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Interference of a short-term exposure to nitrogen dioxide with allergic airways responses to allergenic challenges in BALB/c mice.

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Abstract

Nitrogen dioxide (NO₂) is a common indoor and outdoor air pollutant whose role in the induction of asthma is unclear. We investigated the effects of NO₂ on the development of asthma-like responses to allergenic challenge in BALB/c mice. Ovalbumin (OVA)-immunized mice were intranasally challenged with OVA or saline solution just before starting a 3 h exposure to 5 or 20 ppm NO₂ or air. 20 ppm NO₂ induced a significant increase of bronchopulmonary hyperreactivity in OVA-challenged mice and of permeability according to the fibronectin content of the bronchoalveolar lavage fluid (BALF) 24 h after exposure, as compared to air or 5 ppm. Eosinophilia (cell counts in the BALF and eosinophil peroxidase of lung tissue) was detected at 24 and 72 h with similar levels for air and 20 ppm NO₂ whereas, unexpectedly, a marked reduction was observed for 5 ppm. At 24 h, IL-5 in the BALF was markedly reduced at 5 ppm compared to 20 ppm NO₂ and was also more intense for 20 ppm than for the air group. In contrast to specific IgG1 titers, anti-OVA IgE titers and IL-4 in the BALF were not affected by NO₂ exposure. Irrespective of the concentration of NO₂, OVA-challenged mice did not develop late mucosal metaplasia compared to those exposed to OVA-air. These results indicate that a short exposure to NO₂ can exacerbate or inhibit some features of the development of allergic disease in mice and may depend to concentration of pollutant.

Keywords: Mouse model of asthma; nitrogen dioxide; air pollutant; bronchopulmonary hyperreactivity; lung permeability; eosinophilia; mucus.
Abbreviations

Bovine serum albumin, BSA
Bronchoalveolar lavage fluid, BALF
Bronchopulmonary hyperreactivity, BHR
Enzyme-linked immunosorbent assay, ELISA
Eosinophil peroxidase, EPO
Intranasally, i.n.
Interleukin, IL
Methacholine, Mch
Optical density, OD
Ovalbumin, OVA
Phosphate-buffered saline, PBS
Room temperature, RT
Introduction

Asthma is an allergic respiratory disease that has captured a great deal of attention for several years. One of its perplexing aspects is that its prevalence has increased steadily during this century, doubling in the last 20 years in most industrialized countries (Hartert et al., 2000). Although asthma is familial and genome-wide searches having identified genetic loci predisposing to the disease, it is unlikely that the genetic makeup of stable populations can change significantly in less than one century. The probable cause of the epidemic must therefore relate to the environment (Hartert et al., 2000). Several recent studies have shown an association between air pollution during episodes of smog and asthma exacerbations, and hospital visits for asthma (Anderson et al., 1998; Morgan et al., 1998; Sheppard et al., 1999). Although this finding does not address causality, it supports that air pollution has an effect on acute asthmatic episodes. Current evidence also suggests that asthmatics are more sensitive to the effects of air pollutants (Koren, 1995).

Asthma is characterized by acute bronchoconstriction, late bronchopulmonary hyperreactivity, pulmonary eosinophilic inflammation, excessive mucus production and increased serum IgE titers (Djukanovic et al., 1990), induced by a variety of stimuli. Its pathology seems to be directly linked to the presence in the airways of eosinophils and of Th2 CD4+ lymphocytes (Nakajima et al., 1992; Robinson et al., 1992), which produce IL-4 and IL-5. These Th2 cytokines are said to play a central role in the initiation and perpetuation of asthma (Bochner et al., 1994). IL-5 regulates the growth, differentiation and activation of eosinophils and provides an essential signal for their recruitment to the lungs during allergic inflammation (Lopez et al., 1988). IL-4 induces the differential development of Th0 into Th2 cells (Abehsira et al., 1992), and stimulates B lymphocytes to produce IgE (Paul, 1991).
Nitrogen dioxide (NO₂), a major potent oxidant pollutant, is a well-known airway irritant (American Thoracic Society, 1996). In contrast to other pollutants, NO₂ is a widespread contaminant of outdoor and of indoor environment. Its indoor levels can exceed those found outdoor and are provided by gas cooking appliances and tobacco smoke (Goldstein et al., 1988; Norman et al., 1965). By contrast, the main sources of NO₂ in outdoor air are motor vehicle emissions and fossil-fuel burning industries (American Thoracic Society, 1996). Acute exposures to high concentrations of NO₂ produce changes in pulmonary function, increase airway responsiveness (Mohsenin et al. 1987), and induce pulmonary edema (Hajela et al., 1990). NO₂ also may cause release of inflammatory mediators, and induce mast cell and lymphocyte infiltration (Sandstrom et al., 1991). Animal studies have demonstrated that exposure to NO₂ can increase susceptibility to infection, presumably through its effect on lung defense mechanisms, mucociliary clearance (Hubbard et al., 1994), and alveolar macrophage function (Davis et al., 1992; Robison et al., 1993). NO₂ is tissue-soluble, unsaturated bonds in membrane lipids of the airway and respiratory epithelium, as well lining fluid being its prime targets (Postlethwait et al., 1994). Several studies have associated morbidity of asthma with elevated concentrations of NO₂ (Ostro et al., 1994; Tunicliffe et al., 1994). Based on epidemiological studies, it has been suggested that NO₂ increases the risk for exacerbations of asthma (Castellsague et al., 1995; Rossi et al., 1993). Clinical observations have demonstrated that exposure to NO₂ correlates with exacerbations of asthma and with the potentiation of airway reactivity in asthmatics (Strand et al., 1997; Strand et al., 1996), in contrast to other studies, which have failed to show an association between exposure to NO₂ and increased incidence of asthma (Samet et al., 1993). These discrepancies show that the role of NO₂ for allergic disease is still unclear.
Since NO₂ can increase the bronchial responsiveness to non-specific stimuli such as histamine and methacholine, it may hypothetically affect bronchial responsiveness and other features of asthma to inhaled allergen as well. The existence of such an interaction between NO₂ and allergen resulting in an augmented allergic reaction has been suggested (Molfino et al., 1991; Strand et al., 1997; Tunicliffe et al., 1994). In this study, we investigated the role of NO₂ in the exacerbation of asthma in an ovalbumin-immunized mouse model. For that, we studied whether a short-term exposure to low or high concentrations of NO₂ can potentiate the responses to allergenic challenge in OVA-immunized mice, in order to define its possible adjuvant role in the development of some features of asthma, such as bronchopulmonary responsiveness, eosinophilic pulmonary inflammation, production of Th2 cytokines, and of specific IgE and mucus secretion.
Materials and Methods

Animals
Male strain BALB/c mice (6-7 weeks of age, 23 ± 2 g body weight) purchased from the Centre d’Elevage R. Janvier (Le Genest Saint-Isle, France) were housed (10 mice per cage) in the INERIS animal-care unit, a facility accredited by the Departmental Direction of Veterinary Services. The animals were maintained on a 12 h light/dark cycle, at 20-24°C with relative humidity of 40-70 %, and had free access to conventional laboratory feed and water. Animals were handled in accordance with French State Council guidelines for the care and use of laboratory animals (Decree n°87-849, October the 19th 1987), and was approved by the Institutional Animal Care and Use Committee at the INERIS.

Antigen immunization and challenge
BALB/c mice were immunized by the subcutaneous injection of 10 μg ovalbumin (OVA, ICN Biomedicals, Inc., Aurora, OH), dispersed in 1.6 mg Al(OH)₃ (Merk, Darmstadt, Germany) in 0.4 ml of 0.9 % NaCl (saline) at Days 0 and 7. At Day 14, one week after the second injection, immunized mice were intranasally (i.n.) challenged with 10 μg OVA in 50 μl of saline for about 10 s under anesthesia by intravenous injection of ketamine (Imalgene®1000, 35 mg/kg, Merial, France). Control mice were challenged with the same volume of saline solution.

Exposure system
The whole body exposure system used to generate NO₂ and expose the animals was developed in the laboratory of INERIS. Unrestrained, and conscious mice challenged with OVA or saline were individually placed in a whole body glass chamber of 0.5 l and were
exposed to 5 or 20 ppm NO₂ or to air for 3 h. In each glass chamber, NO₂ was delivered with a flow rate of 5 l/min allowing to have a renewal, and was calibrated at the exact desired concentration in synthetic air. The airflow of NO₂ in each chamber was monitored by a mass flow-meter during all the period of exposure. The relative pressure of the glass exposure chamber was controlled by a manometer. Concentrations of 5 or 20 ppm of NO₂ were obtained from cylinders of NO₂ gas prepared and certified by the supplier (Air Liquide, France).

**Evaluation of bronchopulmonary hyperreactivity**

BHR was evaluated with a barometric plethysmography method. Unrestrained, conscious mice were placed in a whole body plethysmographic chamber (EMKA Technologies, Paris, France) which measured the respiratory waveforms. Animals were exposed to an aerosol of methacholine (Mch, Aldrich, Milwaukee, WI) for 20 s at 0.1 M delivered by an aerosolator. The index of airway obstruction was expressed as enhanced pause (Penh), in response to inhaled Mch (Hamelmann et al., 1997). Penh was calculated as: Penh = [Te (expiratory time)/Tr (relaxation time)-1] × [Pef (peak expiratory flow)/Pif (peak inspiratory flow)]. For the graphic representation, each value was expressed every minute and was calculated from the average of three values of Penh recorded every 20 s. In order to simplify the interpretations, the area under the curve (A.U.C) was calculated for 15 min. The graphics in terms of A.U.C represent the quantitative expression of BHR.

**Serum sample preparations and bronchoalveolar lavage fluid (BALF)**

Mice were anesthetized by the intraperitoneal injection of urethane (ethylcarbamate, 2 g/kg, Sigma, St Louis, MO) and the abdominal cavity was opened. Blood samples were collected from the post vena cava and serum was collected after centrifugation at 500 × g for
10 min and stored at -20°C until used. After exsanguination, the lungs were flushed via the cannulated trachea with $4 \times 0.5$ ml of a sterile PBS solution (phosphate buffer 10 mM, pH 7.4). The total cell numbers were counted automatically (Coulter Counter ZM, Coultronics, Margency, France). BALF was cytocentrifuged for 10 min (Cytospin, Shandon, England). Slides were stained with a May Grünwald Giemsa-derived method (Diff Quick, Baxter Dade AG, Duedingen, Switzerland), and a total of 200 cells was counted for each sample by light microscopy, the percentage of each cell population being calculated. The BALF was centrifuged for 10 min at 1850 × g, 4°C (Jouan, Saint Herblain, France) and the supernatants were removed and stored at -20°C until used.

**Evaluation of lung eosinophil peroxidase activity**

To quantify the lung sequestration of eosinophils, eosinophil peroxidase (EPO) activity in the lung was evaluated in 96-well plates by a cytochemical enzyme assay (Van Oosterhout et al., 1995). Briefly, lungs were removed and homogenized (Potter-Elvejem glass homogenizer, Thomas, Philadelphia, PA) in 0.05 M Tris-HCl buffer pH 8 containing 0.1% Triton X-100 solution. Lung homogenates were centrifuged for 15 min at 1600 × g, 4°C (Sigma, Bioblock Scientific 2K15, Illkirsh, France). EPO activity was measured in the supernatant, based on the oxidation of o-phenylenediamine (Sigma) by EPO in the presence of peroxide hydrogen. Incubations in duplicate were carried out in the absence or presence of the peroxidase inhibitor 3-amino-1,2,4-triazole (AMT, Sigma). Plates were read with an automatic microplate reader (Dinatetech MR 5000, Dinatech Laboratories, Saint Cloud, France) at 490 nm and results were expressed as optical density (OD).
**Evaluation of cytokines**

IL-4 in the BALF was evaluated by ELISA. Briefly, 96-well plates were coated with rat anti-mouse IL-4 (BVD4-1D11, Perbio Sciences, Erembodegem-Aalst, Belgium) at 2 µg/ml diluted in 0.1 M carbonate buffer pH 8.2 and incubated overnight at 4°C. Plates were washed with PBS/0.1% Tween 20 and blocked with PBS containing 1% of bovine serum albumin (BSA) overnight at 4°C. After washing, dilutions of recombinant murine IL-4 (rIL-4, Perbio Sciences) (15.6 to 1,000 pg/ml) or samples were applied overnight at 4°C. Then, biotinylated rat anti-IL-4 antibody (BVD6-24G2, Perbio Sciences) was added at 0.5 µg/ml for 2 h at 4°C. Plates were incubated with ExtrAvidin® peroxidase conjugate (1:2,000 to each well, Sigma) for 45 min at room temperature (RT). Plates were developed with tetramethylbenzidine substrate (TMB, Kiregaard Perry Laboratories, Maryland). The reaction was stopped with sulfuric acid 2N and the plates were read at 450 nm with a microplate reader (Dinatech MR 5000, Dinatech Laboratories). The lower limit of detection of this assay is ≈ 10 pg IL-4/ml sample.

IL-5 in the BALF was quantified using an immunometric assay as described previously (Eum et al., 1995). Briefly, 96-well plates were coated with 10 µg/ml of rat anti-mouse IL-5 (TRFK-4). To these, were added dilutions of recombinant IL-5 (rIL-5) standard (7.6 to 1,000 pg/ml) or of the sample was added, followed by an acetylcholinesterase-labelled rat anti-mouse IL-5 antibody (TRFK-5) at 10 Ellman units/ml. Absorbance was read at 405 nm with a microplate reader (Dinatech MR 5000; Dinatech Laboratories). The lower limit of detection of this assay is ≈ 5 pg IL-5/ml sample.

**Evaluation of anti-OVA specific IgE and IgG1**

The specific anti-OVA specific IgE and IgG1 in the serum were measured by ELISA.
For the determination of specific IgE, 96-well plates were coated with rat anti-mouse IgE (EM 95-3) at 5 µg/ml diluted in 0.5 M carbonate buffer pH 9.6 and incubated overnight at 4°C. The next day, plates were washed with PBS/0.1% Tween 20 and blocked with PBS containing 1% of BSA for 2 h at RT. After blocking, plates were washed and serum samples were added and incubated overnight at 4°C. The plates were then washed and 10 µg/ml of biotinylated OVA was added. The remaining steps were performed exactly as described for IL-4.

For the evaluation of specific IgG1, 96-well plates were coated with OVA (ICN Biomedicals) at 10 µg/ml diluted in 0.1 M carbonate buffer pH 8.2 and incubated overnight at 4°C. Plates were washed with PBS/0.1 % Tween 20 and blocked with PBS containing 1 % of BSA for 1 h at 37°C. After blocking and washing, serum samples were added and incubated for 1 h at 37°C. Plates were washed and incubated with Goat anti-mouse IgG1 alkaline phosphatase conjugated (1:2,000 to each well, Caltag Laboratories, Burlingame, CA) for 1 h at 37°C. The colorimetric reaction was initiated with p-nitrophenylphosphate (1 mg/ml) (Sigma) at 37°C.

As a positive control, serum pooled from OVA-immunized and challenged mice was used. Data from serum samples were expressed according to the absorbance of positive control serum after subtracting the buffer-only blank data from both. An index was calculated as: IgE or IgG1 index = [OD sample- OD buffer only]/[OD positive control- OD buffer only].

**Evaluation of fibronectin**

In order to evaluate the intensity of exudation through the airways, fibronectin in the BALF was measured by the indirect competitive ELISA method described by Rennard et al. (1980). Briefly, 96-wells plates were coated with murine fibronectin (Anawa, Wangen, Switzerland) at 1 µg/ml diluted in 0.02 M carbonate buffer pH 9.6 and incubated overnight at 4°C.
another 96-wells plates, BALF samples and standard fibronectin were incubated with a polyclonal rabbit anti-fibronectin antibody (1:10,000, Anawa). The next day, plates coated with fibronectin were washed with PBS/0.1% Tween 20 and blocked with PBS containing 1% of BSA for 2 h at RT. Then, plates were rinsed with PBS/0.1% Tween 20 and 100 μl of antigen-antibody were applied to plates and incubated for 2 h at RT. After washing, the anti-fibronectin antibody that did not bind with BALF fibronectin content was detected with a biotinylated secondary anti-rabbit antibody (1:1,500, Amersham Pharmacia Biotech, France) and incubated for 2 h at RT. Plates were washed and incubated with ExtrAvidin® peroxidase conjugate (1:20,000 to each well, Sigma) for 45 min at RT. The remaining steps were performed exactly as described for IL-4. The lower limit detection of this assay is ≈ 0.078 μg fibronectin/ml sample.

**Lung histology**

After exsanguination, the lungs were removed and fixed by intratracheal instillation with 10% neutral phosphate-buffer formalin. The whole lung was embedded in paraffin, sectioned at a thickness of 5 μm and stained with periodic acid Schiff (PAS) to examine mucus cells in the airway walls.

**Statistical analysis**

The results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between groups were made using ANOVA. Multiple comparisons between all groups were performed by Fisher’s least-significant difference test. A value of \( P \leq 0.05 \) was considered to be significant.
Results

In order to investigate the influence of NO₂ on the responses to the i.n. administration of OVA in immunized mice, two concentrations of NO₂ that are relatively high as compared to those encountered in the environment were used, since the actual amounts delivered to the lung are quite below the concentrations inhaled (Hatch et al., 1994; Kleeberger et al., 1997). We have developed a murine model of allergen-induced pulmonary inflammation sharing the essential features of asthma, in which the two time points of 24 and 72 h after allergenic challenge allowed to evaluate the parameters of the asthma phenotype. One week after the booster injection of antigen, OVA-immunized mice were challenged i.n. with 10 μg of OVA or saline just before the exposure to air or to NO₂ at 5 or 20 ppm for 3 h. The time points of 24 and 72 h were studied after NO₂ exposure. The peak of BHR, the initial phase of pulmonary eosinophil entrapment, and the peak of production of Th2 cytokines were studied at 24 h, whereas at 72 h, the increase of pulmonary eosinophilic inflammation, the late mucosal metaplasia development and specific immunoglobulins in serum were determined.

Bronchopulmonary responsiveness to methacholine

At Day 15, i.e., 24 h after the exposure to 20 ppm NO₂ or to air, immunized BALB/c mice challenged with OVA expressed significant BHR as compared to those exposed to 5 ppm NO₂ (Figure 1). Mice challenged with saline exposed to air or to NO₂ did not develop BHR. In OVA-challenged mice exposed to 20 ppm NO₂, BHR was significantly increased as compared to those exposed to air and to 5 ppm. At Day 17, 72 h after exposure, OVA-challenged mice exposed to air, or to NO₂ failed to develop BHR (Figure 1).
**Fibronectin exudation into the BALF**

To evaluate the exudation through the airways, the concentration of fibronectin was measured in the BALF. OVA-challenged mice exposed to air or to 20 ppm NO₂ released significantly increased amounts of fibronectin in the BALF, whereas those levels were very low in animals exposed to 5 ppm NO₂ (Figure 2). In contrast, exposure to 20 ppm NO₂ augmented by 6-7 fold the fibronectin concentrations in the BALF of OVA-challenged mice after 24 h, as compared to the OVA-air group. NO₂ at 20 ppm also induced a marked fibronectin exudation in saline-challenged mice, as compared to the saline-air or saline-NO₂ 5 ppm groups. Seventy two hours after exposure, BALF fibronectin content persisted in saline- or OVA-challenged mice exposed to 20 ppm NO₂. At this time point, in OVA-challenged mice exposed to 5 ppm NO₂ or to air, the release of fibronectin was delayed, as compared to mice exposed to 20 ppm NO₂ (Figure 2). Indeed, 24 h after exposure to air or to 5 ppm NO₂, no or low fibronectin was detected in the BALF of OVA-challenged mice. But, 72 h after exposure, increased amounts of fibronectin were found, contrary to mice exposed to 20 ppm NO₂, in which exudation started at 24 h.

**BALF cell infiltration and lung sequestration of eosinophils**

At 24 h, neutrophil counts were increased in the BALF of OVA-challenged mice exposed to air or to NO₂. These counts were found significantly increased and in OVA-challenged mice exposed to 20 ppm, as compared to OVA-air mice. The increase in neutrophil counts in saline-challenged animals exposed to 20 ppm NO₂ was also significantly above that found in saline-air or saline-5 ppm NO₂ mice (Figure 3A). At 72 h, neutrophil counts were normalized in all groups of mice (Figure 3A). Eosinophils in the BALF were detected at 24 h and increased at 72 h, with similar numbers in OVA-challenged mice exposed to air or to 20 ppm NO₂, as compared to those exposed to 5 ppm, in which these counts were markedly reduced.
after 24 and 72 h (Figure 3B). The EPO activity in the lung increased significantly in OVA-challenged mice exposed to air at 24 and 72 h. OVA-challenged mice exposed to 5 ppm NO₂ showed a marked reduction of EPO activity at 24 and 72 h, as compared to the OVA-air group, which correlated with the reduced eosinophil counts in the BALF. Significant reduction of EPO activity was also noted in saline-challenged mice exposed to 5 ppm NO₂, as compared to those exposed to air. This contrasts with results in mice exposed to 20 ppm NO₂ or to air, in which EPO titers were increased to similar levels (Figure 3C).

**Th2 cytokine production in the BALF**

OVA-challenged mice exposed to air or to NO₂ released IL-5 and IL-4 in the BALF at 24 h. In connexion with eosinophilia (cell counts and EPO in the lungs), the production of IL-5 in the BALF was significantly reduced in OVA-challenged mice 24 h after exposure to 5 ppm NO₂, as compared to those exposed to 20 ppm NO₂ or to air (Figure 4A). However, the production of IL-5 was increased 3-fold in OVA-challenged mice exposed to 20 ppm NO₂, as compared to the OVA-air group, and was augmented by 10-fold, as compared to those exposed to 5 ppm (Figure 4A). By contrast, the production of IL-4 in the BALF was increased to the same extent in the three groups 24 h after exposure (Figure 4B). No IL-4 nor IL-5 were detected in the BALF at the 72 h point (Figures 4A and 4B).

**Production of IgE and IgGl anti-OVA antibody in the serum**

Saline- and OVA- challenged mice exposed to air or to NO₂ produced specific IgE and IgGl detected in the serum 24 and 72 h later. The anti-OVA IgE titers increased to a similar extend in the three groups after 72 h (Figures 5A), and the both concentrations of NO₂ failed to affect their production. Specific IgGl titers were markedly increased as compared to IgE, without significant differences between saline and OVA-challenged mice. Mice exposed to 5 ppm
NO₂ and challenged with saline or OVA showed a significant increase of IgG1 titers as compared to those exposed to air or to NO₂ at 20 ppm (Figure 5B).

**Histological analysis**

As expected, the bronchial epithelium of saline-challenged immunized mice exposed to air was mucus-free (Figure 6A), under conditions where that of immunized and OVA-challenged mice was markedly enriched in mucosal cells (Figure 6B). Saline- or OVA-challenged immunized mice exposed to NO₂ at 5 or 20 ppm did not develop mucosal metaplasia as compared to OVA-challenged mice exposed to air (Figures 6D and 6F).
Discussion

Studies in asthmatics have shown that air pollutants such as ozone (Molfino et al., 1991), or NO₂ augment the allergic responses (Jenkins et al., 1999; Strand et al., 1998; Strand et al., 1997; Tunicliffe et al., 1994). At high concentrations, NO₂ is a well-known airway irritant that can cause bronchial constriction in normal subjects (Hajela et al., 1990; Mohesin, 1988), and enhance airway responsiveness to histamine (Bylin et al., 1988) or methacholine (Mohesin, 1987) in asthmatic patients, which are more sensitive than healthy subjects. By contrast, few studies have addressed to the interaction between NO₂ and lung allergy in experimental animals. As shown here, a short-term exposure to NO₂ produces contrasting effects on the development of asthma-related responses in an OVA-immunized mouse model, which depend on the dose of NO₂. Thus, the high dose of 20 ppm potentiated BHR, exudation and release of IL-5 in the BALF after OVA challenge, under conditions where the low dose of 5 ppm failed to modify BHR, and reduced significantly pulmonary eosinophilic inflammation and the production of IL-5 in the BALF. Since a single exposure to NO₂ was used here, both doses were relatively high as compared to those encountered in the environment. It has been reported nevertheless that the final amounts delivered to the lungs are quite below the concentrations inhaled (Hatch et al. 1994; Kleeberger et al., 1997).

The potentiation of BHR by 20 ppm NO₂ in allergic mice may be accounted for by an increased vascular/epithelial permeability, facilitating the allergen availability and accelerating the inflammatory process. NO₂ is a potent tissue-soluble oxidant, which can induce pulmonary edema at high concentrations. In our experiments, immunized animals treated with saline and exposed to 20 ppm NO₂ had increased titers of fibronectin in the BALF, which was used as an indicator of permeability, as compared to saline-air or to saline-
5 ppm NO₂ groups. The high concentration of NO₂ potentiated the effects of OVA, since the fibronectin BALF content was significantly increased in OVA-challenged mice exposed to 20 ppm, as compared to OVA-air or to OVA-5 ppm NO₂ groups, and correlated with the increased BHR. Even though BHR disappeared with time, the increased permeability persisted 72 h after exposure to 20 ppm, whereas a more delayed increase in exudation was observed in OVA-challenged mice exposed to air or to 5 ppm of NO₂. The low concentration of NO₂ had an effect similar to that of OVA alone. Since our mice underwent a single exposure to NO₂, it is possible that such low concentrations of NO₂ become effective upon repeated exposures. In this context, the most likely mechanism for NO₂-induced increment in bronchial sensitivity to inhaled allergens is the damage of epithelial cells mediated by its oxidative activity, which may increase the cell permeability to the allergen, thus increasing its delivered dose, as occurs in cultured human epithelial cell monolayers exposed to NO₂ over short periods (Devalia et al., 1993). This would account for the augmented fibronectin titers in the BALF, after its exudation from the plasma. It is also possible that NO₂ acts as a permissive agent, by allowing other factors to exacerbate asthma or that underlying factors such as the intensity of allergy or inflammation may be a prerequisite for the expression of the detrimental effects of the gas. Finally, NO₂ can reduce the mucociliary activity of the airways in vivo (Helleday et al., 1995) and in vitro (Devalia et al., 1993), further enhancing the accessibility of the allergen to the epithelial cells, owing to its decreased clearance from the airways.

In our experiments, eosinophilia (cell counts and EPO lung content) and IL-5 in the BALF were significantly reduced in OVA-challenged mice exposed to 5 ppm. By contrast, 20 ppm NO₂ did not affect eosinophilia, under conditions where the production of IL-5 in the BALF was significantly increased as compared to the OVA-air group. Recently, Morris et al. (2001)
reported that the exposure to 0.7 ppm NO\textsubscript{2} reduces eosinophilic inflammation in allergic mice, but the IL-5 levels were not measured. The mechanisms of the decreased eosinophilic inflammation and IL-5 production in mice exposed to 5 ppm NO\textsubscript{2} are unknown. They may result from alterations in the regional deposition patterns of OVA and of NO\textsubscript{2} in the airways, or in absorbance, pulmonary clearance or antioxidant defenses. Indeed, NO\textsubscript{2} has a low solubility, and is poorly absorbed by the airway mucosa (Chitano et al., 1995). It is also a very reactive molecule whose uptake in the respiratory system is extremely high (Chitano et al., 1995). The use of mathematical dosimetry models suggests that the uptake of NO\textsubscript{2} between the trachea and the respiratory zone occurs to a similar extent, and peaks at the terminal bronchioles (Chitano et al., 1995). It is also possible that the biphasic effect of 5 and 20 ppm NO\textsubscript{2} may result from pharmacodynamic alterations. Some studies have indeed demonstrated that NO\textsubscript{2} affects lung defense mechanisms, including mucociliary clearance, alveolar macrophages (AM), and the immune system (O’Neill et al., 1995). The decreased OVA-induced IL-5 production and eosinophilia after an exposure to 5 ppm NO\textsubscript{2} may result from an alteration of AM function, including differences in antigen presentation by AM, or from a decreased expression of antigen-derived peptides on their surface, which may be hampered by exposure to NO\textsubscript{2}. Indeed, Kineast et al. (1996) and Erroi et al. (1996) have shown that NO\textsubscript{2} exposure of LPS-stimulated human AM results in a functional impairment of AM. Furthermore, Robison et al. (1993) demonstrated that exposure rates to 0.5 ppm NO\textsubscript{2} for 0.5-10 days, reduces the arachidonate metabolism and superoxide production in response to external stimuli. Thus, NO\textsubscript{2} may reduce the capacity of AM to respond to immunologic stimuli, which might explain the decreased allergic responses in animals exposed to 5 ppm, in particular eosinophilia and production of IL-5.
Airway inflammation is accompanied by mucus secretion, which contributes to airway obstruction. In our experiments, irrespective of the concentration of NO₂, OVA-challenged mice did not develop mucosal metaplasia, in contrast to those exposed to air, which is probably related to mucus denaturation by NO₂-induced oxidation. The mucous layer forms a protective barrier of the airways against the effects of oxidants, which eliminates and/or scavenges the toxic components of NO₂ prior to their diffusion into the airway epithelium. The lipid content of the mucous layer, in particular esterified unsaturated fatty acids constitutes the primary scavenging oxidants (Cavanagh et al., 1987). The latter demonstrated that the phospholipids of the mucous layer cannot offer a significant protection against inhaled NO₂ and that exposure to 40 ppm NO₂ in rats induces in lipid peroxidation correlated with the apparent lack of oxidant scavenging species in the mucous lining the airways.

In summary, a short-term exposure to NO₂ modifies the asthma-like responses to allergenic challenge in BALB/c mice with contrasting effects according to its concentration. Despite the relatively high concentrations of NO₂ used in our investigation, as compared to those encountered in the environment, this study provides new informations concerning the subtle interactions between an air pollutant and allergic disease.
Acknowledgments

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**Figure legends**

**Figure 1.** *Bronchopulmonary hyperresponsiveness is significantly augmented in immunized mice exposed to 20 ppm*

At Day 14, immunized mice were challenged i.n. with saline (□) or OVA (■) and were exposed to 5 or 20 ppm NO$_2$ or to air for 3 h. At Day 15 (24 h) and 17 (72 h), BHR in response to Mch was evaluated. The graphic in terms of A.U.C represents the quantitative expression of BHR. Data are expressed as mean ± SEM. *$P < 0.05$, OVA-challenged mice compared with their saline control. **$P < 0.05$, OVA-Air group compared with NO$_2$-OVA group (5 or 20 ppm). *$P < 0.05$, saline-Air group compared with NO$_2$-saline group (5 or 20 ppm). $P < 0.05$, 5 ppm NO$_2$-OVA group compared with 20 ppm NO$_2$-OVA group. Δ$P < 0.05$, 5 ppm NO$_2$-saline group compared with 20 ppm NO$_2$-saline group.

**Figure 2.** *BALF fibronectin content after short exposure to NO$_2$*

At Day 14, immunized mice were challenged i.n. with saline (□) or OVA (■) and were exposed to 5 or 20 ppm NO$_2$ or to air for 3 h. At Day 15 (24 h) and 17 (72 h) after exposure, mice were sacrificed. Fibronectin levels were evaluated in the BALF by ELISA. Data are expressed as mean ± SEM. *$P < 0.05$, OVA-challenged mice compared with their saline control. **$P < 0.05$, OVA-Air group compared with NO$_2$-OVA group (5 or 20 ppm). *$P < 0.05$, saline-Air group compared with NO$_2$-saline group (5 or 20 ppm). $P < 0.05$, 5 ppm NO$_2$-OVA group compared with 20 ppm NO$_2$-OVA group. Δ$P < 0.05$, 5 ppm NO$_2$-saline group compared with 20 ppm NO$_2$-saline group.
Figure 3. BALF cell infiltration after exposures to NO2
At Day 14, immunized mice were treated as in Figure 2 (Saline (□) or OVA (■)). Differential BALF cell counts and EPO of the lungs were evaluated. A, neutrophils; B, eosinophils; C, eosinophil peroxidase. Data are expressed as mean ± SEM. *P ≤ 0.05, OVA-challenged mice compared with their saline control. #P ≤ 0.05, OVA-Air group compared with NO2-OVA group (5 or 20 ppm). *P ≤ 0.05, saline-Air group compared with NO2-saline group (5 or 20 ppm). P ≤ 0.05, 5 ppm NO2-OVA group compared with 20 ppm NO2-OVA group. ^P ≤ 0.05, 5 ppm NO2-saline group compared with 20 ppm NO2-saline group.

Figure 4. BALF Th2 cytokine levels after exposures to NO2
At Day 14, immunized mice were treated as in Figure 2 (Saline (□) or OVA (■)). Specific antibodies were evaluated in the serum by ELISA. BALF IL-4 and IL-5 content were evaluated by ELISA. A, IL-5; B, IL-4. Data are expressed as mean ± SEM. *P ≤ 0.05, OVA-challenged mice compared with their saline control. #P ≤ 0.05, OVA-Air group compared with NO2-OVA group (5 or 20 ppm). *P ≤ 0.05, saline-Air group compared with NO2-saline group (5 or 20 ppm). P ≤ 0.05, 5 ppm NO2-OVA group compared with 20 ppm NO2-OVA group. ^P ≤ 0.05, 5 ppm NO2-saline group compared with 20 ppm NO2-saline group.

Figure 5. Specific anti-OVA IgE and IgG1 titers in the serum after exposures to NO2
At Day 14, immunized mice were treated as in Figure 2 (Saline (□) or OVA (■)). Specific antibodies were evaluated in the serum by ELISA. A, IgE; B, IgG1. Data are expressed as mean ± SEM. *P ≤ 0.05, OVA-challenged mice compared with their saline control. #P ≤ 0.05, OVA-Air group compared with NO2-OVA group (5 or 20 ppm). *P ≤ 0.05, saline-Air group
compared with NO2-saline group (5 or 20 ppm). \( P \leq 0.05, \) 5 ppm NO2-OVA group compared with 20 ppm NO2-OVA group. \( ^{\Delta} P \leq 0.05, \) 5 ppm NO2-saline group compared with 20 ppm NO2-saline group.

Figure 6. **PAS-stained histologic sections of lungs from allergic mice exposed to NO2**

At Day 14, immunized mice were treated as in Figure 2. Seventy hours after exposure, lungs were collected for histology. Goblet cells were stained with PAS in lung sections. A: Lung section of saline-challenged immunized mice exposed to air. B: Lung section of OVA-challenged immunized mice exposed to air. Note intense goblet cell hyperplasia (arrow). C: Lung section of saline-challenged immunized mice exposed to NO2 at 5 ppm. D: Lung section of OVA-challenged immunized mice exposed to NO2 at 5 ppm. Lung section of saline-challenged immunized mice exposed to NO2 at 20 ppm. Lung section of OVA-challenged immunized mice exposed to NO2 at 20 ppm. Note the absence of goblet cell hyperplasia in saline-challenged mice exposed to air or NO2 in A, C and E, and in OVA-challenged mice exposed to irrespective of the concentration of NO2 in D and F (final magnification: x 200).

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