not change total protein in BFx or BAL 24 h post-exposure (20). The *in vivo* oxidant capabilities of inhaled NO_2 have been indicated, following exposure to 2.0 ppm NO_2 for 4 h, by a decrease in uric and ascorbic acids, in both the bronchial fraction (BFx) and BAL, although reduced glutathione was increased in BFx (33). Exposure to 2.3 ppm NO_2 for 5 h decreased alveolar permeability, assessed using Tc-DTPA clearance, at 11 h, but not 1 or 18 h post-exposure (34). Additionally, NO_2 decreases ciliary beat frequency, and therefore may decrease the clearance of pathogens from the respiratory tract (35).

In healthy subjects, exposure to NO_2 over a range of concentrations and durations results in low degree and variable responses in spirometric pulmonary function, and airway resistance and reactivity (Reviewed; 36-38). Exposure to NO_2 results in no change, or a decrease in spirometric pulmonary function (36-38). Similarly, NO_2 exposure produces no change, an increase, or an decrease in airway resistance (36-38). Also, NO_2 exposure has resulted in no change, or an increase in airway reactivity (36-38). The variability across the results of these experiments appears to be a function of the concentration or total dose of NO_2 to which the subjects were exposed, higher concentrations/doses tending to produce the changes.

Cumulatively, the reviewed data indicate that there is a decreased inflammatory response following multiple-day, compared to single-day NO_2 exposure, and that there is some degree of re-distribution of lymphocyte sub-sets following NO_2 exposure. There also appears to be a difference in the bronchial and bronchoalveolar regional cellular responses to inhaled NO_2. Whether lymphocytes are activated following NO_2 exposure is unknown. This experiment was designed to test the hypothesis that serial-day exposure to 2.0 ppm NO_2 would change the distribution of lymphocyte sub-sets and decrease lymphocyte activation.

METHOD

Design

This experiment used a repeated-measures, counter-balanced, single-blind design. Each subject was exposed, during separate exposure periods, to a control condition of filtered air (FA), and to 2.0 ppm NO_2 in filtered air. Subjects were unaware of the exposure condition. The exposure periods were for 4 h per day for three consecutive days. Total and differential cell counts, lymphocyte sub-set cell counts and activation, and total protein and lactate dehydrogenize (LDH) were measured in BAL post-exposure. Lymphocyte sub-set cell counts and activation were also measured in peripheral blood pre- and post-exposure. Spirometric pulmonary function, and airway resistance and responsiveness were measured throughout the exposure periods. A minimum of three weeks separated the two exposure condition periods.

Subjects

The subject group consisted of 15 healthy volunteers described by physical characteristics, spirometric pulmonary function, and airway resistance and responsiveness (Table 1.). All subjects completed a medical history questionnaire, denied a history of pulmonary dysfunction, were non-smokers, and had no respiratory-tract illness in the three weeks preceding, or during, the experiment. Subjects abstained from strenuous exercise for 12 h, and caffeine for 4 h prior to each session. Each subject was informed of the risks of the experiment and provided informed consent prior to participation. The procedures for this experiment were approved by the institutional Committee on Human Research.

Equipment and Measurements

Pulmonary Function and Airway Resistance and Responsiveness

Forced expired spirometry for the determination of forced vital capacity (FVC) and forced expired volume in one second (FEV_1), was performed using a dry-rolling-seal spirometer (Anderson Instruments; Spirotech Division, Model No. S400), using standardized procedures (39). Specific airway resistance (SRaw) was calculated as the product of total airway resistance and thoracic gas volume, which were both measured using a constant-volume body plethysmograph (Warren E. Collins). Airway responsiveness was determined by the FEV_1 response to inhalation of nebulized (Devilbiss, Model No. 646) phosphate-buffered saline and doubling doses (0.26, 1.53, 4.09, 10.48 µmol) of methacholine delivered via a dosimeter (Rosenthal) at the rate of 0.01 ml breath (40). For initial characterization, the airway responsiveness value was taken as either: the cumulative dose of methacholine that produced a 20% decrease in FEV_1 from baseline (log-linear interpolation); or when FEV_1 did not decrease by 20%, as the maximum dose of 10.48 µmol. For within and between condition comparisons the dose-response curve was used (41).

Exposure Chamber and Atmospheric Monitoring

The exposure sessions were performed in a custom built steel and glass exposure chamber (Nor-Lake Inc., Model No. W00327-3R), which is 2.5 m x 2.5 m x 2.4 m in size, and has an average airflow rate of 300 ft^3 min. The chamber air supply was sourced from ambient air which was filtered by passing through purifying (Purafil Model No. 6239) and high efficiency particle (Aeropac Model No.53

HEPA 95) filters. The filtered air was dehumidified by passing through a drier (Cargocaire Engineering Corp. Model No. HC-575). Subsequently, the air temperature was decreased with a chilled-water coil, and the humidity increased with steam (Nortec Model No. NHMC-050), to obtain the pre-set temperature (20.0 °C) and relative humidity (50%) conditions in the chamber. The temperature and relative humidity inside the chamber were monitored (3-min intervals) and controlled throughout the exposures (Table 2: Johnson Controls, Model No. DSC 8500). For the NO_2 exposures, NO_2 was supplied from gas cylinders containing 250-500 ppm NO_2 in air (Liquid Carbonic Corp.) which was piped through teflon tubing directly into the intake duct of the chamber. The NO_2 concentration in the chamber was monitored continuously (30-s intervals) throughout the exposures (Table 2.), chamber air being piped through teflon tubing directly to a chemiluminescent NO_x analyzer (Monitor Labs Inc. Model No. 8840). The NO_x analyser was calibrated using a dynacalibrator (VICI Mentronics Model No. 340) using ultrapure air (NO_x < 0.001 ppm) and NO_2 of known concentration.

Exercise and Pulmonary Ventilation

During each exposure exercise was utilized to induce mouth breathing and to increase minute ventilation. The exercise consisted of either walking/running on a treadmill (Precor, Model No. M9.1) or pedalling a cycle-ergometer (Monark, Model No. 90818e). The exercise intensity was adjusted for each subject to a target expired minute ventilation (V_E) of 25 l min m^2 body surface area. During exercise, V_E was calculated from tidal volume and breathing frequency measured using a pneumotachograph (Fleish, Model No. 3) at the 10- and 20-min interval of each 30 min exercise period. There was no significant difference in the mean V_E between the FA and NO_2 conditions ($\bar{x}$ ± SD; 47.8 ± 5.6 l min^{-1} vs 47.5 ± 4.8 l min^{-1}).

Bronchoalveolar Lavage

The bronchoscopies were performed in a dedicated room at San Francisco General Hospital. Vital signs were measured pre- and post-bronchoscopy. Throughout the procedure, intravenous access was maintained, and arterial hemoglobin:oxygen (O_2) percent saturation (Ohmeda, Model No. Biox 3700) and the electrocardiograph were monitored. Atropine, to decrease airway secretions, and if required, midazolam, to maintain subject comfort, were administered intravenously. The posterior pharynx was anesthetized using a 4% lidocaine gargle, a 1% lidocaine spray, and 4% lidocaine-soaked, cotton-tipped plegets applied to the mucosa over the ninth cranial nerve. Supplemental O_2 was delivered via a nasal canula at 2 l min. The bronchoscope (Pentax, Model No. FB 18x), tipped with lidocaine jelly, was introduced through the mouth, and the larynx and airways were anesthetized using 1% lodocaine solution as required. The bronchoscope was initially directed and wedged into the right middle lobe orifice (3 x 50 ml lavage), and subsequently the lingula (1 x 50 ml lavage). The purpose of the lingula lavage was to obtain alveolar macrophages for functional assays which will be the subject of another report. The lavages were performed using 0.9% saline heated to 37°C. The first 15 ml of lavage fluid returned was designated the bronchial fraction (BFx). The PB collections were performed immediately before each exposure period and pre-bronchoscopy.

Cell Enumeration
All lavage samples were immediately placed on ice following collection. A 1 ml aliquot was removed for the total and differential cell counts. For the BAL and BFx fluids, total cell counts were performed using a hemacytometer (Fisher Scientific, Cat. No. 0267110), and differential cell counts were performed from cytospins subsequently stained (Diff-Quik, Baxter, Cat. No. B4132-1). Total and differential (400 cells) cell counts were performed in duplicate by two counters. Total and differential cell counts in PB were performed by a certified commercial laboratory (Metwest).

In both the BAL and PB, flow cytometry was used to enumerate lymphocyte sub-sets on the basis of CD as follows; B cells (CD19+), T-helper cells (CD3+CD4+), T-suppressor cells (CD3+CD8+), and NK cells (CD3-CD16+CD56+); and to examine the expression of the activation markers CD25 and CD69 on T and NK cells. For both BAL and PB, 100 ul aliquots of cells were stained with saturating concentrations of anti-human leukocyte monoclinal antibodies for 30 min in the dark at 4°C. The cells were then washed using PBS, lysed, fixed using 500 ml of 1% paraformaldehyde for 5 min at 21°C, washed again using PBS, and stored in the dark at 4°C. The flow cytometer (FACScan, Model No. 440) uses a dual-laser and is equiped with an argon laser (Becton-Dickson). Fluorescein and phycoerythrin are excited by argon laser light at 488 nm and emissions are detected by the instrument in 520 nm and 578 nm channels, respectively. Data collection (cell counting; FACSort) and analyses (cell grouping; CELLQuest) were performed using commercial software (Becton-Dickson).

Biochemical Assays
The BFx and BAL fluids were centrifuged to remove cells and debris, 1 ml aliquots of each fluid was removed for the LDH measurement, and the remaining supernatants frozen at -80°C. The LDH assay was performed within 30 min of the lavage using a commercial kit (Sigma, No. 228-10) and a spectrophotometer (Beckman, Model No. DU 65). Total protein was measured in the previously frozen aliquots using the modified Lowry assay (42).

Symptoms
A symptom questionnaire was administered immediately following each exposure. The questionnaire consisted of a 5-point rating scale (0 = not noticeable to 4 = severe) for the symptoms; anxiety, chest discomfort or chest tightness, chest pain on deep inspiration, cough, eye irritation, headache, nasal irritation, nausea, phlegm or sputum production, shortness of breath, throat irritation, and weezing.

Procedure
Each subject completed nine laboratory sessions. Session one was for screening and characterization, subjects performing pulmonary function and pre-exposure airway responsiveness tests. Sessions two-four were the first exposure condition (FA or NO_2) period, and session five was post-exposure airway responsiveness the and the bronchoscopy which was performed 18 h ± 1 h following the end of session four. Following the minimum three week inter-condition period, sessions six-nine were performed as for the first condition period, using the other exposure (FA or NO_2). Each exposure condition period consisted of 4-h exposures performed at the same time on three consecutive days. For each of the exposure sessions, pulmonary function tests were performed immediately pre- and

post-exposure, and peak flow was monitored during the exposure at the end of each exercise period. During the exposure sessions, subjects alternated 30-min periods of rest and exercise. Following the bronchoscopy sessions, subjects were transferred to the General Clinical Research Center, where they were observed and released when recovered.

Statistical Analyses

The majority of the cell count, and biochemical data were not normally distributed. Therefore, all BFx, BAL, and PB, across-condition (FA vs NO_2) comparisons were performed using the Wilcoxon Signed Rank Test. For the PB data, the pre- to post-exposure change within each condition was used to compare across the two conditions. For the FVC and FEV1, and SRaw data, the pre- to post-exposure change was used to compare across the two conditions for each of the three days using the Wilcoxon Signed Rank Test, and the NO_2 minus FA difference was used to compare across three days using the Friedman ANOVA. Airway responsiveness data was compared within and across conditions using the Wilcoxon Signed Rank Test. Statistical significance was set at $P < 0.05$.

RESULTS

Bronchoalveolar Lavage

The volumes of lavage fluid returned for the BFx and BAL in the FA and NO_2 conditions were: median (range): BFx: 15.0 (13.5 - 15.3) ml and 15.0 (11.2 - 15.6) ml; BAL: 75.0 (47.4 - 85.2) ml and 63.6 (35.4 - 87.6) ml, respectively.

Total and Differential Leukocytes

All differential cell counts for the BFx, BAL, and PB were expressed as a percentage of total leukocytes. In both the BFx and BAL, there was no significant difference in the total leukocyte count between the FA and NO_2 conditions (Table 3.). In the BFx, there was a significant increase in the percent of neutrophils after NO_2 exposure, as compared to FA (Table 3.). In the BFx and BAL, there were no other significant differences in total leukocytes, or the percent of neutrophils, macrophages, eosinophils, or lymphocytes between the two conditions (Table 3.). In the PB, there was no significant difference in the total cells or total leukocytes, or percent of macrophages, neutrophils, lymphocytes, or eosinophils between the FA and NO_2 conditions (Table 4.).

Lymphocyte Sub-Sets and Activation

In the BAL, there was a significant decrease in the percent of T-helper (CD3+CD4+) cells after NO_2 exposure, as compared to FA (Table 5.). In the BAL, there were no significant differences in B cells (CD19+), T-suppressor cells (CD3+CD8+), and NK cells (CD3-CD16+CD56+), between the two conditions (Table 5). There were also no significant differences for either of the two cell activation markers CD25 and CD69 on T and NK cells between the two conditions (Table 3.). In the PB, there were no significant differences in any of the lymphocyte subsets, or in activation of T and NK cells, between the FA and NO_2 conditions (Table 6.).

Total Protein and Lactate Dehydrogenase
In the BFx, there was a significant decrease in total protein after NO_2 exposure, as compared to FA (Table 7.). In the BAL, there was no difference in total protein between the exposure conditions. In both the BFx and BAL, there was no difference in lactate dehydrogenase between the two conditions (Table 7.).

Spirometric Pulmonary Function and Airway Resistance and Reactivity
There were no within or between condition changes in FVC, FEV_1, or airway resistance (Table 8.). Also, there was no significant changes in airway reactivity [median (range) slope of dose-response function: Pre-FA = -0.57 (-43.13 - -0.02); Post-FA = -0.59 (-29.84 - 0.17); Pre-NO_2 = -0.58 (-102.56 - 0.16); Post-NO_2 = -0.55 (-32.98 - 0.13)].

Symptoms
There were no within or between condition differences in any of the symptoms scores.

DISCUSSION

The results of this experiment demonstrate that exposure to 2.0 ppm NO_2 for 4 h per day on 3 consecutive days produces an increase in neutrophils in the BFx and a decrease in CD3+CD4+ cells in the BAL. These results indicate that this level of NO_2 exposure can induce mild bronchial airway inflammation and a small change in cellular immune function.

An increase in neutrophils in the BFx has also been found following a single NO_2 exposure, although the latter increase was in the total neutrophil count (20), as opposed to the differential percent. The increase in the percent of neutrophils following NO_2 in the current experiment of 5.3% to 10.6%, is larger than the values of 2.0% to 4.0%, (no significant difference) following the single exposure (20). This difference is potentially being due to the higher total dose (concentration x time), of NO_2 delivered in the current. Following other multiple-day exposures (22-24), the number of neutrophils were not changed, but the total dose of NO_2 was lower than in the current experiment. Therefore, the NO_2-induced increase in bronchial neutrophils, appears to be a function of the total dose of NO_2, as opposed to the exposure format. The NO_2-induced increase in neutrophils in the BFx was not found in the BAL, suggesting a regional difference in NO_2 exposure (delivery), absorbance, clearance, or antioxidant defenses.

In previous experiments using single-day exposure to NO_2 increases in lymphocytes and/or decreases in macrophages have been found (18-20). There were no changes in the percent of macrophages or lymphocytes in either the BFX or BAL in the current serial-day exposure experiment. Similarly, other multiple-day exposures did not change lymphocyte or macrophage numbers (22-24). Hence, the single-day-induced inflammatory response appears to be attenuated following multiple-day exposures. This difference may be due to an increase in the clearance of NO_2 from the airway epithelium or upregulation of antioxidant defenses.

The decrease in CD3+CD4+ cells after NO_2 exposure in the BAL observed in the current experiment has not been previously been reported, although other lymphocyte sub-sets have been changed following NO_2 exposure (20, 22-24). The CD19+, CD3+CD8+, and CD3-CD16+CD56+ sub-sets were not changed in the current experiment and have been variably changed in other experiments (20, 22-24). Taken together, the results of controlled human NO_2 exposure experiments show no consistent effect on a specific lymphocyte sub-set or group of subsets over a range of NO_2 exposure conditions. Although it is not known if different lymphocyte sub-sets have different specific sensitivities or responses to NO_2 exposure, it is probable that any oxidative or free-radical effects of NO_2, would be similar across all lymphocyte sub-sets given the similarities in cell structure. The CD3+CD8+/CD3+CD4+ ratio may be an important indicating variable of cellular immune function. The decrease in CD3+CD4+ cells did not result in any significant change in the ratio, suggesting that the current NO_2 exposure did not result in a function change in cellular immunity. This is also apparent by the lack of change in activation of any of the lymphocytes sub-sets following NO_2 exposure. The finding in this experiment of only a small decrease in one cell population involved in cellular immune responses may help to explain why no NO_2-induced changes in response to experimental infection have been found in controlled human exposure experiments (28, 29). The finding of no change in any of the leukocytes or lymphocyte sub-sets in the peripheral blood indicates that the inhaled NO_2 was not being transferred to the blood in levels high enough to have any toxic effects at the cellular level. Therefore, it is unlikely that this level of NO_2 exposure will have any systemic effect.

The findings of increased neutrophils and decreased total protein in the BFx, but not in the BAL, suggest regional differences in the response to inhaled NO_2. This differential response may be due to differences in NO_2 exposure (delivery), absorbance, clearance, or antioxidant defenses. Therefore it would be informative to include analysis of lymphocytes sub-sets in the BFx, in future experiments.

In summary, exposure to NO_2 under the conditions used in the current experiment resulted in an increase in neutrophils in the BFx and a decrease in CD3+CD4+ cells in the BAL. Although chronic exposure (multiple years) or higher concentrations (> 2.0 ppm) of NO_2 could conceivably produce larger changes in airway inflammation and cellular immune function, the minor changes observed in this experiment are unlikely to produce the increase in NO2-associated respiratory tract illness observed in epidemiological studies (8-12, 16).

REFERENCES

1) Speding D.J. Air Pollution. Oxford chemistry series. Clarendon Press. 1974. pp:76-82.

2) Goldstein I.F, Lieber K., Andrews L.R., Foutrakis G., Kazembe F., Huange P., Hayes C. (1988) Acute respiratory effects of short-term exposures to nitrogen dioxide. Arch. Environ. Health 43:138-142.

3) Norman V., Keith C.H. (1965) Nitrogen oxides in tobacco smoke. Nature 205:915.

4) Office of Planning and Standards: United States Environmental Protection Agency. (1991) National air pollution emissions estimates 1940-1990. Research Triangle Park. 450/4-91/026.

5) Spengler J., Ryan P.B., Schwab M., Colome S., Wilson A.L., Billick I., Becker E. (1990) An overview of the Los Angeles personalmonitoring study. Total Exposure Assessment Methodology. Proc. EPA/Air Waste Manage. Asssoc. 66-85.

6) Wagner H.M. (1970) Absorption von NO und NO_2 in KIK- und MAK-konzentrationen bei der inhalation. [Absorption of NO and NO_2 in mic- and mic-concentrations during inhalation]. Staub. Reinhalt. Luft. 30:380.

7) Mohsenin V. (1994) Human exposure to oxides of nitrogen at ambient and supra-ambient concentrations Toxicology 89:301-312.

8) Speizer, F.E., and Ferris, B.G. (1973) Exposure to automobile exhaust. Arch. Environ Health 26:313-324.

9) Helsing, K.J., Comstock, G.W., Meyer, M.B., Tockman, M.L. (1982) Respiratory effects of household exposures to tabacco smoke and gas cooking on non-smokers. Environ. Int. 8:365-370.

10) Detels, R., Sayre, J.W., Coulson, A.H., Rokaw, S.N., Massey, F.J.Jr., Tashkin, D.P., *et al.* (1981). The UCLA population studies of chronic obstructive respiratory disease: IV. Respiratory effects of long-term exposure to photochemical oxidants, nitrogen dioxide, and sulfates on current and neversmokers. Am. Rev. Respir. Dis. 124:673-680.

11) Yokoyama, Y., Nitta, H., Maeda, K., Aoki, S. (1985) What interaction does indoor nitrogen dioxide have on the effect of the automobile exhaust. Tokai. J. Exp. Clin. Med. 4:379-384.

12) Koo, L.C., Ho, H.H-C., Ho, C-Y., Matsuki H., Shimizu, H., Mori, T. *et al.* (1990) Personal exposure to nitrogen dioxide and its association with respiratory illness in Hong Kong. Am. Rev. Respir. Dis. 141:1119-1126.

13) Cohen, C.A., Hudson, A.R. Clausen J.L., Kuelson J.H. (1972) Respiratory symptoms, spirometry, and oxidant air pollution in non-smoking adults. Am. Rev. Respir. Dis. 105:251-261.

14) Keller, M.D., Lanese, R.R., Mitchell, R.I., Cote, R.W. (1979) Respiratory illness in households using gas and electricity for cooking. Environ. Res. 19:495-515.

15) Euler, G.L., Abbey, D.E., Hodgkin, J.E., Magie, A.R. (1988) Chronic obstructive pulmonary disease symptom effects of long-term cumulative exposure to ambient levels of total oxidants and nitrogen dioxide in California Seventh-day Adventist residents. Arch. Environ. Health. 43:279-285.

16) Hasselbad V., Eddy D.M., Kotchmar D.J. (1992) Synthesis of environmental evidence: nitrogen dioxide epidemiological studies. J. Air Waste Manage. Assoc. 42:662-671.

17) Samet J.M., Utell M.J. (1990) The risk of nitrogen dioxide: what we ahve learnt from epidemiological and clinical studies.. Toxicol. Ind. Health 6:242-262.

18) Sandstrom T., Anderson M.C., Kolmodin-Hedman B., Stjernberg N., Angstrom T. (1990) Bronchoalveolar mastocytosis and lymphocytosis after nitrogen dioxide exposure in man: a time-kinetic study. Eur. Respir. J. 3:138-143.

19) Sandstrom T., M.C., Stjernberg N., Eklund A., Ledin M-C., Bjermer L., Kolmodin-Hedman B., Lindstrom K., Rosenhall L., Angstrom T. (1991) Inflammatory cell response in bronchoalveolar lavage fluid after nitrogen dioxide exposure of healthy subjects: a dose-response study. Eur. Respir. J. 3:332-339.

20) Helleeday R., Sandstrom T., Stjernberg N. (1994) Differences in bronchoalveolar cell response to nitrogen dioxide exposure between smokers and nonsmokers. Eur. Respir. J. 7:1213-1220.

21) Mohsenin V., Gee B.L. (1987) Acute effect of nitrogen dioxide exposure on the functional activity of alpha-1-protease inhibitor in bronchoalveolar lavage fluid of normal subjects. Am. Rev. Respir. Dis. 136:646-650.

22) Rubenstein I., Reiss T.F., Bigby B.G., Stites D.P., Boushey H.A. (1991) Effects of 0.60 ppm nitrogen dioxide on circulating and bronchoalveolar lavage lymphocyte phenotypes in healthy subjects. Environ. Res. 55:18-30.

23) Sandstrom T., Ledin M-C., Thomasso L., Helleeday R., Stjernberg N. (1992) Reductions in lymphocyte subpopulations after repeated exposure to 1.5 ppm nitrogen dioxide. Br. J. Ind. Med. 49:850-854.

24) Sandstrom T., Helleeday R., Stjernberg N., Bjermer L. (1992) Effects of repeated exposure to 4.0 ppm nitrogen dioxide on bronchoalveolar lymphocyte subsets and macrophages in healthy men. Eur. Respir. J. 5:1092-1096.

25) Cebrian M., Yague E., Rincon M., Lopez-Botet M., de Landazuri M.O., Sanchez-Madrid F. (1988) Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. J. Exp. Med. 168:1621-1637.

26) Walker C., Bode E., Boer L., Hansel T.T., BlaserK., Virchow J. (1992) Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. Am. Rev. Respir. Dis. 146:109-115.

27) Ancochea J., Gonzalez A., Sanchez M.J., Aspa J., Lopez-Botet M. (1993) Expression of lymphocyte activation surface antigens in bronchoalveolar lavage and peripheral blood cells from young healthy subjects. Chest 104:32-37.

28) Frampton M.W., Smeglin A.M., Roberts N.J., Jr., Finkelstein J.N., Morrow P.E., Utell M.J. (1989) Nitrogen dioxide exposure *in Vivo* and human alveolar macrophage inactivation of influenza viris *in Vitro*. Environ. Res. 48:179-192.

29) Giongs S.A.J., Kulle T.J., Bascom R., Sauder L.R., Green D.J., Hebel J.R., Clements M.L. (1989) Effect of nitrogen dioxide exposure on the susceptibility to influenza A virus infection in healthy adults. Am. Rev. Respir. Dis 139:1075-1081.

30) Menzel D.B. The role of free radicals in the toxicity of air pollutants (nitrogen oxides and ozone). In: Pryor W.A. ed. Free radicals in biology: Vol. II. New York: Academic Press. 181-202.

31) Johnson D.A., Frampton M.W., Winters S., Morrow P.E., Utell M.J. (1990) Inhalation of nitrogen dioxide fails to reduce the activity of human lung $alpha_1$-proteinase inhibitor. Am. Rev. Respir. Dis. 142:758-762.

32) Frampton M.W., Finkelstein J.N., Roberts N.J., Jr., Smeglin A.M., Morrow P.E., Utell M.J. (1989) Effects of nitrogen dioxide exposure on bronchoalveolar lavage proteins in humans. Am. J. Respir. Cell Mol. Biol. 1:499-505.

33) Kelly F.J., Blomberg A., Frew A., Holgate S.T., Sandstrom T. (1992) Antioxidant kinetics in lung lavage fluid following exposure to nitrogen dioxide. Am. J. Respir. Crit. Care Med. 154:1700-1705.

34) Rasmussen T.R., Kjaergaard S.K., Tarp U., Pederson O. (1992) Delayed effects of NO_2 exposure on alveolar permeability and glutathione peroxidase in healthy in healthy humans. Am. Rev. Respir. Dis. 146:654-659.

35) Helleeday R., Huberman D., Blomberg A., Stjernberg N., Sandstrom T., (1995) Nitrogen dioxide exposure impairs the frequency of mucociliary activity inhealthy subjects. Eur. Respir. J. 8:1664-1668.

36) Bylin, G. (1993) Controlled studies on Humans. Scand. J. Work Environ. Health 19 (Suppl. 2):37-43.

37) Sandstrom T. (1995) Respiratory effects of air pollutants: experimental studies in humans. Eur. Respir. J. 8:976-995.

38) Bascom *et al.*, (1996) Health effects of outdoor air pollution. Am. J. Respir. Crit. Care Med. 153:477-498.

39) American Thoracic Society. (1995) Standardization of spirometry 1994 update. Am. J. Respir. Crit. Care Med. 152:1107-1136.

40) Kanner R.E., Connett J.E., Altose M.D., Buist S., Lee W.W., Tashkin D.P., Wise R.A. for the Lung Health Study Research Group (1994) Gender differences in airway hyperresponsiveness in smokers with mild COPD. The lung health study. Am. J. Respir. Crit. Care Med. 150:956-961.

41) O'Connor G., Sparrow D., Taylor D., Segal M., Weiss S. (1987) Analysis of dose -response curves to methacholine. An approach suitable for population studies. Am. Rev. Respir. Dis. 136:1412-1417.

42) Markwell M.K., Hass S.M., Bieber L.L., Tolbert N.E. (1978) A moderfication of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87:206-210.

PUBLICATIONS AND PRESENTATIONS

Solomon C., Chen L.L., Christian D.L., Welch B.S., Erle D.J., Dunman E., Kleinman M.T., Balmes J.R. (1997) The effect of exposure to NO_2 on lymphocyte subsets and activation. Am. J. Respir. Crit. Care Med. 155 (4 part 2).

TABLES AND FIGURES

Table 1. Individual subjects physical, spirometric lung function and airway responsiveness characteristics.

Subj.	Gender	Age (yr)	Height (cm)	Mass (kg)	FVC (l)	FEV_1 (l)	FEV_1/FVC (%)	SR_{aw} (l x cm H_2O l s)	PD_{20} (µmol)
1	M	25	176	63.6	6.20	4.97	80	3.7	10.48
2	M	40	177	78.2	5.02	4.23	84	3.0	10.48
3	F	32	175	78.2	4.02	3.43	85	2.6	10.48
4	M	24	189	94.1	6.93	5.60	81	3.7	10.48
5	M	33	178	77.7	7.36	5.42	74	4.2	6.01
6	M	33	171	88.6	4.93	4.26	87	0.75	10.48
7	M	26	177	77.3	5.56	4.28	77	3.6	4.09
8	F	26	169	66.8	4.21	3.15	75	4.8	7.29
9	F	34	163	65.0	3.74	3.43	92	2.1	10.48
10	M	25	173	68.2	5.63	4.55	81	2.3	10.48
11	M	33	174	68.2	5.58	4.36	78	3.4	10.48
12	F	31	165	72.7	4.32	3.42	79	4.6	0.90
13	M	23	180	79.5	5.80	5.24	87	1.9	10.48
14	M	28	179	72.3	5.76	4.31	75	6.0	10.48
15	M	26	185	80.9	5.89	5.04	86	2.5	10.48
Mean ± SD		29.3 4.8	175.4 6.8	75.4 8.6	5.40 1.04	4.38 0.78	81.4 5.3	3.3 1.3	8.90 2.99

FVC, FEV_1, FEV_1/FVC%, SR_{aw}, and PD_{20} data are individual means of pre-exposure data for the FA and NO_2 conditions. PD_{20} = methacholine dose (cumulative), at which FEV_1 decreased 20%.

Table 2. Exposure Atmospheric Characteristics

	Exposure Condition	
	FA	NO_2
NO_2 (ppm)	--	1.95
±		0.14
Temperature (°C)	19.9	20.2
±	0.1	0.4
Relative Humidity (%)	50.0	56.4
±	7.3	6.6

Values are means ± SD. FA = filtered air; NO_2 = nitrogen dioxide.

Table 3. Leukocytes in bronchial fraction and bronchoalveolar lavage, post-exposure to filtered air and nitrogen dioxide.

Cell	BFx		BAL	
	FA	NO_2	FA	NO_2
Leukocytes (%)	88.8	91.7	96.3	92.3
Range	64.3 - 96.5	46.8 - 96.5	83.3 - 99.5	64.8 - 99.3
Macrophages (%)	77.1	88.7	92.2	90.2
Range	46.3 - 95.8	71.3 - 97.9	69.4 - 99.2	51.4 - 97.0
Neutrophils (%)	5.3	10.6*	2.4	3
Range	1.0 - 14.3	2.2 - 43.6	0.5 - 7.2	0.5 - 19.3
Eosinophils (%)	0.3	1.1	0.5	0.6
Range	0.0 - 20.6	0.0 - 13.1	0.0 - 20.4	0.0 - 14.3

Values are median and range. Leukocytes = percent of total cells; all others = percent of leukocytes. * = significantly different from filtered air ($P = 0.005$). BFx = bronchial fraction; BAL = bronchoalveolar lavage; FA = filtered air; NO_2 = nitrogen dioxide.

Table 4. Leukocytes in peripherial blood, pre-post exposure difference for filtered air and nitrogen dioxide.

Cell	FA	NO_2
Monocytes (%)	-1.0	-1.0
Range	-2.0 - 2.0	-2.0 - 6.0
Neutrophils (%)	1.0	2.5
Range	-10.0 - 16.0	-15.0 - 8.0
Eosinophils (%)	0.0	-0.5
Range	-2.0 - 2.0	-16.0 - 3.0

Values are median differences and range. FA = filtered air; NO_2 = nitrogen dioxide.

Table 5. Lymphocyte sub-sets and activation in bronchoalveolar lavage, post-exposure to filtered air and nitrogen dioxide.

Cell	FA	NO_2
Lymphocytes (%)	13.0	12.8
Range	3.1 - 35.1	4.3 - 36.2
CD3+ (%)	93.3	93.8
Range	84.7 - 97.3	88.5 - 97.5
CD3+CD4+ (%)	61.6	55.9*
Range	42.0 - 85.9	29.2 - 73.9
CD3+CD8+ (%)	28.9	31.8
Range	11.5 - 46.6	5.4 - 53.5
CD3+CD4+/CD3+CD8+ (Ratio)	2.3	1.7
Range	0.9 - 6.7	0.5 - 11.0
CD3-CD19+ (%)	1.3	1.3
Range	0.0 - 4.3	0.0 - 4.0
CD3-CD16+CD56+ (%)	0.3	0.2
Range	0.0 - 1.7	0.0 - 1.0
CD3+CD4+CD25+ (%)	30.6	30.7
Range	10.0 - 39.8	12.5 - 46.3
CD3+CD8+CD25+ (%)	7.5	8.9
Range	2.1 - 15.3	3.7 - 15.8
CD3-CD16+CD56+CD25+ (%)	20.8	25.6
Range	2.3 - 46.8	4.6 - 45.3
CD3+CD4+CD69+ (%)	71.5	68.5
Range	54.3 - 85.8	54.6 - 86.2
CD3+CD8+CD69+ (%)	78.9	81.5
Range	62.7 - 93.1	69.8 - 94.2
CD3-CD16+CD56+CD69+ (%)	47.2	54.0
Range	5.2 - 76.2	25.1 - 73.7

Values are median and range. * = significantly different from filtered air ($P = 0.022$). FA = filtered air; NO_2 = nitrogen dioxide.

Table 6. Lymphocyte subsets and activation in peripherial blood, pre-post exposure to filtered air and nitrogen dioxide.

Cell	FA	NO_2
Lymphocytes (%)	-1.5	0.0
Range	-14.0 - 11.0	-0.6 - 15.0
CD3+ (%)	1.6	2.4
Range	-3.2 - 11.9	-4.6 - 7.4
CD3+CD4+ (%)	3.4	2.3
Range	-12.9 - 8.4	-12.2 - 7.8
CD3+CD8+ (%)	0.0	0.5
Range	-4.7 - 13.8	-6.7 - 8.4
CD3+CD4+/CD3+CD8+ (Ratio)	0.1	0.0
Range	-1.5 - 1.1	-0.6 - 2.7
CD3-CD19+ (%)	1.1	0.4
Range	-2.7 - 7.5	-2.4 - 3.3
CD3-CD16+CD56+ (%)	-2.3	1.7
Range	-10.2 - 3.6	-6.5 - 7.4
CD3+CD4+CD25+ (%)	0.2	2.4
Range	-9.5 - 5.6	-6.0 - 6.7
CD3+CD8+CD25+ (%)	0.1	1.3
Range	-3.1 - 8.2	-4.5 - 3.9
CD3-CD16+CD56+CD25+ (%)	0.7	0.2
Range	-3.5 - 4.9	-3.0 - 8.4
CD3+CD4+CD69+ (%)	-0.6	0.7
Range	-2.6 - 2.3	-2.7 - 1.8
CD3+CD8+CD69+ (%)	-0.2	0.2
Range	-3.7 - 6.4	-5.3 - 2.9
CD3-CD16+CD56+CD69+ (%)	-0.4	-0.2
Range	-4.9 - 2.2	-6.5 - 4.8

Values are median differences and range. FA = filtered air; NO_2 = nitrogen dioxide.

Table 7. Total protein and lactate dehydrogenase in bronchial fraction and bronchoalveolar lavage, post-exposure to filtered air and nitrogen dioxide.

	BFx		BAL	
	FA	NO_2	FA	NO_2
TP (mg ml)	0.171	0.117*	0.114	0.105
Range	0.068 - 0.538	0.054 - 0.225	0.072 - 0.262	0.055 - 0.351
LDH (μ l)	14.2	9.3	8.6	6.9
Range	4.9 - 20.9	0.4 - 22.3	2.2 - 33.5	3.3 - 18.3

Values are median and range. * = significantly different from filtered air ($P = 0.004$). BFx = bronchial fraction; BAL = bronchoalveolar lavage; FA = filtered air; NO_2 = nitrogen dioxide; TP = total protein; LDH = lactate dehydrogenase.

Table 8. Forced vital capacity, forced expired volume in one second and specific airway resistence, pre-post exposure differences for filtered air and nitrogen dioxide.

	FA Day 1	2	3	NO_2 Day 1	2	3
FVC (l)	-0.04	0.03	0.03	-0.06	0.0	-0.03
Range	-0.34 - 0.23	-0.21 - 0.22	-0.22 - 0.34	-0.18 - 0.21	-0.16 - 0.21	-0.20 - 0.33
FEV_1 (l)	0.11	0.10	0.09	0.01	0.12	0.02
Range	-0.21 - 0.37	-0.08 - 0.39	-0.07 - 0.37	-0.15 - 0.38	-0.06 - 0.36	-0.19 - 0.40
SR_{aw} (l x cm H_2O l s)	-0.9	-0.7	-0.6	-1.1	-0.8	-0.5
Range	-2.4 - 1.0	-1.6 - 0.5	-1.7 - -0.2	-2.1 - 0.6	-1.3 - 0.9	-2.5 - 0.2

Values are median difference and range. FVC = forced vital capacity; FEV_1 = forced expired volume in one second; SR_{aw} = specific airway resistance; FA = filtered air; NO_2 = nitrogen dioxide.