FINAL REPORT

Effects of Nitrogen Dioxide on Airway Inflammation in Allergic Asthmatic Subjects

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Abstract

Nitrogen dioxide (NO_2) can enhance both early and late airway narrowing after inhaled antigen in allergic asthmatic subjects. We hypothesized that NO_2 may also increase airway inflammation during the late response. Nitrogen dioxide has been shown to cause airway inflammation in healthy subjects without asthma. We also hypothesized that individuals with asthma may have increased non-allergic airway inflammation after exposure to NO_2. To test these hypotheses, we designed two experiments with the following specific aims: *Experiment 1)* To determine the effect of a single exposure to NO_2 on allergen-induced airway inflammation. *Experiment Two:* To determine the effect of NO_2 exposure on non-allergic airway inflammation.

Experiment One: 15 house dust mite (HDM)-sensitive asthmatic subjects were exposed for 3 hours to filtered air or 0.4 ppm NO_2 followed immediately by inhalation of HDM allergen. Lung function was measured before and after each exposure and after allergen challenge, hourly for 6 hours. Sputum (airway lining fluid) was obtained at 6 hours and 26 hours after allergen challenge and assessed for inflammatory cells and biochemical markers of inflammation. There was no significant effect of NO_2 exposure on early or late airway narrowing after HDM allergen for the group as a whole. However, three subjects did have substantially greater early airway narrowing with HDM allergen inhalation after NO_2. A significant decrease in a type of inflammatory cell (eosinophils) was observed in sputum obtained 6 hours after NO_2, but there was no significant NO_2-related difference for any other measure of inflammation. These results suggest that in most asthmatic individuals a 3-hour exposure to a high ambient concentration of NO_2 does not increase the late airway inflammatory response to inhaled allergen. There may, however, be a subset of allergic asthmatic individuals in whom NO_2 exposure does increase early airway narrowing. Future research should be directed towards understanding the determinants of why some asthmatic individuals are more susceptible to NO_2.

Experiment Two: 10 asthmatic subjects were exposed for 3 hours to filtered air or 0.4 ppm NO_2 followed by sputum sampling. Lung function was measured before and after each exposure. Sputum was obtained at 6 hours and 26 hours after exposure and assessed for inflammatory cells and biochemical markers of inflammation. There was no significant effect of NO_2 exposure on lung function or any measures of inflammation in sputum. These results suggest that in mild asthmatic individuals a 3-hour exposure to a high ambient level of NO_2 does not cause either airway narrowing or non-allergic airway inflammation.

Executive Summary

Introduction: Nitrogen dioxide (NO_2) is a common environmental air pollutant that is primarily generated by the combustion of fossil fuels. Outdoor levels are predominantly associated with traffic density and can reach peak concentrations of 0.2-0.6 ppm, although indoor levels often exceed outdoor levels. Inhaled NO_2 is absorbed along the entire respiratory tract, with the greatest dose to airway tissue delivered to the small airways in the deep lung. Although the exact mechanism by which NO_2 reacts with tissue is not known, it is suspected to cause oxidative damage to cell membranes and inflammatory mediator release from cells in the airways. Epidemiological studies suggest that exposure to NO_2 is associated with increased risk of respiratory symptoms and lower respiratory illness. Asthmatic individuals are typically considered more susceptible to the respiratory effects of exposure to ambient NO_2 because their airways are characterized by both a tendency to narrow and inflammation. Several human studies have demonstrated that NO_2 exposure can increase early airway narrowing after inhaled antigen in allergic asthmatic subjects. Taken together, the results of these studies suggest that both the level and duration of NO_2 exposure affects the amount of airway narrowing that occurs after subsequent allergen inhalation. One group of investigators has observed increased late airway narrowing after allergen with pre-exposure to a level of NO_2 as low as 0.26 ppm and a duration of exposure as short as 30 minutes. Late phase airway narrowing occurs 4-8 hours after allergen inhalation and is thought to be due primarily to inflammation. Late phase inflammation is characterized by increased inflammatory cells (eosinophils and neutrophils) and biochemical markers in airway lining fluid (e.g., sputum). Given that NO_2 exposure has been shown to enhance the late phase response to allergen, we hypothesized that NO_2 exposure would also increase the airway inflammatory response to allergen in asthmatic individuals. To test this hypothesis, we designed a controlled exposure study that used sputum induction to sample airway lining fluid following NO_2 exposure and allergen challenge.

Objective and Specific Aims: The overall objective of this project was to investigate the effects of NO_2 exposure on airway inflammation in individuals with allergic asthma. *Specific Aim One:* To determine whether NO_2 exposure increases the airway inflammatory responses of asthmatic subjects during late-phase reactions to inhaled allergen. *Specific Aim Two:* To determine whether asthmatic subjects have significant non-allergic airway inflammation following exposure to a level of NO_2 not reported to cause lower airway inflammation in normal subjects.

Materials and Methods: This project consisted of two separate controlled human exposure experiments. *Experiment One* involved 15 asthmatic subjects who were allergic to house dust mite (HDM). *Experiment Two* involved 10 subjects with mild intermittent or mild persistent asthma who were not required to be sensitive to HDM. For both experiments, the exposure conditions were single 3-hour exposures to either filtered air (FA) or NO_2 at a concentration of 0.4 ppm. For *Experiment One*, the NO_2 exposure was followed by inhalation of HDM allergen. For *Experiment Two*, there was no inhalation of HDM following the exposures. Lung function was measured before and

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after each exposure and hourly for 6 hours after allergen challenge in *Experiment One*. Sputum was obtained at 6 hours and 26 hours after allergen challenge in *Experiment One* and at 6 hours after NO_2 exposure in *Experiment Two*. The primary outcomes measured were as follows: 1) lung function; 2) symptoms; 3) inflammatory cells in sputum samples; 4) and inflammatory protein levels in sputum samples.

Results: *Experiment One*: There was no significant effect of NO_2 exposure on lung function and symptoms compared to the FA control exposure. There was also no significant effect of NO_2 exposure on the early or late lung function response to HDM allergen for the group as a whole. However, three subjects did require a substantially lower allergen dose to cause a 20% decrease in lung function after NO_2. A significant decrease in a type of inflammatory cell (eosinophils) was observed in sputum obtained 6 hours after NO_2 exposure, but there was no significant NO_2-related difference for any other measure of inflammation. *Experiment Two:* There was no significant effect of NO_2 exposure on lung function and symptoms compared to the FA control exposure. There was also no significant effect of NO_2 on inflammatory cells or proteins in sputum samples compared to FA control.

Discussion: The results of *Experiment One* suggest that in most asthmatic individuals a 3-hour exposure to a high ambient concentration of NO_2 does not increase late phase inflammation after inhaled allergen. There was, however, a subset of allergic asthmatic individuals in whom NO_2 exposure did increase early phase airway narrowing, suggesting that there may be a subgroup of asthmatics who are more susceptible to combined NO_2 and allergen exposures. Future research should be directed towards understanding the determinants of why some asthmatic individuals are more susceptible to NO_2. The results of *Experiment Two* suggest that in mild asthmatic individuals a 3-hour exposure to a high ambient level of NO_2 does not cause either airway narrowing or non-allergic airway inflammation.

Introduction

Nitrogen dioxide (NO_2) is a gaseous air pollutant that is generated directly from the combustion of fossil fuels and from the conversion of nitric oxide (NO) by oxidation in the atmosphere. The concentrations of NO_2 in the ambient air of California have decreased by approximately 50% since 1980 due to more stringent controls on both mobile and stationary sources. In 1997, maximum 1-hour values for NO_2 were highest in Riverside and Imperial counties, 0.307 ppm and 0.286 ppm, respectively (Office of Air and Radiation, 1998). These short-term, peak exposures are somewhat unusual. More commonly, exposures to NO_2 are at much lower concentrations, but mildly elevated levels due to heavy traffic during inversion conditions may persist for many hours and even days. Because NO_2 concentrations are related to traffic density, commuters in heavy traffic may be exposed to higher concentrations than those indicated by regional monitors (Sexton et al., 1983; Spengler et al., 1990). In one study involving personal monitoring of Los Angeles commuters (Baker et al., 1990), mean in-vehicle NO_2 concentrations during commute times over 1 week of travel, ranged from 0.028 to 0.170 ppm with an overall mean of 0.078 ppm. This was 50% higher than ambient concentrations measured at local sites. Indoor NO_2 levels, in the presence of an unvented combustion source, may exceed those found outdoors. Natural gas or propane cooking stoves release NO_2, as do kerosene heaters. Peak levels exceeding 2.0 ppm have been measured in homes with gas stoves (Leaderer et al., 1984). Outdoor NO_2 levels provide a background for the higher peaks that may occur indoors. Thus, higher outdoor levels may drive higher peaks indoors, with outdoor levels contributing approximately 50% to indoor levels (Marbury et al., 1988). Distance of residences from and traffic density on roadways appear to influence indoor NO_2 levels (Nakai et al., 1995; Roorda-Knape et al., 1999).

Nitrogen dioxide is a relatively water-soluble gas that is highly reactive as an oxidizing agent, but less reactive than ozone. A large percentage of inhaled NO_2 is absorbed in the respiratory tract (up to 90%). Absorption occurs along the entire tracheobronchial tree and in the alveoli, but the greatest dose to tissue is delivered to the peripheral airways at the junction between the conducting and respiratory (i.e., gas-exchange) airways (Miller et al., 1982). The primary determinant of NO_2 uptake is surface reactivity, i.e., direct interaction with airway lining fluid constituents and/or cellular components (Postlethwait et al., 1990). While NO_2 does not penetrate through the airway epithelium unreacted, the specific substrates with which it reacts are not known with certainty. It may dissolve in the airway lining fluid to form nitric and nitrous acids, which could then cause toxicity due to hydrogen ions or through formation of nitrite ion. Nitrogen dioxide may also react directly with lipids and/or proteins in cells, including cell membranes, producing nitrite ions or free radicals. Respiratory toxicity is likely related to the effects of NO_2 and its reaction products on alveolar macrophages and airway epithelial cells (Krishna & Holgate, 1999).

Studies in animal models show that exposure to NO_2 can cause airway and alveolar injury that can lead to morphological changes. The region of the respiratory tract which appears most sensitive to injury from inhaled NO_2 is that where the greatest dose is

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deposited, the transition zone between conducting and gas-exchange airways. Within this region, the earliest changes observed occur within 24 to 72 hours of continuous exposure and include macrophage aggregation, desquamation of type 1 alveolar epithelial cells and ciliated bronchiolar cells, and accumulation of fibrin in small airways. With continuous NO_2 exposure, repair of injured tissue can begin as early as 24 hours from the start of exposure with new bronchiolar cells generated from nonciliated cells and new type 1 cells generated from type 2 cells. The newly generated cells are relatively resistant to any further effects of inhaled NO_2. An increase in the rate of division of type 2 cells is observed within 12 hours after initial NO_2 exposure, peaks by about 48 hours, and returns to baseline by about 6 days, even with continued exposure (Schlesinger, 1998).

Epidemiological Studies
There are abundant epidemiological data that suggest that persons with asthma are more sensitive to the respiratory effects of ambient NO_2 than nonasthmatics. A population-based study of adults in the Netherlands (Boezen et al., 1998) showed an increased risk for respiratory symptoms in association with NO_2 only in those subjects with pre-existing airway hyperresponsiveness or excessive peak expiratory flow variability (both features characteristic of asthma). Somewhat similarly, data from the Southern California Children's Health Study showed an association between NO_2 levels and respiratory symptoms only in children with asthma (McConnell et al., 1999 and 2003). Other studies have shown associations between NO_2 levels and respiratory symptoms in people with asthma (Linaker et al., 2000; Shima and Adachi, 2000; Smith et al., 2000; Gehring et al., 2002; Nicolai et al., 2003; Chauhan et al., 2003; Belanger et al., 2003; Delfino et al., 2003; Just et al. 2003). Multiple studies from several different countries have demonstrated associations between NO_2 levels and emergency department visits or hospitalizations for asthma (Castellsague et al., 1995; Lipsett et al., 1997; Tenias et al., 1998; Garty et al., 1998, Hajat et al., 1999; Tobias et al., 1999; Ostro et al., 2001; Wong et al. 2001; Ye et al., 2001; Lee et al. 2002; Kuo et al., 2002; Masjedi et al., 2003; Galan et al., 2003; Lin et al., 2004).

In an Australian study of indoor NO_2 exposure and gas stove use (Garrett et al., 1998), atopic children had a greater risk for respiratory symptoms associated with gas stove use than non-atopic children. This finding is consistent with reports of increased responsiveness to allergen challenge of sensitized individuals following experimental NO_2 exposure (see below). It may be the segment of the general population with allergies that is at greatest risk of adverse respiratory health effects after NO_2 exposure at ambient concentrations.

Animal Toxicological Studies
Several studies using experimental animals have shown effects of NO_2 exposure on lymphocytes, cells that play key roles in immune responses, including those involved in allergic asthma. Richters and colleagues, in a series of experiments (Richters & Damji, 1988; Damji & Richters, 1989; Kuraitis & Richters, 1989; Richters & Richters, 1989), found that mice exposed to NO_2 at levels as low as 4 ppm for 8 hours have reductions in splenic CD8+ (cytotoxic/suppressor) lymphocytes. Whether exposure to NO_2

enhances or suppresses immune responses may depend on exposure concentration/dose. For example, antibody responses in monkeys chronically exposed to NO_2 were enhanced at a concentration of 1 ppm, but suppressed at 5 ppm (Fenters et al., 1971; Fenters et al., 1973).

There are also data to support the concept that NO_2 exposure can enhance specific allergic responses in sensitized animals (Gilmour, 1995; Kitabatake et al., 1995). In a rat model of house-dust-mite sensitivity, a 3-hour exposure to 5 ppm of NO_2 increased the specific immune responses to subsequent inhaled house dust mite antigen (Gilmour, 1995). Particularly relevant to the proposed research, allergic inflammation was increased in the lungs of the exposed animals. Nitrogen dioxide exposure also enhanced lymphocyte proliferative responses to allergen in both spleen and mediastinal lymph nodes.

Controlled Human Exposure Studies
In contrast to ozone, NO_2 at concentrations <2.0 ppm does not cause an influx of polymorphonuclear cells (PMNs) into the airways and alveolar spaces. However, prolonged exposure (4-6 hours) to NO_2 at a concentration of 2.0 ppm has been demonstrated to cause mild airway inflammation in several studies (Blomberg et al., 1997; Azadniv et al., 1998). Two studies assessed the inflammatory effects of repeated 4-hour exposures to 2.0 ppm on 3-4 consecutive days and both found similar mild NO_2-induced increases in PMNs in the bronchial wash fraction of bronchoalveolar lavage (BAL) fluid (Blomberg et al., 1999; Solomon et al., 2000).

Multiple studies in normal human subjects have demonstrated changes in total lymphocyte number or in lymphocyte subsets in either BAL or peripheral blood after NO_2 exposure (Sandstrom et al. 1990; Sandstrom et al., 1991; Rubenstein et al., 1991; Sandstrom et al., 1992a; Sandstrom et al., 1992b; Helleday et al., 1994; Blomberg et al., 1997; Azadniv et al., 1998; Solomon et al., 2000). The dose of NO_2 (concentration, duration, single vs. repeated exposure) used in these studies varied widely, which perhaps explains the conflicting results reported.

Two studies have investigated responses to NO_2 exposure in asthmatic subjects. One study using induced sputum to assess airway inflammation found no effect of inhalation of 0.3 ppm NO_2 for 1 hour on cell distribution 2 hours after exposure in eight asthmatic subjects (Vaggagini et al., 1996). A second study using BAL fluid parameters to assess airway inflammation found no effect of 1.0 ppm NO_2 for 3 hours on cell distribution 1 hour after exposure in either normal (n=8) or asthmatic subjects (n=12), although there was some evidence of NO_2-induced increases in several prostanoids in the asthmatic subjects as compared to the normal subjects (Jorres et al., 1995).

Similar to what has been observed with studies of another oxidant pollutant, ozone (Molfino et al., 1991; Jorres et al., 1996), NO_2 exposure has been shown to enhance the lung function responses of allergic asthmatic subjects to subsequent challenge with specific allergen. Exposure of mild allergic asthmatic subjects to 0.4 ppm NO_2 for only 1 hour at rest caused increased bronchoconstrictor responses (both early and late) to a

fixed dose of house-dust-mite antigen (Tunnicliffe et al., 1994). Several studies involving exposure of house-dust-mite sensitive asthmatics to a mixture of 0.4 ppm NO_2 and 0.2 ppm SO_2 showed a lower $PD_{20-FEV1}$ (provocative dose causing a 20% decrease in forced expiratory volume in 1 second) on *Dermatophygoides pteronyssinus* antigen inhalational challenge after exposure to the mixture as compared to after filtered air (Devalia e al., 1994; Rusznak et al., 1996). These investigators also studied dose- and time-dependent effects of exposure to ozone and NO_2, alone and in combination, on allergen $PD_{20-FEV1}$. They found that exposure to a threshold concentration may be more important than the total amount of pollutant inhaled over a period of time. For example, exposure to 0.4 ppm NO_2 for 3 hours led to a decreased allergen $PD_{20-FEV1}$, while exposure to 0.2 ppm NO_2 for 6 hours did not (Jenkins et al., 1999). In these British studies, allergen challenge was performed immediately after NO_2 exposure. Strand and colleagues from Sweden have reported the results of two studies of the effects of inhaled NO_2 on both early and late bronchoconstrictor responses in allergic asthmatic subjects (Strand et al., 1997; Strand et al., 1998) that used exposure protocols in which the timing of the allergen challenge was 4 hours after the exposure. In the first of these Swedish studies, a lower concentration of NO_2 (0.26 ppm) and a shorter duration of exposure (30 minutes) still resulted in an enhancement of the late bronchoconstrictor response to inhaled allergen (Strand et al., 1997). In the second study, a 30-minute exposure to 0.26 ppm NO_2 was repeated on 4 consecutive days followed by an allergen inhalational challenge 4 hours after the last exposure; both the early and late bronchoconstrictor responses to allergen were enhanced by NO_2 exposure (Strand et al., 1998). This group of investigators have also reported the results of a novel study using exposure to ambient air pollution in a road tunnel (Svargtengren et al., 2000). They had subjects with mild allergic asthma sit inside a car in a Stockholm traffic tunnel for 30 minutes followed by an allergen inhalational challenge 4 hours later. The median NO_2 concentration during exposure was 163 ppb (the PM10 and PM2.5 concentrations were 170 and 95 µg/m3, respectively). Subjects exposed to tunnel NO_2 levels >156 ppm had significantly greater early and late bronchoconstrictor responses to inhaled allergen.

Allergic Airway Inflammation
As noted above, allergen inhalational challenge can induce both early and late-phase bronchoconstrictor responses in sensitized individuals. Early-phase bronchoconstriction occurs within 1 hour of the challenge and is due to release of pre-formed mediator substances that directly act on airway smooth muscle (e.g., histamine) from cells in the airways (e.g., mast cells) that have specific IgE antibody to the inhaled allergen on their surface. Late-phase bronchoconstriction occurs 4-8 hours after allergen inhalation and is felt to be due to acute airway inflammation as a result of cytokine [e.g., interleukin (IL)-5, IL-8, RANTES (regulated upon activation, normal T-cell expressed and secreted), GM-CSF (granulocyte and macrophage colony-stimulating factor)] and other mediator release from airway mast cells and alveolar macrophages with specific IgE antibody on their cell surfaces (Arshad, 2000; Bousquet et al., 2000). Th2-like cytokine release from sensitized T-lymphocytes may also play a role in the late-phase inflammatory response (Arshad, 2000; Bousquet et al., 2000). Induced sputum or BAL fluid samples obtained during late-phase reactions show increases in PMNs and eosinophils, as well as the

products of their degranulation [e.g., myeloperoxidase (MPO) and eosinophilic cationic protein (ECP), respectively]. Given that NO_2 exposure has been repeatedly found to cause enhancement of late-phase lung function changes, one would expect that enhancement of airway inflammatory responses should occur as well. In fact, although no studies of the effects of NO_2 on lower airway or late-phase inflammatory responses to inhaled allergen have been published, Wang and colleagues have shown an exposure-induced increase in ECP, but not in MPO, during the early-phase response to nasal allergen provocation (Wang et al., 1995a; Wang et al., 1995b; Wang et al., 1999). Epidemiological data often indicate that exposure to high ambient NO_2 is associated with adverse respiratory effects in asthmatic individuals with a lag time of 24 hours or greater. This finding suggests that NO_2 exposure may be affecting late-phase inflammatory responses more than early-phase bronchoconstriction. If this is indeed the case then strategies for protecting sensitized asthmatic individuals from adverse health effects of exposure to high ambient NO_2 should consider the timing of late-phase responses to inhaled allergen.

Summary
The epidemiological data reviewed above suggest that persons with asthma may be more sensitive to NO_2 exposure than normal, healthy persons. The animal toxicological data provide evidence that NO_2 exposure can affect immune function, including enhancement of allergic inflammatory responses in the lungs. Controlled human exposure studies have clearly confirmed that NO_2 exposure can enhance both the early and late bronchoconstrictor responses to inhaled antigen in allergic asthmatic subjects. Controlled exposure studies of normal subjects have shown that inhaled NO_2 can cause mild, non-specific proximal airway inflammation; asthmatic subjects have been inadequately studied.

Overall Objective and Specific Aims

The overall objective of this project was to investigate the effects of NO_2 exposure on airway inflammation in individuals with asthma.

Specific Aim One: To determine whether NO_2 exposure enhances the specific lower airway inflammatory responses of asthmatic subjects during late-phase reactions to inhaled allergen.

Specific Aim Two: To determine whether asthmatic subjects have significant non-specific, lower airway inflammation following exposure to a concentration of NO_2 not reported to cause lower airway inflammation in normal subjects.

Hypotheses

Our primary hypothesis was that pre-exposure to NO_2 would enhance subsequent inhaled allergen-induced changes in airway leukocyte distribution, as well as cytokine and cell degranuation product release, compared to pre-exposure to filtered air. Our secondary hypothesis was that NO_2 exposure alone would induce changes in airway leukocyte distribution, as well as cytokine release, compared to control exposure to filtered air.

Materials and Methods

<u>Design</u>

This project consisted of two separate controlled human exposure experiments. *Experiment One* used 15 asthmatic subjects who were allergic to house dust mite (HDM); *Experiment Two* used 10 subjects with mild intermittent or mild persistent asthma who were not required to be sensitive to HDM. The exposure condition for both experiments was a single 3-hour exposure to NO_2 at a concentration of 0.4 ppm. For both experiments, a control exposure condition of filtered air (FA) was used. To allow recovery from preceding sessions, a minimum of 3 weeks separated each of the exposure conditions within each experiment.

For both experiments, each subject attended the laboratory for one characterization session, and subsequently for two exposure sessions. The characterization session was used to collect physical and pulmonary characteristics, and to familiarize each subject with the procedures of the experiment. Each of the experiments utilized a repeated measures design, with each subject completing each condition within the experiment. The order of the experimental conditions was counter-balanced/randomized within each experiment.

The major difference in study protocol between the two experiments was the administration of an inhalational challenge to HDM allergen immediately post-NO_2 or FA exposure in *Experiment One* (see Fig. 1). For both experiments, spirometry was performed and symptom questionnaires completed immediately before and after each exposure. For *Experiment One*, each exposure was immediately followed by an inhalational allergen challenge with doubling doses of HDM allergen until a 20% fall in FEV_1 was achieved. Spirometry was performed and symptom questionnaires were administered for 6 hours after the allergen challenge, with additional spirometry prior to each sputum induction, and at intervals overnight. Sputum induction was performed at 6 (S-6) and 26 (S-26) hours after allergen challenge. For *Experiment Two*, no allergen challenge was performed so no additional spirometry was performed after each exposure condition except prior to the two sputum inductions, S-6 and S-26 (see Fig. 2).

Independent Variables:
The independent variables were the exposure conditions as follows:
      1) FA
      2) NO_2 (0.4 ppm).

Dependent Variables:
The dependent variables measured were as follows:
      1) Spirometric pulmonary function (FVC, FEV_1, FEF_{25-75}, FEF_{75}): for both experiments these parameters were obtained pre- and post NO_2 and FA exposures; for *Experiment One*, FEV_1 was obtained serially during and hourly for 6 hours post-allergen challenge after each exposure
      2) Symptoms (general and respiratory)

3) Cells in induced sputum: total and differential cell counts (macrophages, lymphocytes, neutrophils, eosinophils, epithelial cells)
4) Inflammatory mediator protein levels in induced sputum -- *Experiment One*: interleukin-5 (IL-5), interleukin-8 (IL-8), granulocyte/macrophage-colony stimulating factor (GM-CSF), eosinophilic cationic protein (ECP), and total protein; *Experiment Two* : interleukin-6 (IL-6), IL-8, and total protein.

Subjects

All subjects were informed of the risks of the experiment and provided informed consent prior to participation. The procedures for this experiment were approved by the University of California, San Francisco, Institutional Review Board, the Committee on Human Research.

All subjects completed a medical history questionnaire, were current non-smokers, had no history of excessive smoking (defined as cumulative history <1 pack-yr, >3 mo abstinence), and had no serious health problems. Female subjects were not pregnant throughout the project. Subjects had no respiratory-tract illness in the three weeks preceding, or during, each session. Subjects were characterized by age, gender, height, weight, spirometric lung function, methacholine responsiveness, and allergy skin prick testing. All subjects had a physician diagnosis of asthma confirmed by methacholine challenge (defined as a methacholine PC_{20} <8mg/ml). Subjects refrained from inhaled steroids for 2 weeks; long-term bronchodilators for 2 days; short-term bronchodilators for 8 hours; non-steroidal anti-inflammatory agents, antihistamines and other allergy medications for 3 or 5 days and caffeine for 4 hours prior to each visit. Subjects had no asthma exacerbations, respiratory tract infections or more than usual exposure to allergy provoking agents for 3 weeks prior to each visit.

Experiment One:

Fifteen mild intermittent to mild persistent atopic asthmatics with specific sensitivity to house dust mite (HDM) were recruited. House dust mite allergy was confirmed by skin prick testing to *D. pteronyssinus* (*D. pter*) allergen. Subject characteristics are listed in Table 1.

Experiment Two:

Ten subjects with mild intermittent to mild persistent asthma who did not need to have specific sensitivity to HDM were recruited. Subject characteristics are listed in Table 2.

Equipment and Procedures

Laboratory:

All sessions, excluding the post-allergen challenge observation period (6 hours), were conducted in the Human Exposure Laboratory at the Lung Biology Center, San

Francisco General Hospital (SFGH) campus, University of California San Francisco. The subjects were observed for 6 hours post-allergen challenge at the General Clinical Research Center (GCRC) at the SFGH campus.

Spirometry:

Spirometry was performed using American Thoracic Society (ATS) guidelines (American Thoracic Society, 1995). At the initial visit for both experiments, spirometry was performed using a dry rolling seal spirometer (Collins Medical, Inc). For *Experiment One*, spirometry on the exposure days was performed using a hand-held EasyOne spirometer (ndd Medical Technologies) to allow subject monitoring at the GCRC (Mortimer et al., 2003). Each subject used the same EasyOne spirometer for both exposure visits. The best of at least two consistent efforts based on FEV_1 was recorded for each time point. For *Experiment Two*, pre- and post-exposure spirometry was performed using the dry rolling seal spirometer.

Methacholine Challenge:

Methacholine inhalation challenge was performed following a protocol modified from the ATS guidelines (American Thoracic Society, 2000), using a nebulizer (DeVilbiss) and dosimeter (Rosenthal) set to deliver 9 μL per breath. Subjects inhaled aerosol from the nebulizer in five breaths, (one every 12 seconds over a 1-minute period) and spirometry was measured 3 min after each dose. The next dose was administered within 30 seconds of completing the spirometry. Increasing doses of methacholine (0.0625, 0.25, 1, 2, 4, 8 mg/mL) were given, until a 20% decrease in FEV_1 from saline FEV_1 was achieved. Methacholine PC_{20} (MPC_{20}) was calculated from linear interpolation of the last two values on the log dose-response curve (O'Connor et al., 1987). A positive methacholine test was defined as a MPC_{20} <8 mg/mL (Kanner et al., 1994).

Allergy Skin Testing:

Epicutaneous skin-prick testing with nine local aeroallergens [*Dermatophygoides. pteronyssinus* (*D. pter*) plus *aspergillus fumigatus*, birch mix, chinese elm, cat, dog, mountain cedar, mugwort sage, olive tree, perennial rye] and controls of saline/50% glycerol and histamine were performed on the volar forearm to determine atopic status. Sensitivity was be defined as a >2 mm skin wheal response.

For *Experiment One*, additional dilutional skin testing for *D. pter* sensitivity was performed on the arm not used for the screening skin-prick testing. Dilutions of *D. pter* allergen were prepared by diluting stock solution in sterile normal saline. Skin sensitivity to *D. pter* was determined using dilutions ranging from 0.057 to 30,000 allergy units (AU)/mL, and was defined as the dilution that produced a >2 mm weal after 15 minutes.

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Exposure Chamber:

The exposure sessions were conducted in a custom-built stainless 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 is sourced from ambient air, which is filtered by passing through purifying (Purafil Model No. 6239), and high efficiency particle (Aeropac Model No.53 HEPA 95) filters. The filtered air is dehumidified by passing through a drier (Cargocaire Engineering Corp.). HC-575), and the air temperature is decreased with a chilled-water coil. Subsequently, temperature and humidity are increased with steam (Nortec Model No. NHMC-050), to obtain the pre-set temperature (20 ^{O}C) and relative humidity (50%) conditions in the chamber. The temperature and relative humidity inside the chamber are monitored [LabView 2; (3-minute intervals)] and controlled throughout the exposures (Johnson Controls, Model No. DSC 8500).

Nitrogen Dioxide Exposure System:

Nitrogen dioxide from a gas cylinder (Airgas, certified 5000 ppm, balance air) was supplied to the inlet duct of the chamber using a dual regulator/flow meter system. During exposure, NO_2 concentration inside the chamber was continuously monitored with a chemiluminescent NO_x analyzer (Monitor Labs, Model No 8840, Englewood, CO, USA), with sampling via Teflon tubing in the breathing zone of the subject and was recorded every 10 minutes.

Exposures:

Exposures were performed with intermittent exercise (first 30 minutes of each hour). Breathing frequency and tidal volume were measured at 10 and 20 min during each exercise period using a pneumotachograph (Fleish, Model No 3) and minute ventilation was calculated. Exercise was adjusted to approximate the calculated target ventilation (body surface area in m^2 x 25 L/min).

Allergen Challenge:

Predicted Allergen PC_{20} (APC_{20}) was calculated according to Cockcroft (Cockcroft et al., 1987) using the formula:
$$\log_{10} (APC_{20}) = 0.68 \log_{10} (MPC_{20} \times SS).$$
MPC_{20} = the provocative concentration of methacholine that caused a 20% decrease in in FEV_1 from saline baseline; SS = the dilution of *D. pter* (AU/mL) that produced a >2 mm weal after 15 minutes.

The starting dose for allergen challenge was four doubling doses below the predicted APC_{20} dose. The range of allergen doses was 0.057 to 30,000 AU/mL, and doses were thawed to room temperature before inhalation. Using the same nebulizer and dosimeter from the methacholine challenge, subjects inhaled normal saline (one breath every 12 seconds over 1 minute), performing spirometry 10 minutes after inhalation. Increasing

doses of allergen were then inhaled until a 20% decrease in FEV_1 from saline baseline was observed or all doses were given. Spirometry was performed at 10, 20, 30, 45, and 60 minutes after the allergen challenge and subsequently hourly until 6 hours after the challenge.

Cumulative allergen PD_{20} was calculated by linear interpolation of the last two values on the log dose-response curve. The maximum early phase response (EPR) was recorded as the greatest drop in FEV_1 in the first hour following allergen challenge. A late phase response (LPR) was characterized by a fall of >15% in FEV_1 from baseline 4-6 hours after allergen challenge.

Symptom Questionnaire:

Subjects completed a symptom questionnaire before and after each exposure, and hourly during the 6-hr monitoring period after allergen challenge. Symptoms included six pulmonary (chest tightness, chest pain, cough, phlegm, shortness of breath, and wheezing) and six general (headache, nausea, anxiety, eye irritation, nasal irritation and throat irritation) symptoms and were rated from 0 (none) to 4 (severe).

Sputum Induction:

Sputum induction (SI) was performed at 6 and 26 hours post-allergen challenge in an isolation booth (Biosafety, Aerostar). Spirometry was performed pre- and post-sputum induction. After spirometry, subjects were pretreated 15 minutes prior to SI with 400 µg albuterol. Subjects inhaled nebulized (DeVilbiss Ultra-Neb 99) 3% sterile saline for 20 minutes; at 2-minute intervals saliva was cleared from the mouth prior to coughing and collection of sputum. The sample was diluted in an equal volume of 0.1% dithiothreitol (Sputalysin, Behring Diagnostics, Inc) and incubated in a 37ºC shaking water bath for 15 minutes with sample manipulation to ensure complete homogenization. An aliquot for total cell count and cytospin preparation was separated, and then the sample was centrifuged at 1000 rpm for 15 minutes at 4ºC. The supernatant was re-centrifuged at 3000 rpm for 15 minutes at 4ºC and stored in aliquots at –80ºC until analysis.

Cell Counts:

Total cell counts were performed using a hemocytometer (Fisher Scientific) and were expressed as number of cells per mL of sputum. Cytospin slides were prepared using a cytocentrifuge (Thermo Shandon) at 500 rpm for 5 min. Slides were stained with Diff-Quik (Dade Behring). Differential cell counts were obtained by enumeration of 400 non-squamous cells per slide, performed by two counters. Macrophages, lymphocytes, neutrophils, and eosinophils were expressed as a percent of leukocytes. Cell concentrations were calculated from the differential percent and total leukocyte cell count and were expressed as number of cells per mL of sputum.

Biochemical Assays:

Analyses of soluble mediators in sputum were performed on samples after additional centrifugation at 10,000 rpm for 3 minutes at 4^0C. Eosinophilic cationic protein (ECP) was measured using the UniCAP fluoroenzymeimmunoassay (Pharmacia & Upjohn Diagnostics AB) with a detection limit of 2 ug/L. Levels of IL-6 and IL-8 were measured using Quantiglo ELISA (R&D Systems), with a detection limit of 0.8 pg/mL. Interleukin-5 (IL-5) was measured with a Quantikine ELISA (R&D Systems) with a detection limit of 3 pg/mL. Granulocyte/macrophage-colony stimulating factor (GM-CSF) was measured using a Quantikine HS ELISA (R&D Systems) with a detection limit of 0.26 pg/mL. Quantitation of total protein was determined using the BCA Protein Assay kit (Pierce).

Statistical Analysis

Most of the sputum and spirometric data were not normally distributed. Therefore, statistical analysis of the data comparing FA and NO_2 conditions was performed using the non-parametric Wilcoxon Signed Rank Test. Comparisons of responders to non-responders were performed using the Mann-Whitney U test. The analyses were conducted using the statistical program SYSTAT (SPSS Inc). Statistical significance was set at $p < 0.05$.

Figure 1. Study Design for Experiment One
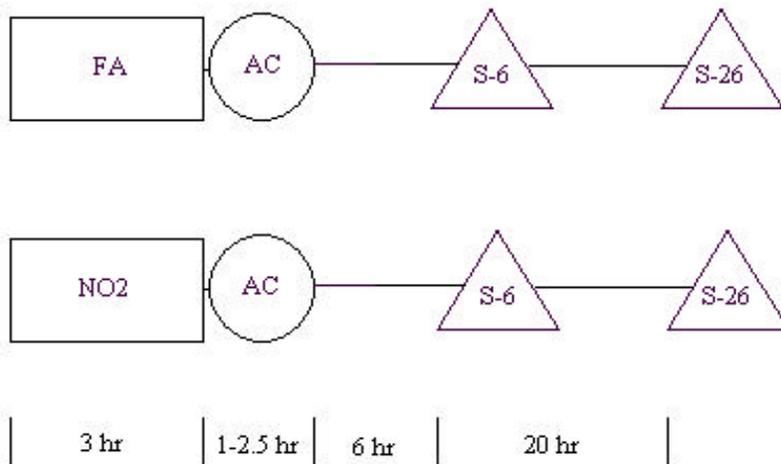
FA: filtered air, NO$_2$: nitrogen dioxide, AC: allergen challenge (*D.pter*), S-6 and S-26: sputum induction 6 and 26 hours after allergen challenge.

Figure 2. Study Design for Experiment Two
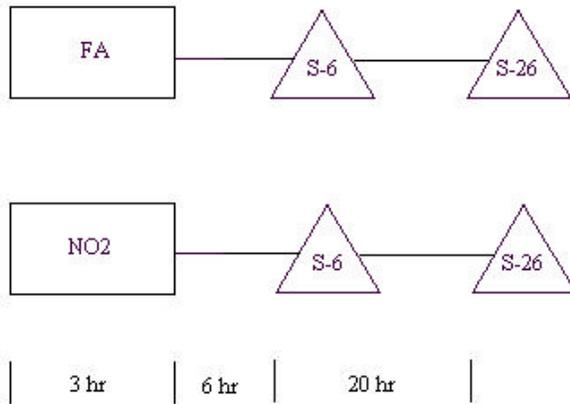
FA: filtered air, NO$_2$: nitrogen dioxide, S-6 and S-26: sputum induction 6 and 26 hours after allergen challenge.

Table 1. Experiment One Subject Characteristics

Subject #	Age (yr)	Sex	Ht (cm)	Wt (kg)	FEV_1 (L)	FEV_1 %pred	MPC_{20}	Meds
1	30	M	175	75	3.71	85.5	4.13	None
2	24	F	163	64	2.38	70.6	0.88	IS, SB, LA, AH
3	21	F	173	84	3.53	93.0	0.47	SB
4	34	M	170	80	3.29	81.7	0.75	IS, SB
5	23	F	173	52	3.01	80.4	0.91	None
6	30	M	183	80	4.05	87.1	4.62	SB
7	37	F	175	67	3.34	96.1	5.60	AH
8	30	F	163	61	1.87	58.1	0.24	IS, LB, SB, LA, NS, AH
9	48	F	170	98	2.07	68.5	0.81	IS, SB
10	43	M	175	68	3.90	97.0	0.70	SB, AH, DE
11	34	F	163	56	3.10	99.5	4.22	LB, SB, LA, NS
12	26	M	183	99	3.78	79.6	0.58	None
13	45	M	185	84	3.45	78.6	4.39	None
14	35	F	165	136	2.04	64.2	1.63	IS, LA, SB
15	21	F	168	61	3.20	88.4	2.44	None
Mean (SD)	32 (8.6)	F=9 M=6	172 (7.5)	78 (22)	3.11 (0.71)	81.9 (12.4)	2.16 (1.88)	

MPC_{20}: methacholine provocative concentration causing 20% reduction in FEV_1, IS: inhaled steroid, LB: long-acting bronchodilator, SB: short-acting bronchodilator, LA: leukotriene antagonist (anti-inflammatory), AH: antihistamines, NS: nasal steroid, DE: decongestant/expectorant.

Table 2. Experiment Two Subject Characteristics

Subject #	Age (yr)	Sex	Ht (cm)	Wt (kg)	FEV_1 (L)	FEV_1 %pred	MPC_{20}	Meds
1	34	M	170	80	3.29	81.7	1.0	IS, SB
2	37	F	175	67	3.34	96.1	8.0	AH
3	21	F	173	84	3.53	93.0	0.5	SB
4	43	M	175	68	3.90	97.0	1.0	SB, AH, DE
5	40	F	163	75	1.83	66.0	0.25	None
6	35	F	165	136	2.04	64.2	2.0	IS, LA, SB
7	29	F	155	47	2.65	84.0	1.0	SB
8	21	F	168	61	3.20	88.4	4.0	None
9	43	M	173	68	2.18	61.4	1.0	IS, SB
10	28	M	183	76	2.96	66.4	0.5	SB
Mean (SD)	33.1 (8.2)	F=5 M=5	170 (7.9)	77 (23)	2.89 (0.69)	79.8 (14.1)	1.9 (2.4)	

MPC_{20}: methacholine provocative concentration causing 20% reduction in FEV_1, IS: inhaled steroid, LB: long-acting bronchodilator, SB: short-acting bronchodilator, LA: leukotriene antagonist (anti-inflammatory), AH: antihistamines

Note: The subject numbers for Experiment Two do not correspond to those for Experiment Two. In other words, the subjects who participated in both experiments have different numbers for each experiment.

Results

<u>Experiment One</u>

Exposure Conditions:

The concentration for the NO_2 exposures was (mean $\pm$ SD) 0.40 $\pm$ 0.01 ppm. The temperature and humidity inside the chamber for FA were 19.8 $\pm$ 0.3°C and 54.4 $\pm$ 3.9%, and for NO_2 exposure were 19.9 $\pm$0.6°C and 51.8 $\pm$ 4.5%, respectively. Minute ventilations for FA and NO_2 were 41.7 $\pm$ 6.7 L and 40.5 $\pm$ 6.8 L, respectively. There was no statistical significant difference in these variables between the two conditions.

Spirometry:

Table 3 displays the FEV_1 responses to the two experimental conditions: FA/allergen challenge and NO_2/allergen challenge. There was no statistically significant difference in FEV_1 before the FA and NO_2 exposures or change in FEV_1 (or in FVC, FEF_{25-75}, or FEF_{75}) with either exposure. After FA exposure, 13 of the 15 subjects had an early phase response (EPR) during the allergen challenge and seven had a LPR. After NO_2 exposure and allergen challenge, 15 had an EPR and five had a LPR. However, there was no statistically significant difference in FEV_1 or %drop in FEV_1 in either phase, comparing the NO_2 to the FA exposure arms.

Allergen $PD_{20\text{-}FEV1}$:

The reproducibility of allergen challenge is considered to be $\pm$ one doubling dose of allergen (25). Of the 15 subjects, 12 had a difference within $\pm$ one doubling dose of allergen between the FA and NO_2 allergen challenges. These subjects were designated non-responders to NO_2 exposure in terms of enhancement of the early-phase bronchoconstrictor response to allergen according to this criterion. The remaining three subjects had a $\geq$ two doubling-dose difference between allergen challenges, and were designated responders as they required less allergen to reach a PD_{20} after NO_2 exposure compared to after FA (Figure 2). There was no statistically significant difference in allergen $PD_{20\text{-}FEV1}$ comparing NO_2 to FA for the 15 subjects as a group.

Subject Symptoms:

There were no statistically significant differences in pulmonary symptoms comparing NO_2 and FA exposure arms.

Sputum Volume:

Sputum volumes for FA and NO_2 S-6 sputum samples were [median (IQR)] 5.9 (3.8 – 7.4) mL and 5.0 (4.7 – 7.2) mL, and for S-26 were 5.1 (3.6 – 6.3) mL and 4.8

(3.9 – 6.3) mL, respectively. There was no statistical significant difference between the two conditions.

Sputum Cell Distribution:

Cell distribution for the sputum samples at 6 and 26 hours following allergen challenge are shown in Table 4. Comparing NO_2 to FA, there was a statistically significant decrease in eosinophil concentration in the S-6 sample (p=0.012). However there was no statistically significant difference in total leukocyte concentration, percent or concentration of macrophages, lymphocytes, and neutrophils for S-6 or S-26 samples comparing NO_2 to FA.

Analysis of cell concentrations, corrected for cumulative allergen dose inhaled during allergen challenge, showed no statistically significant difference for any cell type comparing NO_2 to FA (data not shown).

Sputum Biochemical Analyses:

Biochemical analyses for the sputum samples at 6 and 26 hours following allergen challenge are shown in Table 5. There was no statistical difference in IL-5, IL-8, GM-CSF, ECP, or total protein (TP), comparing the NO_2 condition to the FA condition for either S-6 or S-26 samples.   Analysis of data corrected for the total protein concentration in each sample showed a small but significant increase in IL-5/TP ratio (p = 0.031) after NO_2 exposure.

Correction of concentrations and total protein ratios for cumulative allergen dose inhaled during allergen challenge resulted in no statistically significant difference for any concentration or ratio, comparing NO_2 to FA (data not shown).

Sub-set of Responders:

Table 6 shows the study data stratified by whether the subjects are responders or non-responders to NO_2. Comparison of the three responders to the 12 non-responders showed the following statistically significant differences between the two groups: 1) the responders had higher allergen $PD_{20-FEV1}$, macrophage concentration in sputum samples at both 6 and 26 hr, and IL-5/TP at 26 hr after FA; and 2) they had higher IL- and IL-8/TP at both 6 and 26 hr after. Although the small number of subjects in the responder group limits the statistical power of this analysis, inspection of the values in Table 6 suggests a difference in the inflammatory responses of the two groups. At 6 hr after allergen challenge, there are increased NO_2-related changes in neutrophils, eosinophils, IL-5, IL-8, GM-CSF, and ECP in the responders compared to the non-responders. Most of these NO_2-related increased responses persisted at 26 hr after allergen challenge. Of note, the baseline lung function was higher and both the non-specific and specific allergen airway reactivity of the three responders was lower than the 12 non-responders.

<u>Experiment Two</u>

Exposure Conditions:

The concentration for the NO_2 exposures was (mean $\pm$ SD) 0.40 $\pm$ 0.017 ppm. The exposure chamber is designed to maintain a temperature of 20°C and a humidity of 50% inside the chamber. There was no statistical significant difference in these variables between the two conditions.

Spirometry:

Table 7 displays the FEV_1 responses to the two experimental conditions: FA and NO_2. There was no statistically significant difference in FEV_1 before the FA and NO_2 exposures or change in FEV_1 with either exposure. There were also no statistically significant changes in FVC or FEF_{25-75} with either exposure.

Subject Symptoms:

There were no statistically significant differences in pulmonary symptoms comparing FA and NO_2 exposure arms.

Sputum Volume:

There was no statistical significant difference between the two conditions.

Sputum Cell Distribution:

Cell distribution for the sputum samples at 6 and 26 hours following exposures are shown in Table 8. Comparing NO_2 to FA, there was no statistically significant difference in total leukocyte concentration, percent or concentration of macrophages, lymphocytes, neutrophils and eosinophils for S-6 or S-26 samples

Sputum Biochemical Analyses:

Biochemical analyses for the sputum samples at 6 and 26 hours following exposures are shown in Table 9. There was no statistical difference in IL-6, IL-8, or total protein, comparing NO_2 to FA for either S-6 or S-26 samples.

Figure 3. Experiment One: Effect of 0.4 ppm NO_2 on HDM Allergen PD_{20} in Subjects with Asthma
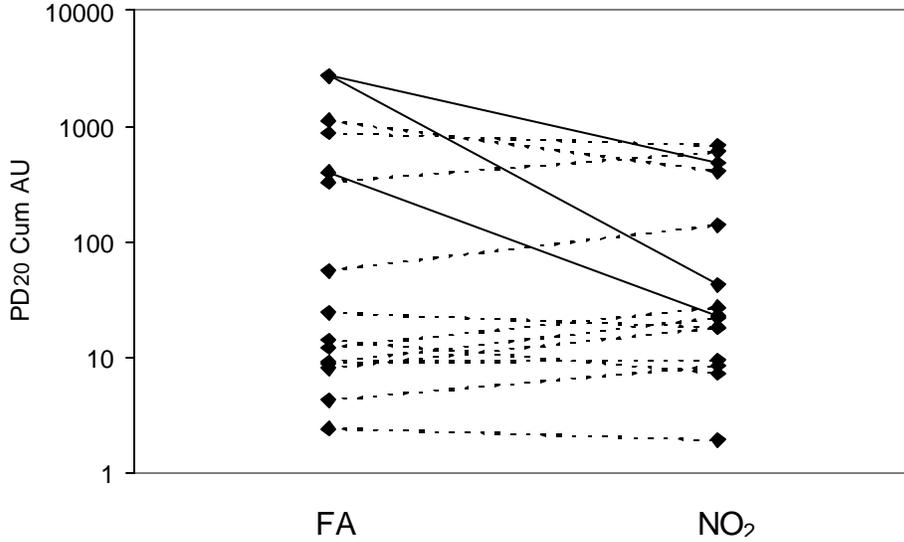
PD_{20} expressed as cumulative allergen units of house dust mite (*D.pteronyssinus*) causing 20% reduction in FEV_1, FA: filtered air, NO_2: nitrogen dioxide.

Table 3.   Experiment One: FEV_1 Responses to Filtered Air/Allergen and Nitrogen Dioxide/Allergen

Subject	Pre Exp FEV_1		Pre AC FEV_1		EPR FEV_1		EPR % change		LPR FEV_1		LP % change		PD_{20}-FEV_1 AU	
	FA	NO₂	FA	NO₂	FA	NO₂	FA	NO₂	FA	NO₂	FA	NO₂	FA	NO₂
1	3.99	3.89	4.01	3.99	3.11	2.86	- 22	- 28	3.93	3.64	- 2	- 9	327	598
2	2.56	2.46	2.71	2.31	1.61	1.80	- 41	- 22	1.98	2.04	- 27	- 12	7.9	18
3	3.52	3.40	3.62	3.89	3.15	2.89	- 13	- 26	3.26	3.45	- 10	- 11	> 2710	42 **R**
4	3.61	3.64	3.71	3.58	2.76	1.72	- 25	- 52	2.24	2.75	- 40	- 23	4.2	8.4
5	3.86	3.31	3.61	3.47	2.34	2.38	- 35	- 31	3.73	3.57	+ 3	+ 3	14	7.2
6	4.04	3.99	4.11	3.99	3.66	1.98	- 11	- 50	3.56	3.26	- 13	- 18	> 2697	480 **R**
7	3.24	3.34	3.40	3.41	2.11	2.24	- 38	- 34	2.05	2.25	- 40	- 34	24	18
8	2.31	1.82	2.10	1.97	1.65	1.53	- 21	- 22	1.86	1.76	- 11	- 11	8.9	22
9	1.97	1.99	1.99	2.22	1.54	1.49	- 23	- 33	1.76	1.85	- 12	- 17	1112	402
10	4.01	4.23	3.98	4.19	3.00	2.56	- 25	- 39	3.01	3.27	- 25	- 22	2.4	1.9
11	2.97	3.07	3.28	3.17	2.14	2.05	- 35	- 35	2.79	2.81	- 15	- 11	401	23 **R**
12	3.66	3.75	3.80	3.97	2.88	3.08	- 24	- 22	3.68	3.67	- 3	- 8	9.0	9.3
13	3.71	3.91	3.67	3.78	2.58	2.66	- 30	- 30	3.08	3.31	- 16	- 12	12	27
14	2.27	2.27	2.32	2.13	1.57	1.52	- 32	- 29	1.63	2.00	- 30	- 6	56	139
15	3.11	2.98	3.16	3.07	1.79	2.43	- 44	- 21	3.00	2.93	- 5	- 5	866	674
Median	3.52	3.34	3.61	3.47	2.34	2.24	-25	-30	3.00	2.93	-13	-11	24	23
IQR	2.77-3.79	2.72-3.82	2.94-3.76	2.69-3.93	1.72-2.94	1.76-2.61	-22.5-35	-24-34.5	2.02-3.41	2.15-3.38	-7.5-26	-8.5-17.5	9-634	14-271

FEV_1 (L), AC: allergen challenge, EPR: early phase response (0-1hr), LP: late phase (4-6hr), PD_{20}-FEV_1: provocative dose of HDM allergen causing 20% decrease in FEV_1 (allergen units), FA: filtered air, NO₂: nitrogen dioxide, R: responder (difference $\geq$ 2 doubling dose), IQR: interquartile range.

Table 4. Experiment One: Leukocytes in Induced Sputum at 6 hours and 26 hours after Allergen Challenge with Prior Exposure to Filtered Air or Nitrogen Dioxide

	S-6		S-26	
	FA	NO$_2$	FA	NO$_2$
Total leukocytes cells x 10^6/L	152 93 - 230	140 78 - 220	132 77 - 231	122 67 - 266
% Macrophage	26.1 15.2 - 36.3	24.5 17.3 - 31.7	23.3 19.5 - 34.5	29.1 21.3 - 50.3
% Neutrophil	57.1 50.5 - 68.6	66.9 50.7 - 72.4	66.5 52.7 - 75.5	55.0 39.7 - 68.8
% Eosinophil	7.2 2.5 - 12.6	4.5 1.6 - 7.7	3.5 0.9 - 7.4	2.1 0.1 - 5.9
% Lymphocyte	3.4 2.9 - 5.3	3.3 2.7 - 4.8	4.5 2.9 - 5.8	4.8 2.4 - 7.6
Macrophage cells x 10^6/L	34.6 23.3 - 55.7	26.7 16.9 - 48.9	42.2 16.1 - 62.9	33.3 18.6 – 74.2
Neutrophil cells x 10^6/L	82.6 55.7 - 156.6	76.3 35.8 - 161.3	75.7 50.7 - 146.4	53.2 37.0 - 127.0
Eosinophil cells x 10^6/L	6.2 3.0 - 15.2	3.1 [*] 1.0 - 8.9	5.3 1.3 - 15.8	4.52 0.05 - 5.7
Lymphocyte cells x 10^6/L	6.0 2.4 - 7.7	3.7 1.5 - 11.6	6.7 2.8 - 13.1	4.3 2.2 - 11.7

FA: filtered air, NO$_2$: nitrogen dioxide, S-6 and S-26: sputum induction 6 and 26 hours after allergen challenge. Values expressed as median; interquartile range.   [*] $p = 0.012$.

Table 5. Experiment One: Biochemical Analysis of Induced Sputum at 6 hours and 26 hours after Allergen Challenge with Prior Exposure to Filtered Air or Nitrogen Dioxide

	S-6		S-26	
	FA	NO$_2$	FA	NO$_2$
IL-5 pg/ml	13.4 9.9 - 16.5	18.6 11.4 - 32.1	11.6 6.3 - 14.6	9.0 6.0 - 19.4
ECP pg/ml	270 127 - 566	330 191 - 647	446 159 - 735	362 81 - 860
GM-CSF pg/ml	1.04 0.74 - 2.85	1.46 0.52 - 3.79	0.54 0.52 - 1.56	0.75 0.52 - 0.95
IL-8 pg/ml	2580 1503 - 4055	3640 1224 - 4966	2783 1958 - 7390	4250 1028 - 8106
Total Protein mg/ml	3.19 2.89 – 3.51	2.93 2.59 – 3.50	3.22 2.81 – 3.89	3.07 2.53 – 3.42
IL-5/TP pg/mg	4.2 3.2 - 5.9	6.6* 4.7 - 8.6	3.3 2.1 - 5.8	3.2 2.0 - 7.0
ECP/TP pg/mg	90 36 - 160	102 67 - 217	146 44 - 244	126 27 - 294
GM-CSF/TP pg/mg	0.37 0.22 - 0.59	0.44 0.22 - 1.44	0.19 0.16 - 0.48	0.23 0.21 - 0.34
IL-8/TP pg/mg	809 470 - 1129	1180 437 - 1707	1158 605 - 2103	1481 356 - 3930

*p=0.031

FA: filtered air, NO$_2$: nitrogen dioxide, S-6 and S-26: sputum induction 6 and 26 hours after allergen challenge, IL-5: interleukin-5, ECP: eosinophilic cationic protein, GM-CSF: granulocyte/macrophage colony stimulating factor, IL-8: interleukin-8, TP: total protein. The absolute cytokine values for each sputum sample were divided by the total protein concentration for that sample to normalize for the amount of protein present. Values expressed as median and interquartile range.

Table 6. Experiment One: Responders vs. Non-Responders

	Responders (n=3)		Non-Responders (n=12)	
FEV$_1$ % pred. (baseline)	93		80	
Methacholine PC$_{20}$	4.22		0.90	
	FA	**NO$_2$**	**FA**	**NO$_2$**
Pre-exposure FEV$_1$	3.52	3.40	3.43	3.33
Allergen PD$_{20}$	2697*	42	13	20
EPR % change in FEV$_1$	13	35	28	30
LPR % change in FEV$_1$	13	11	14	12
S6				
Leukocytes cells x 10^6/L	227	422	128	124
% Macrophage	34.3	19.9	25.3	27.4
% Neutrophil	55.7	71.4	61.6	58.6
% Eosinophil	2.7	5.1	8.2	4.0
Macrophage cells x 10^6/L	86.8*	82.3	25.0	23.4
Neutrophil cells x 10^6/L	126.5	292.3	78.1	74.5
Eosinophil cells x 10^6/L	6.2	3.8	7.3	2.7
IL-8 pg/ml	4921	5303*	2490	2835
IL-8/TP pg/mg	1727	2040*	745	812
IL-5 pg/ml	9.50	18.64	14.37	18.27
IL-5/TP pg/mg	2.98	6.62	4.74	6.69
GM-CSF pg/ml	0.95	3.97	1.10	1.44
GM-CSF/TP pg/mg	0.25	1.49	0.38	0.43
ECP	428	580	269	310
ECP/TP pg/mg	137	218	79	101
TP mg/ml	3.12	2.66	3.23	3.07
S26				
Leucocytes cells x 10^6/L	261	375	121	109
% Macrophage	25.8	24.0	22.7	31.0
% Neutrophil	66.5	61.2	66.0	54.2
% Eosinophil	2.0	5.0	4.4	1.4
Macrophage cells x 10^6/L	90.4*	89.7	20.7	25.2
Neutrophil cells x 10^6/L	153.6	229.2	55.7	47.9
Eosinophil cells x 10^6/L	5.3	5.1	5.1	1.3
IL-8	8606	25587*	2781	2726
IL-8/TP	2212	8612*	1081	770
IL-5	6.69	9.86	12.63	6.99
IL-5/TP	1.94*	3.77	4.03	2.65
GM-CSF	0.52	0.95	0.60	0.72
GM-CSF/TP	0.16	0.36	0.20	0.23
ECP	502	804	357	264
ECP/TP	146	245	125	82
TP	3.44	2.67	3.02	3.10

*p<0.05

PC$_{20}$: provocative concentration causing 20% reduction in FEV$_1$, PD$_{20}$: provocative dose causing 20% reduction in FEV$_1$. Values expressed as median.

Table 7. Experiment Two: FEV$_1$ Responses to Filtered Air and Nitrogen Dioxide

Subject #	Pre-Exposure FEV$_1$		Post-Exposure FEV$_1$		Pre-S-6 FEV$_1$	
	FA	NO$_2$	FA	NO$_2$	FA	NO$_2$
1	3.27	3.35	3.54	3.49	3.24	3.47
2	3.39	3.40	3.37	3.38	3.50	3.51
3	3.55	3.45	3.61	3.60	3.72	3.73
4	3.73	3.77	3.84	3.90	3.68	3.65
5	1.81	2.17	1.60	2.16	1.71	2.06
6	2.06	2.21	2.23	2.09	2.05	2.02
7	2.49	2.67	2.57	2.59	2.62	2.47
8	3.13	2.94	3.05	2.92	3.12	3.00
9	1.86	1.99	2.28	1.84	2.29	1.82
10	2.91	2.70	3.27	2.97	3.21	2.84
Median	3.02	2.82	3.16	2.95	3.17	2.92
IQR	2.06-3.39	2.21-3.40	2.28-3.54	2.16-3.49	2.29-3.50	2.06-3.51

FA: filtered air, NO$_2$: nitrogen dioxide, S-6: sputum induction 6 hours after exposure, IQR: interquartile range.

Table 8. Experiment Two: Leukocytes in Induced Sputum at 6 hours and 26 hours After Exposure to Filtered Air or Nitrogen Dioxide

	S-6		S-26*	
	FA	NO$_2$	FA	NO$_2$
Total leukocytes cells x 10^6/L	59.5 45.0 – 144.5	74.5 48.5 – 110.5	58.5 39.8 – 128.5	59.8 50.3 – 91.8
% Macrophage	44.6 34.2 – 64.8	46.2 34.5 – 55.6	40.1 33.6 – 61.5	45.5 35.4 – 48.0
% Neutrophil	50.2 28.8 – 61.2	44.9 35.0 – 64.3	50.7 35.8 – 63.0	52.6 42.3 – 60.5
% Eosinophil	0.7 0.3 – 1.5	0.7 0.5 – 1.9	1.5 0.5 – 2.0	0.3 0.0 – 7.2
% Lymphocyte	1.3 0.0 – 3.3	0.4 0.3 - 1.8	0.8 0.3 – 2.9	0.2 0.0 – 3.5
Macrophage cells x 10^6/L	35.0 13.8 – 75.0	28.6 25.8 – 31.5	23.3 16.7 – 44.9	27.0 13.3 – 33.5
Neutrophil cells x 10^6/L	38.6 16.0 – 83.6	34.6 16.8 – 75.1	27.1 18.1 – 86.2	31.7 21.8 – 65.5
Eosinophil cells x 10^6/L	0.8 0.1 - 1.3	0.7 0.4 – 1.7	1.0 0.3 – 1.5	0.2 0.0 – 2.3
Lymphocyte cells x 10^6/L	0.6 0.0 – 9.9	0.3 0.1 - 1.7	0.4 0.1 – 3.6	0.1 0.0 – 2.1

FA: filtered air, NO$_2$: nitrogen dioxide, S-6 and S-26: sputum induction 6 and 26 hours after exposure. Values expressed as median; interquartile range. * n = 8.

Table 9. Experiment Two: Biochemical Analysis of Induced Sputum at 6 hours and 26 hours after Prior Exposure to Filtered Air or Nitrogen Dioxide

	S-6		S-26*	
	FA	NO_2	FA	NO_2
IL-6 Pg/ml	106.0 82.6 – 251.6	78.1 31.6 – 98.8	64.3 28.4 – 129.2	66.1 31.1 – 171.1
IL-8 Pg/ml	2330 1705 - 5822	3045 2289 - 5250	3348 1774 - 5952	2349 1290 - 4014
Total Protein Mg/ml	1.62 1.61 – 2.34	1.89 1.60 – 7.51	1.51 1.30 – 2.00	2.20 1.44 – 2.75

FA: filtered air, NO_2: nitrogen dioxide, S-6 and S-26: sputum induction 6 and 26 hours after exposure to FA or NO_2. IL-5: interleukin-6, IL-8: interleukin-8, TP: total protein. Values expressed as median and interquartile range. * n = 8.

Discussion

This project was designed to test the hypotheses that exposure to a high ambient concentration of NO_2 would a) enhance the airway inflammatory response to inhaled allergen and b) induce nonspecific airway inflammation in mild allergic asthmatic subjects.

In Experiment One, using an exposure regimen previously reported to have enhanced the early phase bronchoconstrictor response to inhaled allergen in similar groups of subjects (Rusznak et al., 1996; Jenkins et al., 1999), we failed to confirm our hypothesis. In fact, we also failed to observe enhancement of the EPR in most subjects. Our results, placed in the context of previous reports (Tunnicliffe et al, 1994; Devalia et al., 1994; Rusznak et al., 1996; Strand et al., 1997 and 1998; Jenkins et al., 1999; Svartengren et al., 2000) indicate that it is only a subset of asthmatic subjects who respond to ambient NO_2 exposures with increased bronchoconstriction upon inhalational challenge with an allergen to which they are specifically sensitized. In our study, the three responders had lower airway reactivity to HDM allergen than the non-responders so they received a higher dose of HDM after filtered air exposure. They also had higher macrophage concentrations after HDM challenge following filtered air. Both of these factors may be related to the difference we observed in the EPR to HDM challenge after NO_2 exposure.

While we did not find that NO_2 exposure led to enhanced allergen-induced neutrophils or eosinophils in induced sputum obtained 6 hours following inhalational challenge, there were non-significant increases in all four inflammatory mediators measured (IL-5, IL-8, GM-CSF, and ECP) after NO_2 exposure as compared to filtered air. We also found a statistically significant decrease in eosinophils and an increase in IL-5/TP ratio at 6 hr after NO_2. There may be decreased transit of eosinophils across the bronchial mucosa associated with increased activation and cytolysis (Erjefault et al., 1999 and 2000) after mild NO_2-induced oxidant injury (hence explaining the apparent inconsistency between decreased sputum eosinophils and increased ECP after NO_2). Bronchial tissue sampling is required to determine whether this occurs. Two recent toxicological studies using ovalbumin (OVA)-sensitized animal models support the concept that NO_2 exposure might actually cause reduced instead of increased allergen-induced airway eosinophilia. One study using BALB/c mice showed that a 3-hour exposure to 5 ppm NO_2 caused a marked reduction in BAL eosinophils with a subsequent OVA challenge compared to air-OVA controls (Proust et al., 2002), and a second study using C57B1/6 mice showed that 2-hour exposures to NO_2 at either 0.7 or 5 ppm on 3 consecutive days reduced BAL eosinophil levels compared to air-OVA controls (Hubbard et al., 2002).

When we designed and began data collection for this project, there were no published reports addressing the airway inflammatory response to inhaled allergen following exposure to NO_2 in human subjects. Recently, however, the results of such a study have been reported by Barck and coworkers (Barck et al., 2002). These investigators found a NO_2-related increase in neutrophils, but not eosinophils, measured using BAL

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19 hours after allergen challenge. Because a different NO_2 exposure regimen and allergen challenge protocol were used by Barck and coworkers, their results and ours can not be directly compared. A methodological difference between the two studies is that we used a multi-dose allergen challenge protocol to achieve a 20% decrease in FEV_1 rather than a fixed-dose allergen challenge. In addition, we recruited subjects sensitized to *D. pteronyssinus*, while the Swedish investigators recruited subjects sensitized to either one of two pollens (birch and timothy). The timing of the allergen challenge following exposure to NO_2 may also be an issue; in our study subjects underwent allergen challenge immediately after a 3-hour exposure to NO_2 whereas in Barck et al.'s study allergen challenge was performed 4 hours after a 30-minute exposure.

One interesting finding that both Experiment One and the Barck et al. study do share is a failure to observe an overall group enhancement of the EPR to allergen challenge by pre-challenge exposure to NO_2. As noted above, a number of studies by several groups, including members of the Barck study team, have previously demonstrated such an NO_2 -enhancing effect (Tunnicliffe et al, 1994; Devalia et al., 1994; Rusznak et al., 1996; Strand et al., 1997 and 1998; Jenkins et al., 1999; Svartengren et al., 2000). A close examination of the individual data from these studies reveals that enhanced sensitivity to inhaled allergen is not a universal result following NO_2 exposure. In fact, there are "responders" and "non-responders" in every study. Understanding the basis of this differential response to NO_2 among asthmatic individuals should be a focus of future research. One possibility is that genetically determined differences in antioxidant defenses play a critical role in mediating the effects of an oxidant pollutant like NO_2. Previous work has suggested that individuals with the GSTM1 null genotype (involving the complete absence of this antioxidant enzyme) have increased risk of adverse effects from exposure to ambient ozone and diesel exhaust particles (Bergamaschi et al., 2001; Romieu et al., 2004; Diaz-Sanchez et al., 2004). This genotype, as well as other polymorphisms in antioxidant enzyme genes, may also be a determinant of susceptibility to NO_2. Of course, other factors may determine susceptibility such as dietary antioxidant intake, smoking, and polymorphisms in genes involved in innate and acquired immune responses.

A potential limitation of the current study is the method used to sample airway lining fluid, sputum induction. We chose this method because it less invasive and less costly in terms of both time and resources than fiberoptic bronchoscopy with BAL. We have experience with both techniques in assessing the inflammatory response to ozone, and have directly compared them in one study (Arjomandi et al., 2003). There is considerably greater within-subject variability in sputum neutrophils than in BAL neutrophils in asthmatic subjects leading to a greater signal-to-noise ratio with the former technique when used to assess a pollutant-induced inflammatory response. In other words, our sample size may have been too small to have detected a significant effect of NO_2 exposure on the airway inflammatory response to subsequent allergen challenge given the "noise" inherent in using induced sputum data. A larger number of subjects may have allowed us to detect an effect of NO_2. Moreover, if the primary site where NO_2 affects allergen-induced inflammation is the peripheral airways (Miller et al.,

1982), then BAL may be a more appropriate method for sampling these airways than induced sputum.

Other potential limitations of Experiment One relate to the experimental protocol. It is possible that the exposure to NO_2 (0.4 ppm for 3 hours with intermittent exercise) was too low to induce enhancement of the late airway inflammatory response to inhaled allergen. We selected this exposure protocol because it had been used in several studies by other investigators who reported NO_2 enhancement of the early phase response to the allergen we used, *D. pteronyssinus,* but a larger effective dose of NO_2 or a longer duration of exposure may be required to affect the late phase response. However, the NO_2 exposure studied by Barck et al. was actually lower than what we used (Barck et al., 2002). The timing of the allergen challenge in our study also might be an issue. Perhaps a delay of several hours after the NO_2 exposure would allow for development of sufficient non-specific airway inflammation to provide greater enhancement of allergen-induced inflammatory responses.

Another limitation is the allergen challenge dose regimen. Ideally the same dose of allergen would be used for both arms of the study. However, given the suspected enhancement of the bronchoconstrictor response to inhaled allergen by prior exposure to NO_2, and that previous studies had used a multiple dose regime (Rusznak et al., 1996; Strand et al., 1997; Jenkins et al., 1999), we decided against the use of a single dose on the basis of subject safety. In retrospect, the responder group inhaled less allergen after NO_2 exposure, which may have decreased the inflammatory response and made it difficult to find a statistically significant inflammatory effect of NO_2. In addition, allergen challenge performed immediately after NO_2 exposure may not be at the optimal time. Rusznak et al. have shown that the enhancement of airway responsiveness to inhaled allergen in asthmatic subjects may be maximal at 24 hours after combined NO_2 and sulfur dioxide exposure (Rusznak et al., 1996). However, the maximal airway inflammatory response to allergen challenge may occur at a different time point than the maximal bronchoconstrictor response to allergen.

In Experiment Two, using a protocol that provided a similar cumulative NO_2 exposure (0.4 ppm for 3 hours) to what the two previous studies of asthmatic subjects had used (Vaggagini et al., 1996, 0.3 ppm for 2 hours; Jorres et al., 1995, 1.0 ppm for 1 hour), we confirmed that this level of NO_2 exposure does not induce changes in inflammatory cell distribution. We also found no differences in two important cytokines associated with nonspecific airway inflammation, IL-6 and IL-8. Taking our results together with those of the previous studies (Vaggagini et al., 1996; Jorres et al., 1995), it seems unlikely that short-term exposure to peak levels of NO_2 that occur in the ambient air in California induce nonspecific airway inflammation in asthmatic subjects. This conclusion is subject to several caveats, however. All three studies involved small numbers of subjects. Both Experiment Two and that of Vaggagini et al. used induced sputum to assess airway inflammation and, as noted above, this method may not have sufficient sensitivity to detect mild effects of NO_2 exposure in small numbers of subjects. Although the Jorres et al. study did use the more sensitive method of BAL to detect airway inflammation, as well as a higher concentration of NO_2, bronchoscopy was

performed only 1 hour after the 1-hour exposure, which may have been too short an interval for changes in airway inflammatory cells to have developed.

The concentrations of NO_2 used in all of the controlled human exposure studies discussed here were at or above the hourly peak exposures currently observed at regional monitoring stations in California, although it is possible that individuals who live close to major roadways or drive in heavily congested traffic may be exposed to higher levels. The lack of effect of inhaled NO_2 at even these high ambient levels on airway inflammatory cell distribution in controlled exposure studies of asthmatic subjects suggests that another mechanism or factor is responsible for the associations between NO_2 exposure and adverse asthma-related health outcomes consistently observed in epidemiological studies. Perhaps the observation by Jorres and coworkers of increased BAL prostanoid concentrations holds a clue to the pathway by which NO_2 exposure can exacerbate asthma. Another possibility is that NO_2 is not actually playing a role in inducing asthma exacerbations, but is only a marker for exposure to the relevant pollutant.

Summary and Conclusions

In conclusion, 3-hour exposure to a high ambient concentration of NO_2 did not enhance airway responses to a subsequent allergen challenge in most asthmatic subjects studied (Experiment One), nor did it cause nonspecific airway inflammation in a second group of allergic asthmatic subjects (Experiment Two). An important caveat to these results is that the assessment of airway inflammation in both experiments involved the analysis of induced sputum, which may be less sensitive than the analysis of BAL fluid. The most notable finding related to NO_2 exposure was a decrease in allergen-induced eosinophils in sputum obtained 6 hours after inhalational challenge in Experiment One. A subset of subjects also experienced marked enhancement of the early phase response to allergen in Experiment One.

Recommendations

Future research in the area of NO_2 modulation of airway responses to inhaled allergen in asthmatic individuals should include efforts to understand the mechanisms underlying the decrease in airway eosinophils, the characteristics that determine the between-subject variability in response, the effect of longer-term vs. short-term NO_2 exposure, whether the responses of asthmatic children are different than those of adults, the comparability of results when induced sputum vs. BAL is used to sample airway lining fluid, and the specific patterns of response in the "responsive" subgroup. More research is also needed on the mechanism(s) underlying the consistent association between NO_2 exposure and adverse asthma-related health outcomes observed in epidemiological studies.

Table 10. Table of Abbreviations

Exposure conditions:
FA = filtered air
NO2 = NO2 at a concentration of 0.4 ppm

Sputum induction:
SI = sputum induction
S-6 = sputum induction at 6 hours post-allergen challenge in Experiment One
S-26 = sputum induction at 26 hours post-allergen challenge in Experiment One

Allergen:
HDM = house dust mite
D. pter = Dermatophygoides pteronyssinus

Biochemical analyses:
ECP = eosinophilic cationic protein
IL-5 = interleukin 5
IL-6 = interleukin 6
IL-8 = interleukin-8
GM-CSF = granulocyte/macrophage colony stimulating factor

Spirometry:
FVC = forced vital capacity
FEV1 = forced expired volume in 1 s
FEF25-75 = forced expired flow-rate at 25-75% FVC
FEF75 = forced expired flow-rate at 75% FVC

Inhalation Challenges:
MPC20 = methacholine provocative concentration causing 20% reduction in FEV1
APC20 = predicted allergen provocative concentration causing 20% reduction in FEV1
PD20 = cumulative D. pter allergen provocative dose causing 20% reduction in FEV1

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Witten A, Solomon C, Abbritti E, Arjomandi M, Zhai W, Kleinman M, Balmes J. Effects of nitrogen dioxide on allergic airway responses in subjects with asthma. Thorax (submitted).

Abstracts

Witten A, Abbritti E, Kleinman M, Arjomandi M, Balmes J, Solomon C. Effects of nitrogen dioxide on allergic airway responses in subjects with asthma. Am J Respir Crit Care Med 2003, 167, No. 7:A500.

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