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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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Nitrogen dioxide (NO_2) is a common indoor and outdoor air pollutant whose role in the induction of asthma is unclear. We investigated the effects of NO_2 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 NO_2 or air. Twenty parts per million of NO_2 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 with air or 5 ppm NO_2. 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 NO_2, whereas a marked reduction was unexpectedly observed for 5 ppm NO_2. At 24 h, interleukin-5 in the BALF was markedly reduced at 5 ppm compared with 20 ppm NO_2 and was also more intense for 20 ppm NO_2 than for the air group. In contrast to specific IgG1 titers, anti-OVA IgE titers and interleukin-4 in the BALF were not affected by NO_2 exposure. Irrespective of the concentration of NO_2, OVA-challenged mice did not develop late mucosal metaplasia compared with those exposed to OVA-air. These results indicate that a short exposure to NO_2 can exacerbate or inhibit some features of the development of allergic disease in mice and may depend on the concentration of pollutant.

Key words: Mouse model of asthma, Nitrogen dioxide, Air pollutant, Bronchopulmonary hyperreactivity, Lung permeability, Eosinophilia, Mucus

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 past 20 years in most industrialized countries.[1] Although asthma is familial and genome-wide searches have identified genetic loci predisposing to the disease, it is unlikely that the genetic make-up of stable populations can change significantly in less than one century. The probable cause of the epidemic must therefore relate to the environment.[1] Several recent studies have shown an association between air pollution during episodes of smog and asthma exacerbations, and hospital visits for asthma.[2–4] Although this finding does not address causality, it supports air pollution having an effect on acute asthmatic episodes. Current evidence also suggests that asthmatics are more sensitive to the effects of air pollutants.[5]

Asthma is characterized by acute bronchoconstriction, late bronchopulmonary hyperreactivity, pulmonary eosinophilic inflammation, excessive mucus production and increased serum IgE titers,[6] induced by a variety of stimuli. Its pathology seems to be directly linked to the presence in the airways of eosinophils and of T helper 2 (Th2) $CD4^+$ lymphocytes,[7,8] which produce interleukin (IL)-4 and IL-5. These Th2 cytokines are said to play a central role in the initiation and perpetuation of asthma.[9] IL-5 regulates the growth, differentiation and activation of eosinophils and provides an essential signal for their recruitment to the lungs during allergic inflammation.[10] IL-4 induces the differential development of T helper 0 cells into Th2 cells,[11] and stimulates B lymphocytes to produce IgE.[12]

Nitrogen dioxide (NO_2), a major potent oxidant pollutant, is a well-known airway irritant.[13] In contrast to other pollutants, NO_2 is a widespread contaminant of outdoor and of indoor environment. Its indoor levels can exceed those found outdoors, and are provided by gas cooking appliances and tobacco smoke.[14,15] By contrast, the main sources of NO_2 in outdoor air are motor vehicle emissions and

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fossil-fuel burning industries.[13] Acute exposures to high concentrations of NO_2 produce changes in pulmonary function, increase airway responsiveness,[16] and induce pulmonary edema.[17] NO_2 may also cause release of inflammatory mediators, and induce mast cell and lymphocyte infiltration.[18] Animal studies have demonstrated that exposure to NO_2 can increase susceptibility to infection, presumably through its effect on lung defense mechanisms, mucociliary clearance,[19] and alveolar macrophage function.[20,21] NO_2 is tissue soluble, with unsaturated bonds in membrane lipids of the airway and respiratory epithelium, as well lining fluid, being its prime targets.[22] Several studies have associated morbidity of asthma with elevated concentrations of NO_2.[23,24] Based on epidemiological studies, it has been suggested that NO_2 increases the risk for exacerbations of asthma.[25,26] Clinical observations have demonstrated that exposure to NO_2 correlates with exacerbations of asthma and with the potentiation of airway reactivity in asthmatics,[27,28] in contrast to other studies, which have failed to show an association between exposure to NO_2 and increased incidence of asthma.[29] These discrepancies show that the role of NO_2 for allergic disease is still unclear.

Since NO_2 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 NO_2 and allergen resulting in an augmented allergic reaction has been suggested.[24,27,30] In the present study, we investigated the role of NO_2 in the exacerbation of asthma in an ovalbumin (OVA)-immunized mouse model. For that, we studied whether a short-term exposure to low or high concentrations of NO_2 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 in the INERIS animal-care unit, a facility accredited by the Departmental Direction of Veterinary Services. The animals 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 number 87–849, 19 October 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 of OVA (ICN Biomedicals, Inc., Aurora, OH, USA), dispersed in 1.6 mg of $Al(OH)_3$ (Merk, Darmstadt, Germany) in 0.4 ml of 0.9% NaCl (saline) at days 0 and 7. At day 14, 1 week after the second injection, immunized mice were intranasally (i.n.) challenged with 10 μg of OVA in 50 μl of saline for about 10 sec under anesthesia by intravenous injection of ketamine (35 mg/kg Imalgene®1000; Merial, Lyon, France). Control mice were challenged with the same volume of saline solution.

Exposure system

The whole body exposure system used to generate NO_2 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 NO_2 or to air for 3 h. In each glass chamber, NO_2 was delivered with a flow rate of 5 l/min, allowing one to have a renewal, and was calibrated at the exact desired concentration in synthetic air. The airflow of NO_2 in each chamber was monitored by a mass flow-meter during the period of exposure. The relative pressure of the glass exposure chamber was controlled by a manometer. Concentrations of 5 or 20 ppm of NO_2 were obtained from cylinders of NO_2 gas prepared and certified by the supplier (Air Liquide, Le Blanc Mesnil, France).

Evaluation of bronchopulmonary hyperreactivity

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) that measured the respiratory waveforms. Animals were exposed to an aerosol of methacholine (Aldrich, Milwaukee, WI, USA) for 20 sec at 0.1 M delivered by an aerosolator. The index of airway obstruction was expressed as enhanced pause (P_{enh}), in response to inhaled methacholine,[31] calculated as: $P_{enh} = [T_e / T_r - 1] \times [P_{ef} / P_{if}]$, where T_e is expiratory time, T_r is relaxation time, P_{ef} is peak expiratory flow and P_{if} is peak inspiratory flow. For the graphic representation, each value was expressed every minute and was calculated from the average of three values of P_{enh} recorded every 20 sec. To simplify the interpretations, the area under the curve was calculated for 15 min. The graphics in terms of area under the curve represent the quantitative expression of BHR.

Serum sample preparations and bronchoalveolar lavage fluid

Mice were anesthetized by the intraperitoneal injection of urethane (2 g/kg ethylcarbamate; Sigma, St Louis, MO, USA) 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 × 0.5 ml of a sterile phosphate-buffered saline (PBS) solution (phosphate buffer 10 mM; pH 7.4). The total cell numbers were counted automatically (Coulter Counter ZM, Coultronics, Margency, France). Bronchoalveolar lavage fluid (BALF) was cytocentrifuged for 10 min (Cytospin, Shandon, UK). 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.[32] Briefly, lungs were removed and homogenized (Potter-Elvejhem glass homogenizer; Thomas, Philadelphia, PA, USA) 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 (Bioblock Scientific 2K15; Sigma, 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 (Sigma). Plates were read with an automatic microplate at 490 nm and results are expressed as optical density (OD).

Evaluation of cytokines

IL-4 in the BALF was evaluated by enzyme-linked immunosorbent assay (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 overnight at 4°C. After washing, dilutions of recombinant murine IL-4 (Perbio Sciences) (15.6–1000 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 (1:2000 to each well; Sigma) for 45 min at room temperature (RT). Plates were developed with tetramethylbenzidine substrate (Kiregaard Perry Laboratories, MD, USA). The reaction was stopped with 2 N sulfuric acid and the plates were read at 450 nm with an automatic microplate reader. The lower limit of detection of this assay is ∼10 pg of IL-4/ml sample.

IL-5 in the BALF was quantified using an immunometric assay as described previously.[33] 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 standard (7.6–1000 pg/ml) or of the sample, 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 an automatic microplate reader. The lower limit of detection of this assay is ∼5 pg of 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 bovine serum albumin (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 conjugate (1:2000 to each well; Caltag Laboratories, Burlingame, CA, USA) for 1 h at 37°C. The colorimetric reaction was initiated with *p*-nitrophenylphosphate (1 mg/ml) (Sigma) at 37°C. Plates were read at 405 nm with an automatic microplate reader.

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

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.*[34] Briefly, 96-well 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. In another 96-well plates, BALF samples and standard fibronectin were incubated with a polyclonal rabbit anti-fibronectin antibody (1:10000; 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 the 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 (1:1500; Amersham Pharmacia Biotech, Orsay, France) and incubated for 2 h at RT. Plates were washed and incubated with ExtrAvidin® peroxidase conjugate (1:20000 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 of 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 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 analysis of variance. Multiple comparisons between all groups were performed by Fisher's least-significant difference test. $p \leq 0.05$ was considered significant.

Results

To investigate the influence of NO_2 on the responses to the i.n. administration of OVA in immunized mice, two concentrations of NO_2 that are relatively high compared with those encountered in the environment were used, since the actual amounts delivered to the lung are quite below the concentrations

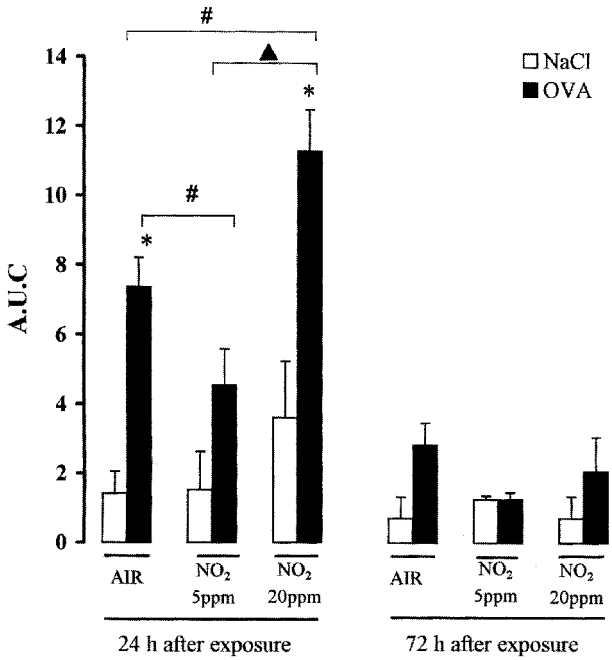

FIG. 1. BHR is significantly augmented in immunized mice exposed to 20 ppm NO_2. At day 14, immunized mice were challenged i.n. with saline (□) or OVA (■) and were exposed to 5 or 20 ppm NO_2 or to air for 3 h. At days 15 (24 h) and 17 (72 h), BHR in response to methacholine was evaluated. The graphic in terms of area under the curve (A.U.C) represents the quantitative expression of BHR. Data are expressed as the mean ± SEM. * $p \leq 0.05$, OVA-challenged mice compared with their saline control; $^{\#}p \leq 0.05$, OVA-air group compared with the NO_2-OVA group (5 or 20 ppm); ▲ $p \leq 0.05$, 5 ppm NO_2-OVA group compared with the 20 ppm NO_2-OVA group.

inhaled.[35,36] 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 allow one 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 NO_2 at 5 or 20 ppm for 3 h. The time points of 24 and 72 h were studied after NO_2 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 NO_2 or to air), immunized BALB/c mice challenged with OVA expressed significant BHR as compared with those exposed to 5 ppm NO_2 (Fig. 1). Mice challenged with saline exposed to air or to NO_2 did not develop BHR. In OVA-challenged mice exposed to 20 ppm

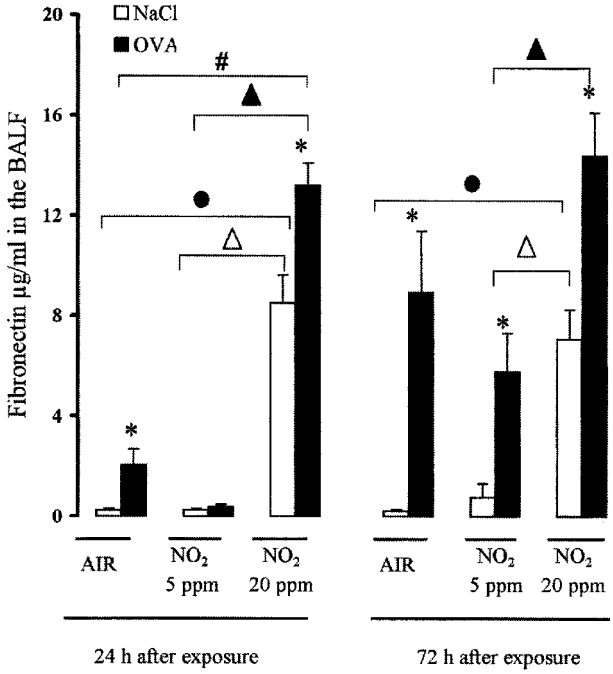

FIG. 2. BALF fibronectin content after short exposure to NO_2. At day 14, immunized mice were challenged i.n. with saline (□) or OVA (■) and were exposed to 5 or 20 ppm NO_2 or to air for 3 h. At days 15 (24 h) and 17 (72 h) after exposure, mice were sacrificed. Fibronectin levels were evaluated in the BALF by ELISA. Data are expressed as the mean ± SEM. $^{*}p \leq 0.05$, OVA-challenged mice compared with their saline control; $^{\#}p \leq 0.05$, OVA-air group compared with the NO_2-OVA group (5 or 20 ppm); $^{\bullet}p \leq 0.05$, saline-air group compared with the NO_2-saline group (5 or 20 ppm); $^{\blacktriangle}p \leq 0.05$, 5 ppm NO_2-OVA group compared with the 20 ppm NO_2-OVA group; $^{\triangle}p \leq 0.05$, 5 ppm NO_2-saline group compared with the 20 ppm NO_2-saline group.

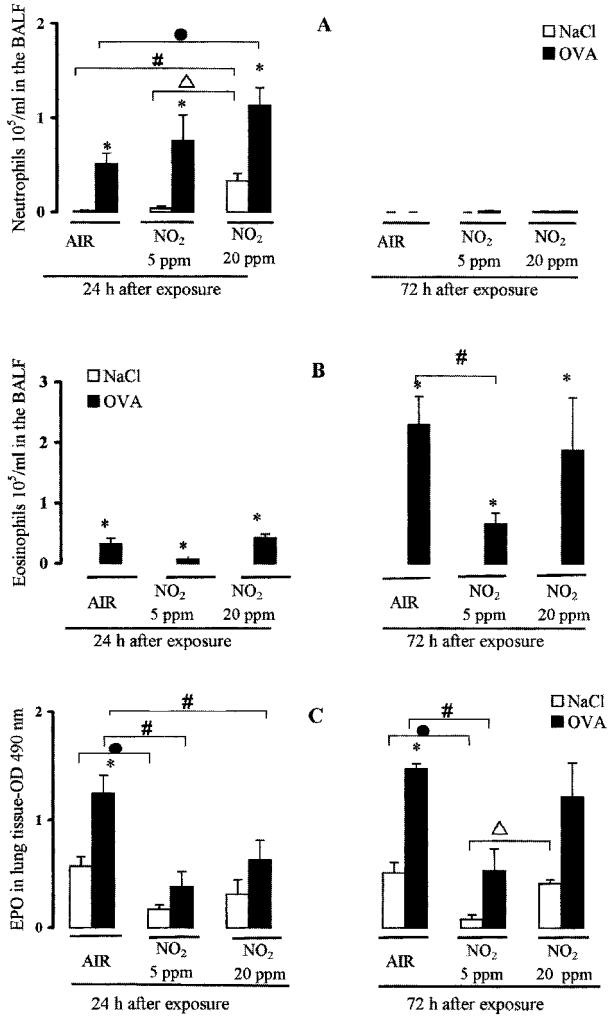

FIG. 3. BALF cell infiltration after exposures to NO_2. At day 14, immunized mice were treated as in Fig. 2 (saline (□) or OVA (■)). Differential BALF cell counts and EPO of the lungs were evaluated. (A) Neutrophils, (B) eosinophils and (C) eosinophil peroxidase. Data are expressed as the mean ± SEM. $^{*}p \leq 0.05$, OVA-challenged mice compared with their saline control; $^{\#}p \leq 0.05$, OVA-air group compared with the NO_2-OVA group (5 or 20 ppm); $^{\bullet}p \leq 0.05$, saline-air group compared with the NO_2-saline group (5 or 20 ppm); $^{\blacktriangle}p \leq 0.05$, 5 ppm NO_2-saline group compared with the 20 ppm NO_2-saline group.

NO_2, BHR was significantly increased as compared with those exposed to air and to 5 ppm NO_2. At day 17, 72 h after exposure, OVA-challenged mice exposed to air or to NO_2 failed to develop BHR (Fig. 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 NO_2 released significantly increased amounts of fibronectin in the BALF, whereas those levels were very low in animals exposed to 5 ppm NO_2 (Fig. 2). In contrast, exposure to 20 ppm NO_2 augmented by six- to seven-fold the fibronectin concentrations in the BALF of OVA-challenged mice after 24 h, as compared with the OVA-air group. NO_2 at 20 ppm also induced a marked fibronectin exudation in saline-challenged mice, as compared with the saline-air or saline-5 ppm NO_2 groups. Seventy-two hours after exposure, BALF fibronectin content persisted in saline- or OVA-challenged mice exposed to 20 ppm NO_2. At this time point, in OVA-challenged mice exposed to 5 ppm NO_2 or to air, the release of fibronectin was delayed, as compared with mice exposed to 20 ppm NO_2 (Fig. 2). Indeed, 24 h after exposure to air or to 5 ppm NO_2, 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 NO_2, 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 NO_2. These counts were found significantly increased and

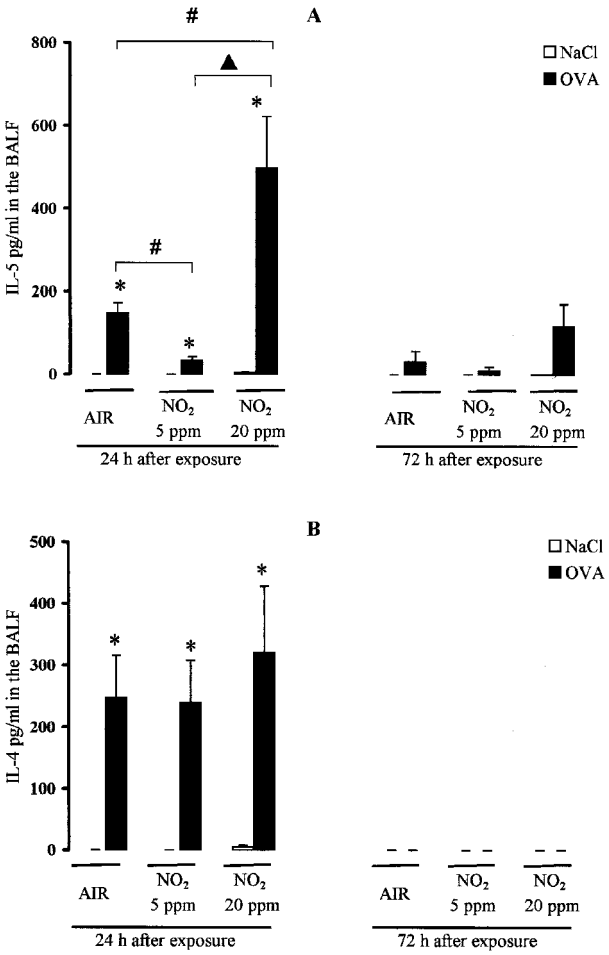

FIG. 4. BALF Th2 cytokine levels after exposures to NO_2. At day 14, immunized mice were treated as in Fig. 2 (saline (□) or OVA (■)). Specific antibodies were evaluated in the serum by ELISA. BALF IL-4 and IL-5 content were evaluated by ELISA. (A) IL-5 and (B) IL-4. Data are expressed as the mean ± SEM. $^{*}p \leq 0.05$, OVA-challenged mice compared with their saline control; $^{\#}p \leq 0.05$, OVA-air group compared with the NO_2-OVA group (5 or 20 ppm); $^{\blacktriangle}p \leq 0.05$, 5 ppm NO_2-OVA group compared with the 20 ppm NO_2-OVA group.

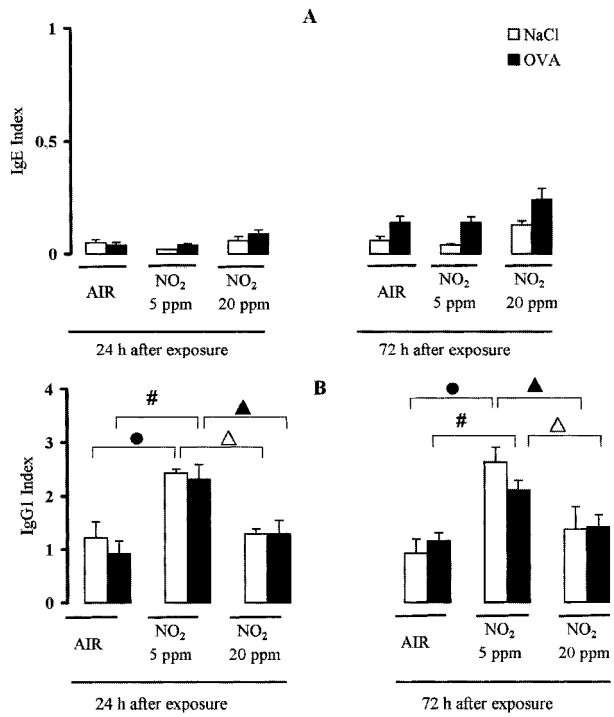

FIG. 5. Specific anti-OVA IgE and IgG1 titers in the serum after exposures to NO_2. At day 14, immunized mice were treated as in Fig. 2 (Saline (□) or OVA (■)). Specific antibodies were evaluated in the serum by ELISA. (A) IgE and (B) IgG1. Data are expressed as the mean ± SEM. $^{\#}p \leq 0.05$, OVA-air group compared with the NO_2-OVA group (5 or 20 ppm); $^{\bullet}p \leq 0.05$, saline-air group compared with the NO_2-saline group (5 or 20 ppm); $^{\blacktriangle}p \leq 0.05$, 5 ppm NO_2-OVA group compared with the 20 ppm NO_2-OVA group; $^{\triangle}p \leq 0.05$, 5 ppm NO_2-saline group compared with the 20 ppm NO_2-saline group.

in OVA-challenged mice exposed to 20 ppm, as compared with OVA-air mice. The increase in neutrophil counts in saline-challenged animals exposed to 20 ppm NO_2 was also significantly above that found in saline-air or saline-5 ppm NO_2 mice (Fig. 3A). At 72 h, neutrophil counts were normalized in all groups of mice (Fig. 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 NO_2, as compared with those exposed to 5 ppm NO_2, in which these counts were markedly reduced after 24 and 72 h (Fig. 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 NO_2 showed a marked reduction of EPO activity at 24 and 72 h, as compared with 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 NO_2, as compared with those exposed to air. This contrasts with results in mice exposed to 20 ppm NO_2 or to air, in which EPO titers were increased to similar levels (Fig. 3C).

Th2 cytokine production in the BALF

OVA-challenged mice exposed to air or to NO_2 released IL-5 and IL-4 in the BALF at 24 h. In connection 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 NO_2, as compared with those exposed to 20 ppm NO_2 or to air (Fig. 4A). However, the production of IL-5 was increased three-fold in OVA-challenged mice exposed to 20 ppm NO_2 as compared with the OVA-air group, and was augmented by 10-fold as compared with those exposed to 5 ppm NO_2 (Fig. 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 (Fig. 4B). No IL-4 nor IL-5 were detected in the BALF at the 72 h point (Fig. 4A,B).

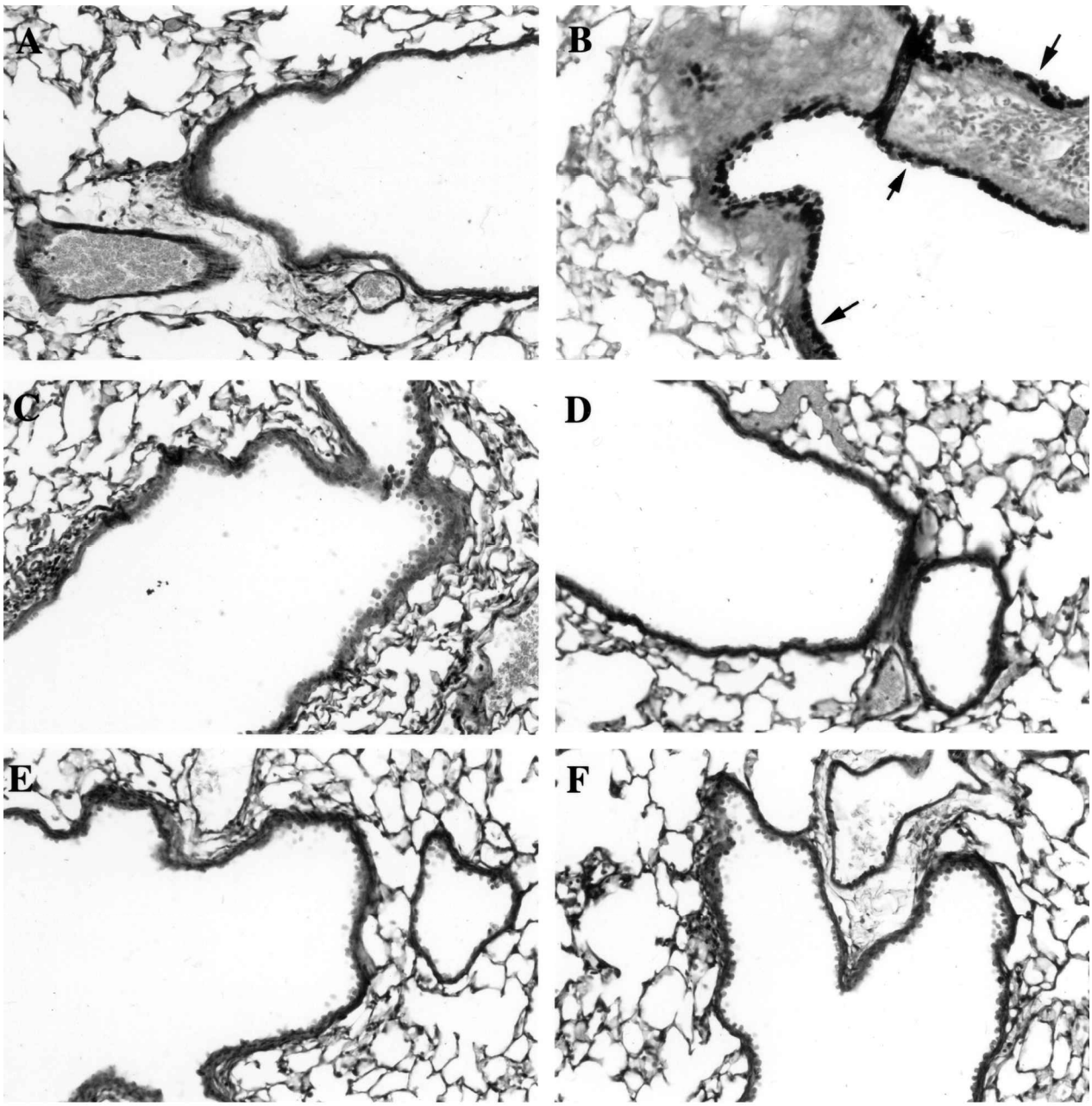

FIG. 6. Periodic acid Schiff (PAS)-stained histologic sections of lungs from allergic mice exposed to NO_2. At day 14, immunized mice were treated as in Fig. 2. Seventy hours after exposure, lungs were collected for histology. Goblet cells were stained with PAS in lung sections. (A) Lung section of saline-challenged 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 NO_2 at 5 ppm. (D) Lung section of OVA-challenged immunized mice exposed to NO_2 at 5 ppm. (E) Lung section of saline-challenged immunized mice exposed to NO_2 at 20 ppm. (F) Lung section of OVA-challenged immunized mice exposed to NO_2 at 20 ppm. Note the absence of goblet cell hyperplasia in saline-challenged mice exposed to air or NO_2 in (A), (C) and (E), and in OVA-challenged mice exposed to irrespective of the concentration of NO_2 in (D) and (F) (final magnification, $\times$ 60).

Production of IgE and IgG1 anti-OVA antibody in the serum

Saline- and OVA-challenged mice exposed to air or to NO_2 produced specific IgE and IgG1 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 (Fig. 5A), and both concentrations of NO_2 failed to affect their production. Specific IgG1 titers were markedly increased as compared with IgE, without significant differences between saline and OVA-challenged mice. Mice exposed to 5 ppm NO_2 and challenged with saline or OVA showed a significant increase of IgG1 titers as compared with those exposed to air or to NO_2 at 20 ppm (Fig. 5B).

Histological analysis

As expected, the bronchial epithelium of saline-challenged immunized mice exposed to air was mucus free (Fig. 6A), under conditions where that of immunized and OVA-challenged mice was markedly enriched in mucosal cells (Fig. 6B). Saline- or OVA-challenged immunized mice exposed to NO_2 at 5 or 20 ppm did not develop mucosal metaplasia as compared with OVA-challenged mice exposed to air (Fig. 6D,F).

Discussion

Studies in asthmatics have shown that air pollutants such as ozone,[30] or NO_2 augment the allergic responses.[24,27,37,38] At high concentrations, NO_2 is a well-known airway irritant that can cause bronchial constriction in normal subjects,[17,39] and enhance airway responsiveness to histamine[40] or methacholine[16] in asthmatic patients, which are more sensitive than healthy subjects. By contrast, few studies have addressed to the interaction between NO_2 and lung allergy in experimental animals. As shown here, a short-term exposure to NO_2 produces contrasting effects on the development of asthma-related responses in an OVA-immunized mouse model, which depend on the dose of NO_2. 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 NO_2 was used here, both doses were relatively high as compared with those encountered in the environment. It has been reported, nevertheless, that the final amounts delivered to the lungs are quite below the concentrations inhaled.[35,36]

The potentiation of BHR by 20 ppm NO_2 in allergic mice may be accounted for by an increased vascular/epithelial permeability, facilitating the allergen availability and accelerating the inflammatory process. NO_2 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 NO_2 had increased titers of fibronectin in the BALF, which was used as an indicator of permeability, as compared with the saline-air or saline-5 ppm NO_2 groups. The high concentration of NO_2 potentiated the effects of OVA, since the fibronectin BALF content was significantly increased in OVA-challenged mice exposed to 20 ppm, as compared with the OVA-air or OVA-5 ppm NO_2 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 NO_2. The low concentration of NO_2 had an effect similar to that of OVA alone. Since our mice underwent a single exposure to NO_2, it is possible that such low concentrations of NO_2 become effective on repeated exposures. In this context, the most probable mechanism for NO_2-induced increase 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 NO_2 over short periods.[41] This would account for the augmented fibronectin titers in the BALF, after its exudation from the plasma. It is also possible that NO_2 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, NO_2 can reduce the mucociliary activity of the airways *in vivo*[42] and *in vitro*,[41] 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 NO_2. By contrast, 20 ppm NO_2 did not affect eosinophilia, under conditions where the production of IL-5 in the BALF was significantly increased as compared with the OVA-air group. Recently, Morris *et al.*[43] reported that the exposure to 0.7 ppm NO_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_2 are unknown. They may result from alterations in the regional deposition patterns of OVA and of NO_2 in the airways, or in absorbance, pulmonary clearance or antioxidant defenses. Indeed, NO_2 has a low solubility, and is poorly absorbed by the airway mucosa.[44] It is also a very reactive molecule whose uptake in the respiratory system is extremely high.[44] The use of mathematical dosimetry models suggests that the uptake of NO_2 between the trachea and the respiratory zone occurs to a similar extent, and peaks at the terminal bronchioles.[44] It is also possible that the biphasic effect of 5 and 20 ppm NO_2 may result from pharmacodynamic alterations. Some studies have indeed demonstrated that NO_2 affects lung defense mechanisms, including mucociliary clearance, alveolar macrophages (AM), and the immune system.[45] The decreased OVA-induced IL-5 production and eosinophilia after an exposure to 5 ppm NO_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_2. Indeed, Kineast *et al.*[46] and Erroi *et al.*[47] have shown that NO_2 exposure of LPS-stimulated human AM results in a functional impairment of AM. Furthermore, Robison *et al.*[21] demonstrated that exposure rates to 0.5 ppm NO_2 for 0.5–10 days reduces the arachidonate metabolism and superoxide production in response to external stimuli. Thus, NO_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 NO_2, OVA-challenged mice did not develop mucosal metaplasia, in contrast to those exposed to air, which is probably related to mucus denaturation by NO_2-induced oxidation. The mucus layer forms a protective barrier of the airways against the effects of oxidants, which eliminates and/or scavenges the toxic components of NO_2 prior to their diffusion into the airway epithelium. The lipid content of the mucus layer, in particular esterified unsaturated fatty acids, constitutes the primary scavenging oxidants.[48] The latter demonstrated that the phospholipids of the mucus layer cannot offer a significant protection against inhaled NO_2 and that exposure to 40 ppm NO_2 in rats induces lipid peroxidation correlated with the apparent lack of oxidant scavenging species in the mucus lining the airways.

In summary, a short-term exposure to NO_2 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 NO_2 used in our investigation, as compared with those encountered in the environment, this study provides new information concerning the subtle interactions between an air pollutant and allergic disease.

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