

Increased Immune and Inflammatory Responses to Dust Mite Antigen in Rats Exposed to 5 ppm NO₂¹

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Immune hypersensitivity to house dust mite antigen (HDM) is a frequent cause of respiratory allergy. The objective of this study was to determine whether exposure to NO₂, a common indoor air pollutant, modulates immune responses to HDM and influences immune-mediated lung disease. Brown Norway rats were immunized ip with 100 µg semipurified antigen and *Bordetella pertussis* adjuvant and challenged 2 weeks later with an intratracheal injection of 50 µg of a crude antigen preparation. Exposure to 5 ppm NO₂ for 3 hr after both immunization and challenge procedures resulted in significantly higher levels of antigen-specific serum IgE, local IgA, IgG, and IgE antibody than air controls, and increased numbers of inflammatory cells in the lungs. Lymphocyte responsiveness to antigen in the spleen and MLN was also significantly higher in NO₂-exposed animals. These data show that exposure to a common air pollutant can upregulate specific immune responses and subsequent immune-mediated pulmonary inflammation. © 1996 Society of Toxicology

Nitrogen dioxide is a major component of photochemical smog (Tucker *et al.*, 1992) and is a common indoor air pollutant (USEPA, 1993). Research over the past three decades has shown that NO₂ exposure can significantly alter host defense mechanisms of experimental animals (Goldstein *et al.*, 1973; Jakab, 1987) and modulate specific immunity to nonreplicating antigens (Ehrlich *et al.*, 1975; Fujimaki *et al.*, 1982, 1987). Experimental studies in rodents have found both enhancement and suppression of immune function after NO₂ inhalation depending upon the concentration, frequency, and duration of exposure, as well as the endpoint and species under study (reviewed in USEPA, 1993).

¹ The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

A possible clinical outcome of elevated immunity by exposure to NO₂ is the development or exacerbation of hypersensitivity disease (i.e., allergy) (Danielle, 1988). There is now substantial evidence that during episodes of high air pollution, hospital admissions by asthmatics and other allergic individuals increases (Bates and Siltso, 1987; Cody *et al.*, 1992). It is not clear whether this rise is caused by an increase in the number of sensitized individuals or by an increase in the severity of attacks in existing populations. A convenient way to address this hypothesis is to study the induction and exacerbation of immune responses in experimental animals and to determine the effect of pollutant exposure on a variety of immunologic and pathological parameters.

Several studies have already demonstrated enhanced allergic responses by exposure to NO₂, O₃, and sulfur compounds. Matsumara (1970a) reported that guinea pigs exposed to high levels of O₃ (5–10 ppm), NO₂ (70 ppm), or SO₂ (330 ppm) for 30 min prior to airway sensitization with ovalbumin antigen had elevated specific antibody titers and increased anaphylactic responses following challenge with aerosolized antigen. Pollutant exposure also enhanced dissemination of the antigen from the lung to the blood stream over a 3-hr period (Matsumara, 1970b), suggesting that increased lung permeability caused a greater flow of antigen to sensitized immune tissue. In a similar fashion, Biagini *et al.* (1986) demonstrated that exposure to 1 ppm O₃ enhanced the development of allergy to inhaled platinum in monkeys. Using rats, Reidel *et al.* (1988) found that daily exposure to SO₂ (0.1–16.6 ppm) increased specific antibody levels to ovalbumin antigen in serum and bronchoalveolar lavage fluid (BAL), and increased antigen-induced bronchoconstriction. Osebold and colleagues (1979) demonstrated that continuous exposure to O₃ (0.5–0.8 ppm) resulted in elevated levels of albumin, IgG, and IgA antibodies in the BAL fluid of mice, and increased numbers of IgA producing B lymphocytes in the lungs. They also reported (Osebold *et al.*, 1980) that intermittent exposure to a combination of sulfuric acid aerosol (1 mg/m³) and O₃ (0.5–0.8 ppm) increased reactivity to subsequent ovalbumin challenge delivered by aerosol

which corresponded to elevated numbers of antigen-specific IgE containing cells in the lungs (Gershwin *et al.*, 1981).

To date, only two published studies have investigated the effect of pollutant exposure on subsequent reactivity to antigen in atopic human volunteers. Molino *et al.* (1991) reported that exposure to 0.12 ppm O₃ significantly increased bronchial responsiveness following antigen challenge in some individuals. Bascomb *et al.* (1990) found no difference in symptoms or inflammatory responses to a nasal challenge of allergen in atopic individuals preexposed to 0.5 ppm O₃ or air; however, they noted that atopic patients had a much stronger inflammatory response to O₃ alone than control, nonallergic volunteers.

Use of Brown Norway rats has increased in allergy research because of their high IgE responder status compared to other strains of rat (Sedgwick and Holt, 1984). We have previously described techniques to sensitize mice or rats to house dust mite antigen (HDM), and have demonstrated increases in pulmonary eosinophilia, serum, and local IgE antibody and airway resistance following intratracheal antigen challenge in sensitized rats (Gilmour *et al.*, 1993). Since NO₂ and house dust mite are two of the most common gaseous and allergenic compounds respectively measured in indoor air, we sought to determine the effects of acute NO₂ exposure (5 ppm, 3 hr) on immune responses and lung inflammatory cells in sensitized rats exposed to house dust mite allergen.

METHODS

Animals. Six-week-old female Brown Norway rats purchased from Charles River Laboratories were kept in an environmentally controlled room for 1 week prior to each experiment. The animals were provided with food and water *ad libitum*, and were maintained in a 12-hr light/dark cycle. Randomly selected animals tested serologically upon arrival, and sentinels monitored throughout the study, were free of Sendai virus, pneumonia virus, mouse hepatitis, and a variety of other rodent viruses as well as *Mycoplasma* sp. Rats were also monitored for and found to be free of ectoparasites and endoparasites. All procedures were approved by the animal care and use committee of the Health Effects Research Laboratory, U.S. EPA before the start of the study.

Immunization procedure. Semipurified extracts of *Dermatophagoides farinae* and *D. pteronyssinus*, purchased from Greer Laboratories (Lenoir NC), were rehydrated in saline to 10 mg/ml, mixed equally, and stored in aliquots at -70°C until use. Crude (particulate) fractions of *D. farinae* and *D. pteronyssinus* (Greer Laboratories) used for intratracheal challenge were stored in a similar manner. Rats were immunized intraperitoneally with 100 µg of semipurified antigen suspended in 0.3 ml saline containing 10⁸ killed *Bordetella pertussis* organisms (Accurate Antibodies, Westbury, NY). Two weeks later, rats were lightly anesthetized by metaflane inhalation and received a single intratracheal instillation of 50 µg crude (particulate) allergen suspended in sterile saline.

NO₂ exposures. Immediately after each immunization procedure, rats were caged individually, placed in Rochester chambers, and exposed to NO₂ or filtered air. Nitrogen dioxide from cylinders (National Welders, Raleigh, NC) was introduced into the chambers and concentration was continuously monitored with chemoluminescent monitors (Bendix Corp., Roncevert, WV). Concentration information was automatically collected

and a computer-controlled feedback loop operated mass flow controllers to modify gas input into chambers as needed. Concentrations were maintained at 5 ppm ± 5% for the duration of the exposure. Temperature and relative humidity ranged between 20.5 and 22.8°C and 40 and 60% respectively for all exposures. After antigen challenge and exposure, the rats were returned to the animal colony. Experimental groups described below were euthanized 7 days after intratracheal challenge with antigen.

Group	Day 0: Intraperitoneal immunization	Day 14: Intratracheal challenge	Day 21: Euthanization
A/A	Air	Air	
A/N	Air	NO ₂	
N/A	NO ₂	Air	
N/N	NO ₂	NO ₂	

Inflammatory responses. Seven days after intratracheal instillation of crude allergen, five rats from each exposure group were euthanized by sodium pentobarbital. The animals were bled for collection of serum, the trachea was cannulated, and the lungs were lavaged with a single volume of warmed saline (35 ml/kg). Cell counts in the lavage fluid were obtained with a hemocytometer and the viability was assessed by trypan blue exclusion. Following dilution, 50,000 cells from each sample were centrifuged onto microscope slides, and stained with Diff Quick (American Scientific Inc., Sewickley, PA). Differential counts were performed on 200 cells for identification of alveolar macrophages, polymorphonuclear leukocytes (PMNs), lymphocytes, and eosinophils. Lavage fluid was assessed for protein by spectrophotometry against an albumin standard using Coomassie Plus assay reagent (Pierce, Rockford, IL).

Antibody analysis. Serum and lavage fluid were assessed for antigen-specific IgG, IgA, and IgE antibody by ELISA. Prior to assay, serum and BAL samples were pooled from each experimental treatment group and serial dilutions of the pooled samples were tested to determine which concentrations would occur on the sloping part of the reaction curve. For the assay, 96-well polystyrene plates were coated with antigen solution containing 100 µg semipurified HDM/ml in 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed five times in 0.1 M phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Each well was filled with a blocking solution of 2% bovine serum albumin (BSA) in PBS without Tween and incubated for 2 hr prior to washing. Test serum samples (IgG 1:100, IgE 1:50) or lavage fluid (1:10 IgE, 1:20 IgA, IgG) were then added to the wells and incubated with the coated antigen overnight at 4°C. Following another wash step, biotin-labeled mouse anti-rat IgG, IgE, or IgA (Accurate Antibodies) was added to the plates at optimal dilutions and incubated for 2 hr at 37°C. Excess antibody was removed by washing and a solution of horseradish peroxidase avidin D (Vector Labs Inc., Burlingame, CA) was added to the wells for 2 hr. After another wash, a solution of TM blue enzyme reagent (TSI-CDP, Milford, MA) was added and the color change was measured at a wavelength of 650 nm with an ELISA plate reader (Molecular Devices, Menlo Park, CA). Values from similar dilutions of normal rat serum incubated on each plate were subtracted from all sample values to control for nonspecific binding effects.

Lymphocyte proliferation in the presence of antigen. Spleens and mediastinal lymph nodes were gently homogenized in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM Hepes buffer, 5 × 10⁻⁵ M 2-mercaptoethanol, and 100 units/ml of penicillin and 100 mg/ml streptomycin (GIBCO, Grand Island, NY). After three centrifugation and lysing steps with 0.89% NH₄Cl and RPMI medium, cells were counted with a hemocytometer and resuspended at a concentration of 5 × 10⁶ cells/ml. One hundred-microliter aliquots of each cell suspension were added in triplicate to wells of 96-well flat-bottomed tissue culture plates. Cells were incubated in the presence of 10 µg of semipurified HDM, BSA, or medium alone for 4 days. Preliminary studies had found this incubation period and

antigen concentration to produce optimal culture conditions. Eighteen hours before the end of the incubation period, 0.5 μ Ci of [³H]thymidine (DuPont, Wilmington, DE) in 25 μ l RPMI medium was added to each well. The cultures were harvested automatically onto filters (Skatron Inc., Sterling, VA) and the incorporated [³H]thymidine was measured after addition of scintillation fluid (Packard, Meriden, CT). Lymphocyte responsiveness was expressed as the difference in radiotracer counts between cells incubated with HDM (stimulated) and those of the cells incubated with irrelevant antigen (BSA).

Statistics. Data from two duplicate experiments were analyzed by three-way ANOVA where air versus NO₂ versus time of inhalation exposure were the variables. In each experiment the same statistically significant differences were found between NO₂ and air-exposed animals for each measurement. Because of interassay variability, however, the data are presented as percentage of control (air-exposed) values. Significant differences between NO₂ treatments and air controls were noted when $p < 0.01$.

RESULTS

Exposure to NO₂ immediately after both the priming and challenge immunizations with HDM resulted in an increased proliferation to antigen by cells from both the mediastinal lymph nodes and spleens compared to cells obtained from air controls (Fig. 1). No significant differences were found between animals given a single NO₂ exposure and the air-exposed controls.

Antigen-specific IgG antibody was increased in the BAL fluid of rats exposed to NO₂ after both immunization procedures compared to air controls (Fig. 2). A single NO₂ exposure after either immunization procedure caused a significant decrease in antigen-specific IgG antibody. Levels of antigen-specific IgG in the serum were not significantly different between any of the treatment groups.

IgE levels did not exceed the assay limit of detection in the serum or BAL fluid of air-exposed animals following immunization and challenge with antigen (Fig. 3). IgE antibody was detected however in the BAL fluid and serum of rats exposed to NO₂ after immunization and challenge. IgE antibody was also detected in animals which had been exposed to NO₂ after the challenge immunization compared to air controls. Similar results (data not shown) were obtained when the serum and BAL samples were tested in an *in vivo* passive cutaneous anaphylaxis assay (PCA).

The double NO₂ exposure caused an increase in antigen-specific IgA levels in BAL fluid (Fig. 4), while in a manner similar to the local IgG data, a significant decrease was observed when the animals were given a single exposure after antigen challenge. No differences in total protein in lavage fluid were found between any of the groups (data not shown).

To assess the influence of NO₂ exposure on antigen-specific lung damage, the number and type of pulmonary inflammatory cells were assessed in NO₂ and air-exposed rats 7 days after antigen challenge. While no differences were found in the total number of lavageable cells, the double NO₂ exposure did cause an increase in the ratio of inflamma-

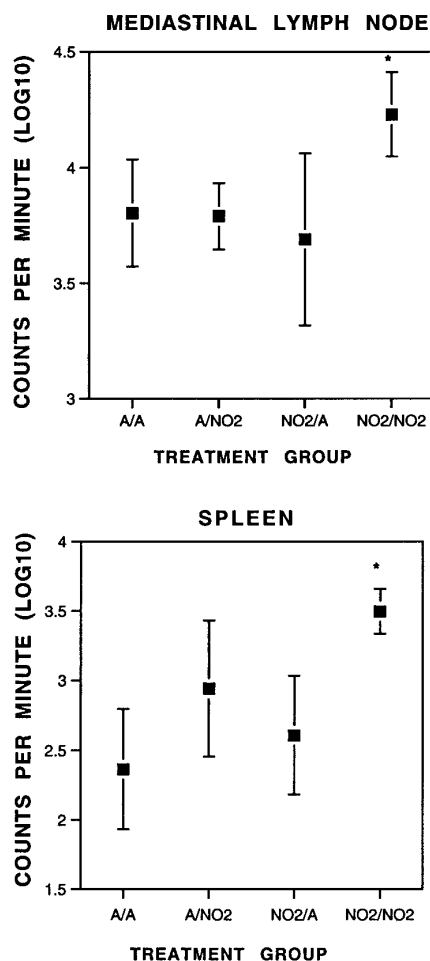


FIG. 1. Lymphocytes (5×10^5) from MLN or spleen were incubated with 10 μ g of HDM antigen for 4 days. Proliferative responses were assessed by [³H]thymidine incorporation over the last 18 hr of incubation. Data were analyzed by three-way ANOVA, $n = 5$.

tory cells to alveolar macrophages compared to controls (Fig. 4). Antigen challenge in air controls resulted in 17% inflammatory cells in the lung which comprised lymphocytes, neutrophils (PMNs), and eosinophils. NO₂ challenge after the primary immunization and challenge enhanced the ration of inflammatory cells to 40%. Single NO₂ exposures resulted in 12 and 35% inflammatory cells, respectively, which did not differ statistically from control values. Similar (5 ppm) NO₂ exposures without the immunization procedure resulted in no inflammatory cells 7 days after exposure (data not shown).

DISCUSSION

The role of air pollutants in the potentiation of lung disease has been a key research question for several decades. NO₂ is present in both photochemical smog and emissions

from kerosene heaters and gas stoves. In that context, its ability to promote or augment allergic lung disease deserves attention. The objective of this study was to determine the effect of NO_2 exposure on immune responses to a common allergen, and to examine whether altered immunity was associated with changes in immune-mediated pulmonary inflammation. We found that a 3-hr exposure to 5 ppm NO_2 after both a priming injection and pulmonary challenge with allergen enhanced specific immune responses to HDM and increased the number of inflammatory cells in the lung. Single NO_2 exposures after either phase resulted in variable responses but no trend toward suppression or enhancement of all immune parameters was discernible.

These data indicate that under these conditions two exposures to NO_2 are necessary to augment immunity to dust mite allergen and that the gas exposure has some influence on systemic immune function. A number of previous studies

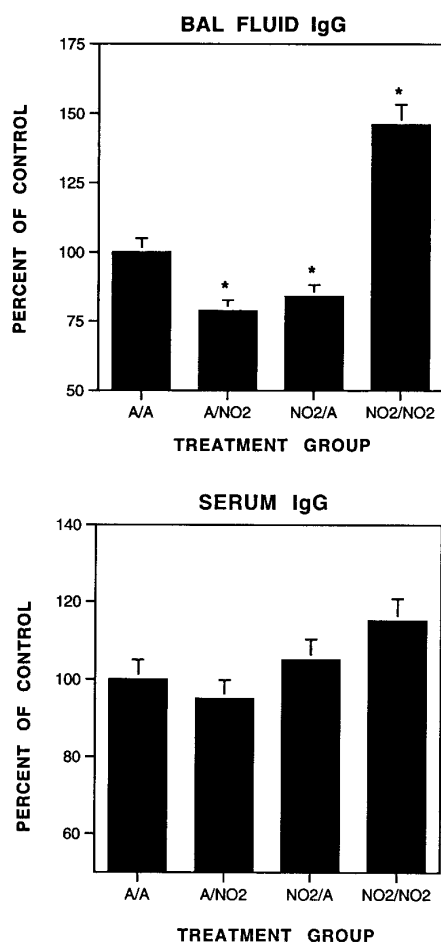


FIG. 2. Specific IgG antibody was measured 7 days after antigen challenge by ELISA at concentrations of 1:40 and 1:200 for BAL fluid and serum, respectively. Results are presented as percentage of control (air-exposed values) for two replicate experiments. Individual experimental data were analyzed by three-way ANOVA, $n = 10$.

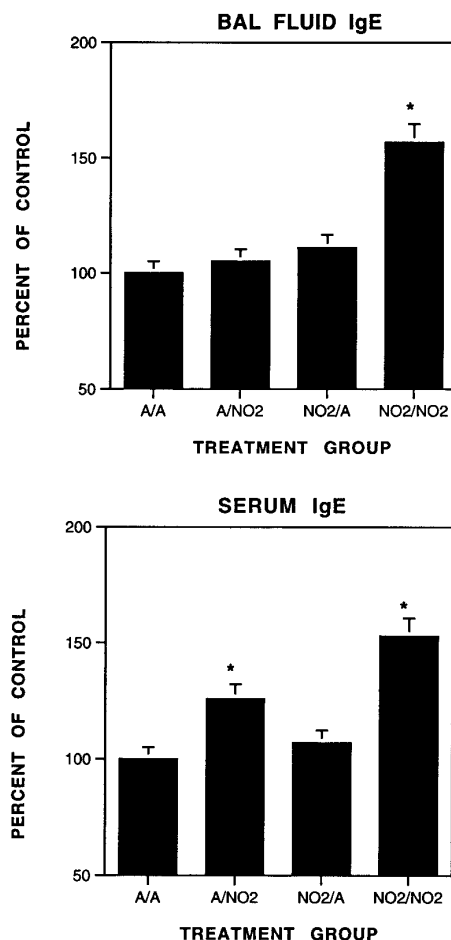


FIG. 3. Specific IgE antibody was measured 7 days after antigen challenge by ELISA at concentrations of 1:10 and 1:50 for BAL fluid and serum, respectively. Results are presented as percentage of control (air-exposed values) for two replicate experiments. Individual experimental data were analyzed by three-way ANOVA, $n = 10$.

have reported NO_2 -induced changes in serum antibody production (Erich *et al.*, 1975; Fujimaki *et al.*, 1982, 1987), splenic lymphocyte populations (Damji and Richters, 1989), and lymphocyte function (Maigetter *et al.*, 1978; Holt *et al.*, 1979). Although the mechanism for such systemic immunomodulation is not known, it may involve production of mediators by pulmonary cells which could then exert effects elsewhere in the body. Recent studies have shown that O_3 exposure can induce the release of proinflammatory cytokines (IL1, IL6) as well as lipid mediators such as prostaglandin E_2 (Devlin *et al.*, 1991). This latter compound has been shown to affect murine peritoneal macrophage function after O_3 inhalation (Canning *et al.*, 1991) and suggests that other products of pollutant exposure could also affect systemic immune function. As yet, however, the indirect effects of air pollutant exposure in sites remote from the lung have received little investigation.

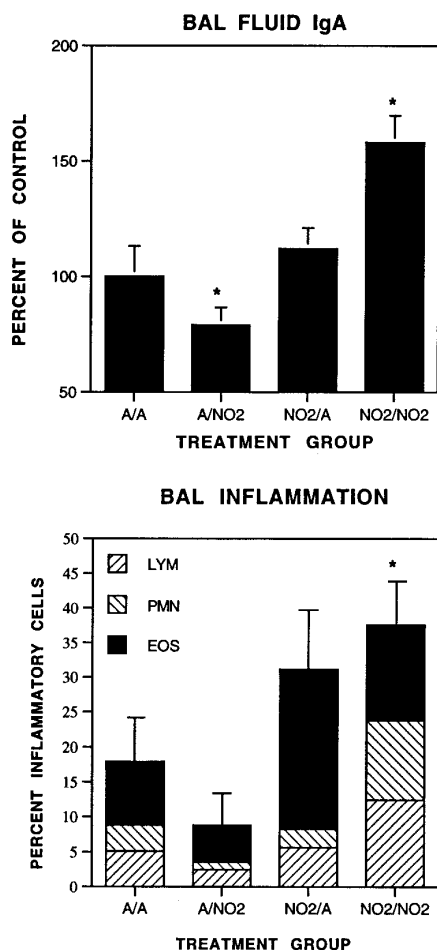


FIG. 4. Specific IgA antibody was measured 7 days after antigen challenge in BAL fluid by ELISA at a concentration of 1:40. Pulmonary cells obtained by bronchoalveolar lavage were centrifuged onto microscope slides and stained with Diff Quick. Differential counts were performed on 200 cells for identification of alveolar macrophages, polymorphonuclear leukocytes (PMNs), lymphocytes, and eosinophils. Results are presented as percentage of control (air-exposed values) for two replicate experiments. Individual experimental data were analyzed by three-way ANOVA, $n = 10$.

It is likely that NO₂ exposure around the time of antigen challenge affected the permeability of the lung and improved translocation of antigen to local lymph nodes and the circulation. Sherwin and Richters (1971) first demonstrated that exposure to NO₂ (2–8 ppm) increased intraalveolar and pulmonary intracellular accumulation of protein. Matsumara (1970) reported that exposure to high levels of O₃, NO₂, or SO₂ for 30 min increased bronchial reactivity to inhaled antigen in guinea pigs and found that the exposure increased translocation of instilled antigen from the lungs to the blood. Hillam *et al.* (1983) later demonstrated that acute exposure to 26 ppm NO₂ for 24 hr prior to intratracheal instillation of sheep red blood cells increased local and systemic immunity and postulated that increased lung permeability could

alter translocation of particulate antigen to immunoreceptive sites in the submucosa and lymphatic tissue.

The immunization regime was designed to maximize secondary immune responses after local challenge with antigen. In preliminary experiments not reported here (unpublished data), the immune responses were not fully developed at 2 days; hence, all immune function and inflammatory data were assessed 7 days postchallenge. Pulmonary inflammation was greatest in rats exposed to NO₂ after both immunization procedures compared to air controls. This increased inflammation seemed to correspond to higher levels of immunity and could have been produced by IgE-mediated mast cell degranulation products or by higher levels of immune complexes leading to increased complement fixation and cellular recruitment by chemoattractants. Alternatively, the increased lymphocyte activity in both the local lymph nodes and the spleen may have produced more cytokines such as IL5 which promotes both recruitment and maturation of eosinophils (Coffman *et al.*, 1989).

In summary, these data show that acute exposure to NO₂ causes an increase in specific immune responses to a known allergen and increased immune-mediated pulmonary inflammation, both of which are associated with allergic lung disease and asthma. These effects were demonstrated at levels 5 to 10 times less than previously reported in experimental studies. Although the mechanism for this pollutant-enhanced immunity and subsequent inflammation is not known, it may involve the release of mediators from the lung tissue as well as increasing antigen translocation to lymphoid tissue underlying the epithelial surfaces of the lung. The numerous epidemiological studies which have shown an association between episodes of high air pollution and increased respiratory symptoms suggest that modulation of allergic immune responses may occur in susceptible individuals. The levels or duration of pollutant exposure where this may occur and the possible mechanisms of modulation must be clarified. It is also important to point out that NO₂ levels are significantly higher in homes with gas stoves than the outdoor concentrations (Drye *et al.*, 1989). Coupled with exposure to perennial allergens such as dust mite, NO₂ and other toxic air pollutants may contribute to the significant rise in allergic asthma over the past 10 years.

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