

Site-specific sensitization in a murine model of allergic rhinitis: Role of the upper airway in lower airways disease

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Background: Allergic rhinitis (AR) is the most common atopic disease with strong links to asthma. We have developed a murine model of AR to study nasal, bronchial, and systemic immune response to local allergen stimulation.

Objectives: The purpose of this study was to develop and characterize a murine model of AR.

Methods: Six- to 8-week-old BALB/c mice were sensitized by means of intranasal (local) application of ovalbumin (OVA) or systemic intraperitoneal injection. They were then challenged with intranasal OVA, and allergic response was assessed.

Results: Intranasal particle deposition was found to be exclusively in the nares. All sensitized animals showed increased levels of OVA-specific serum IgE and IgG after challenge, although the timing to maximal response varied with the route and dose of allergen used. Histology of the upper and lower airways showed marked eosinophilic infiltration, and analysis of bronchoalveolar lavage fluid showed increased IL-5 and PMN infiltrates after challenge.

Conclusion: Using exclusive local sensitization and challenge of mouse nares, we were able to demonstrate inflammatory changes in both the upper and lower airways, even though distribution of allergen particles appeared to be only in the nares of these animals. This provides further evidence for the importance of the upper airway in lower airways disease. We have shown that the route of administration greatly affects the characteristics of the subsequent immune responses. (*J Allergy Clin Immunol* 2002;110:891-8.)

Key words: Allergic rhinitis, asthma, animal models

Allergic rhinitis (AR) is the most common of the atopic diseases, affecting 15% to 20% of the population beginning as early as age 2 years.¹⁻⁶ AR is linked to allergic asthma (AA) and sinusitis,^{1,4,7,8} with AR symptoms and

Abbreviations used

AA: Allergic asthma

AR: Allergic rhinitis

BAL: Bronchoalveolar lavage

OVA: Ovalbumin

skin prick test positivity to common aeroallergen occurring in up to 88% of asthmatic subjects.^{4,8-11} Treatment of AR reduces the frequency and severity of AA symptoms.¹²

These data indicate a close relationship between the upper and lower airways in allergic diseases.^{1-3,6,13-16} The large particle size of aeroallergens suggests primary natural sensitization occurs in the nares.⁹ Development of specific IgE is important in AR and AA pathogenesis in human patients,^{17,18} but regulation of local allergic responses has not been fully explored. Inflammation and airways hyperresponsiveness are characterized by infiltration of the mucosa and airway walls by T cells, eosinophils, and mast cells in both human patients and animals.^{10,13,19} Several animal models of AA have shed light on the cellular and cytokine profiles associated with airway inflammation.¹⁹⁻²⁴ They demonstrate that the relative importance of mast cells, eosinophils, IgE, and the cytokines IL-4 and IL-5 in the development of allergic inflammation varies with the sensitization protocol used and the animal strain.²⁴⁻²⁶

Murine models of AA and the few reported models of AR are generated primarily by means of systemic sensitization through the intraperitoneal or subcutaneous route and subsequent local challenge with nebulized or intranasal allergen.²⁷⁻³² Exclusive local sensitization has been described in only a small number of studies.^{27-29,33} In one study intranasal sensitization resulted in pulmonary eosinophilic infiltrates and hyperresponsiveness that evolved more slowly than that seen in the intraperitoneally sensitized animals.²² Local application of allergen likely results in a differentially regulated allergic response compared with that seen in systemically sensitized animals. This underscores the importance of a suitable well-characterized animal model that can, as closely as possible, mimic in vivo sensitization in human AR and asthma.

Using exclusive local sensitization of the mouse nares, we have developed a model of AR to investigate the effect of site-specific sensitization on the regulation of upper and lower airway allergic responses in vivo.

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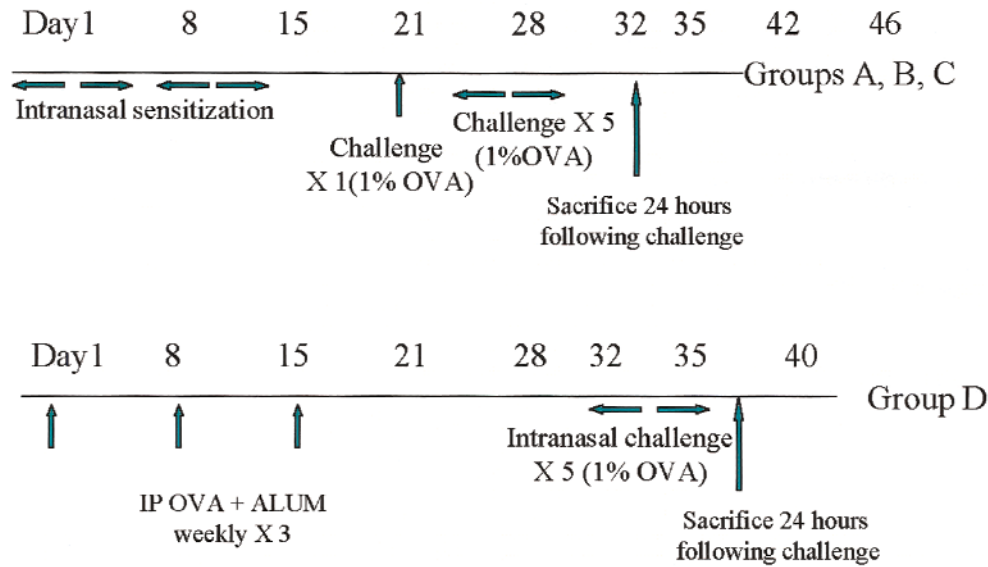


FIG 1. Sensitization and challenge protocols. Schematic of the protocols used to develop allergic sensitization in 6- to 8-week-old BALB/c mice. Groups A (control), B (low dose), and C (high dose) were sensitized exclusively through the intranasal route, whereas Group D (systemic) received 3 intraperitoneal injections, as described in the "Methods" section. *IP*, Intraperitoneal.

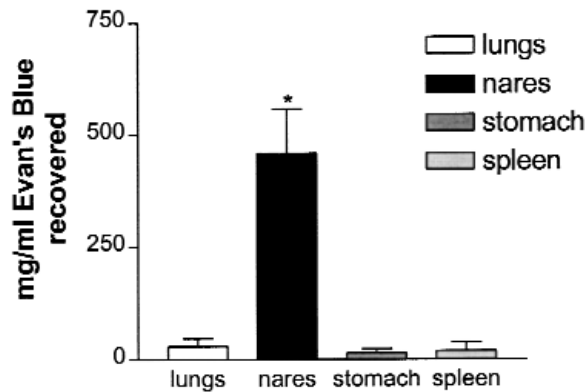


FIG 2. Distribution of Evan's Blue dye in animals inoculated through the intranasal route. Six animals were treated with intranasal Evan's Blue dye, and distribution in various organs was assessed, as describe in the "Methods" section. Data represent means $\pm$ SEM. * $P = .0001$.

METHODS

Animals

Six- to 8-week-old BALB/c male mice were obtained from Harlan-Sprague Dawley (Indianapolis, Ind) and were housed in a conventional animal facility at the Meakins-Christie Laboratories. All studies were evaluated and performed in accordance with the Animal Ethics Committee at McGill University.

Ovalbumin sensitization and challenge

Ovalbumin (OVA; grade V; Sigma Chemical Co, St Louis, Mo) was used in all experiments. Animals were sensitized and chal-

lenged according to the protocol shown in Fig 1. Briefly, awake animals were sensitized daily by means of local instillation of 5 μ L in each of the nares of PBS (group A, sham control), 0.001% OVA in PBS (group B, low dose), or 1% OVA in PBS (group C, high dose). Systemically sensitized animals (group D) received intraperitoneal injection weekly for 3 weeks with 0.4 mL of a solution containing 250 mg/mL OVA mixed with 4 mg/mL AL(OH)₃. Two weeks later, all animals were challenged daily with PBS (group A) or 1% OVA (groups B, C, and D). Animals were killed 24 hours after final challenge by means of anesthetic overdose.

Measurement of OVA-specific IgG and IgE

Serial serum samples were obtained by means of saphenous vein puncture, and OVA-specific IgG and IgE levels were assessed by using ELISA. Briefly, ELISA plates were coated with 20 mg/mL OVA in 0.1 mol/L carbonate buffer, pH 8.3, and blocked with PBS-Tween 20 (0.05%). For OVA-specific IgG levels, purified mouse IgG (Zymed Laboratories, San Francisco, Calif) was used as standard. Serum samples were loaded into each well and incubated for 2 hours. Plates were washed, and biotin-labeled rabbit anti-mouse IgG plus horseradish peroxidase-streptavidin (Zymed Laboratories) was added. After washing, TMB substrate (Zymed Laboratories) was added. The reaction was stopped with 1 mol/L H₃PO₄ and read at 450 nm. OVA-specific IgE measurement was performed with an ELISA kit (Pharmingen OPTeia IgE Kit, San Diego, Calif) with the following modifications: serum was incubated overnight at 4°C with protein G sepharose beads before loading onto ELISA plates. Standards and samples were plated, and ELISA was performed as per the protocol (Pharmingen OPTeia mouse IgE set).

Bronchoalveolar lavage

Immediately postmortem, the trachea was isolated from the upper airway, and a 22-gauge stainless-steel catheter was inserted into the proximal trachea and secured. The lungs were lavaged with 0.5 mL of ice-cold normal saline. The fluid obtained was frozen at

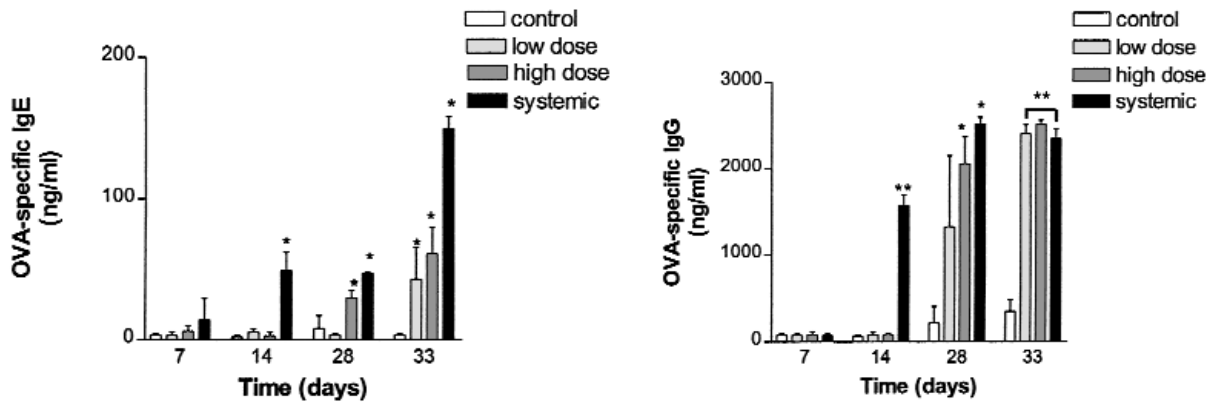


FIG 3. OVA-specific IgE and IgG levels in sensitized and challenged mice over time. Specific anti-OVA antibodies were measured in sera obtained by means of serial saphenous vein puncture by using ELISA, as described in the "Methods" section. Six to 8 animals were used per group, and mean antibody levels $\pm$ SEM were presented. * $P \leq .007$, ** $P = .0005$.

-70°C for later analysis. IL-4, IL-5, and IFN- γ were assayed in bronchoalveolar lavage (BAL) fluid by using OPTEDIA kits, according to protocols provided by the manufacturer (Pharmingen). Cells from the initial lavage fluid were spun onto glass slides and stained by using the Diffquick method. Differential cell counts were obtained manually under light microscopy. Two to 3×100 cells were counted per slide, and means were obtained.

Histologic analysis

Immediately postmortem, the trachea was isolated and cannulated as described above. With a 1-mL formalin-filled syringe, the lungs were slowly inflated with the fixative. They were then isolated and placed entirely in formalin. The skull was also isolated and placed in formalin. The specimens were embedded in paraffin, and 0.5- μm sections were cut. Slides were stained by using Giemsa staining and examined under standard light microscopy.

Distribution of intranasal particles

Ten microliters of a 1 mg/mL solution of Evan's Blue dye in PBS was instilled into each nostril of awake animals, which were then sacrificed by means of anesthetic overdose. The nares, lungs and trachea, stomach, and spleen of each animal were removed, and the Evan's Blue was extracted in formamide. The optical density of the supernatant was assessed at 620 nm by using a spectrophotometer, and concentrations were calculated by using a curve derived from known standards.

Statistical analysis

The results are expressed as means $\pm$ SEM. Statistical significance was measured by means of 1-way analysis of variance, followed by Tukey post hoc testing for individual group comparisons with SPSS software.

RESULTS

Development of the sensitization and challenge protocol

Two concentrations of intranasal allergen were used: the 1% solution (high dose) used in AA models and a 0.001% solution (low dose), as shown in Fig 1. Systemi-

cally sensitized animals receiving intraperitoneal sensitization, as per previously published AA models,¹⁹⁻²⁴ acted as positive controls. All the above animals received 1% intranasal OVA challenge. PBS-sensitized and PBS-challenged mice served as negative controls.

Distribution of particles after local challenge

Awake animals were treated with intranasal application of Evan's Blue dye in normal saline to assess the distribution of allergen. As shown in Fig 2, the particles were distributed primarily in the upper airways. There was 87.8% Evan's Blue dye recovery from the nares (459.5 ± 98.8 mg/mL [$\pm$ SEM], $P = .0001$) compared with 5.6% in the lungs (29.4 ± 17.9 mg/mL [$\pm$ SEM], not significant) and 2.8% in the stomach (14.7 ± 9.0 mg/mL [$\pm$ SEM], not significant). Dye uptake in the lungs and stomach was not significantly different from that measured in the spleen, which served as a negative control (Fig 2). Thus stimulation of the allergic response is potentially greatest in the upper airway.

OVA-specific IgG and IgE levels in animals after sensitization

Serial serum samples were obtained at days 0, 7, 14, 28, and 33 and assayed for OVA-specific IgG and IgE to determine the kinetics of antibody response to OVA-specific sensitization. As shown in Fig 3, both IgG and IgE levels were significantly increased in systemically sensitized animals as early as 14 days. Locally sensitized animals had significant production of IgG by 28 days (Fig 3, B) in both the low- and high-dose groups. OVA-specific IgE reached a significant level in the high-dose, locally sensitized group by day 28, whereas the low-dose group showed significant OVA-specific IgE only after challenge on day 33. These data demonstrate that systemic humoral responses are achieved in locally sensitized animals, with delayed kinetics relative to those seen in systemically sensitized mice.

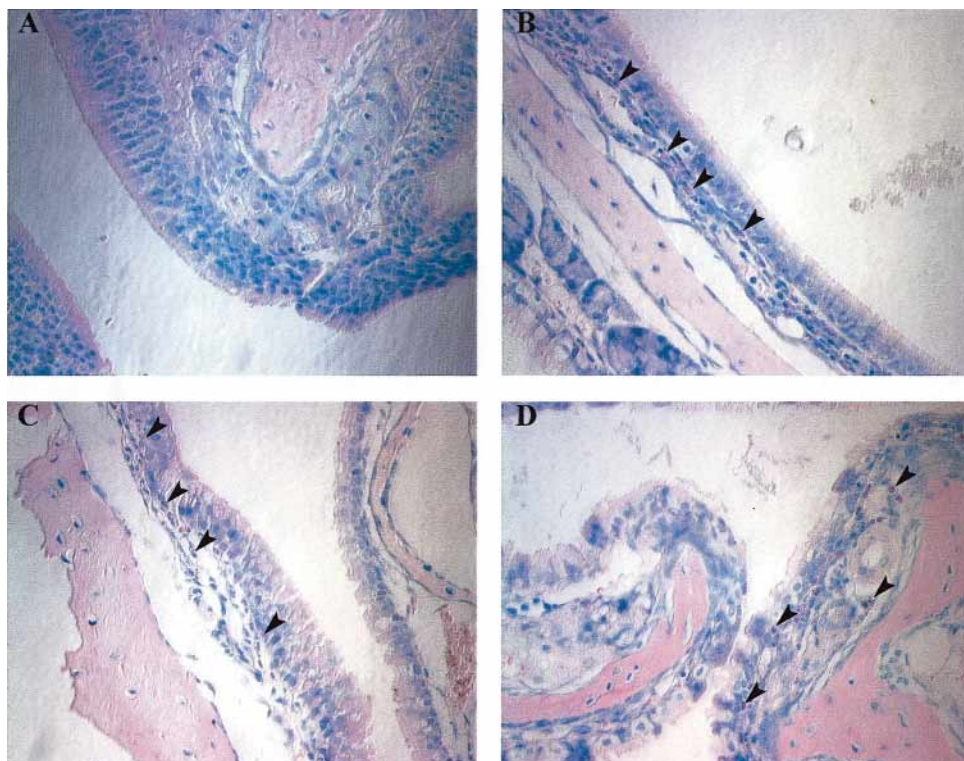


FIG 4. Histology of BALB/c nares after challenge: progressive accumulation of inflammatory cells, particularly eosinophils, in treated groups. **A**, Sham saline control; **B**, local, low dose; **C**, local, high dose; **D**, systemic. Sections were stained with Giemsa stain (original magnification $\times 400$).

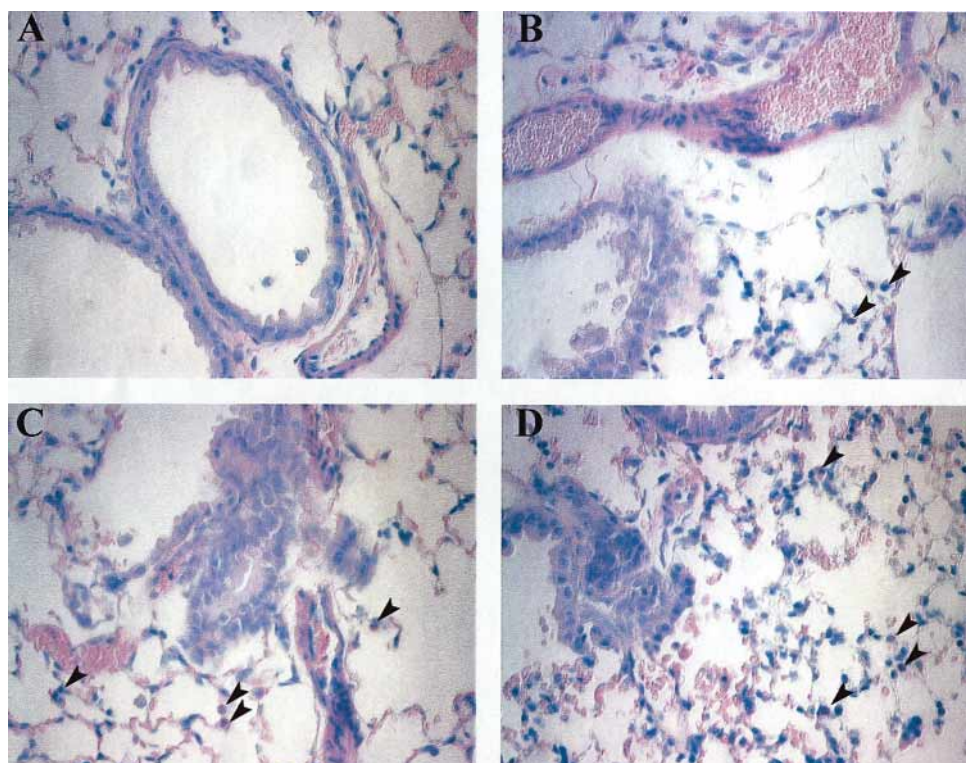


FIG 5. Histology of BALB/c lungs after challenge: progressive inflammation and airway thickening in treated groups. **A**, Sham saline control; **B**, local, low dose; **C**, local, high dose; **D**, systemic. Sections were stained with Giemsa stain (original magnification $\times 200$).

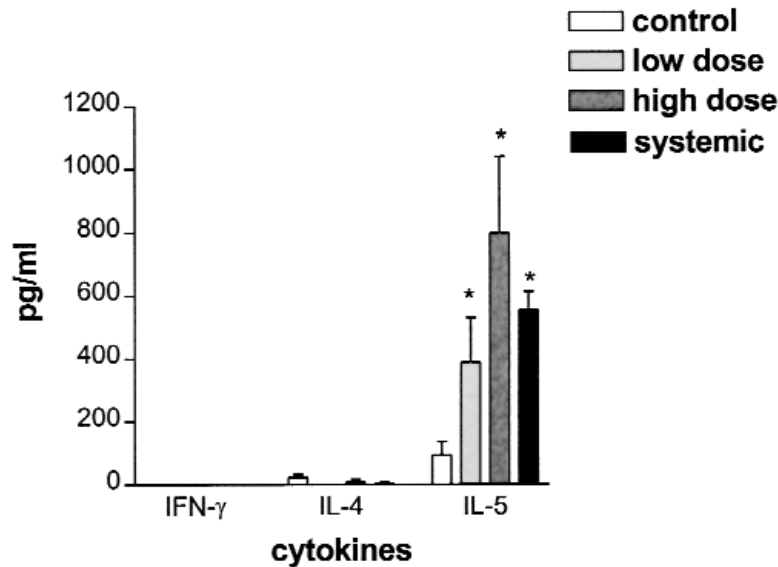


FIG 6. Detection of IL-5 in BAL fluid of sensitized animals after challenge. IL-4, IL-5, and IFN- γ were assayed in BAL fluid by means of ELISA. Four to 6 animals were used per group, and means $\pm$ SEMs were tabulated. * $P = .03$.

Histology of the upper and lower airways

As shown in Fig 4, *B-D*, OVA sensitization and intranasal challenge resulted in inflammation of the sinuses characterized by mucous cell hyperplasia, recruitment and influx of eosinophils and lymphocytes, and disruption of the brush border in all groups. The degree of inflammation appears to parallel the levels of OVA-specific IgE shown in Fig 3, *A*, with the intraperitoneal route of sensitization producing the greatest inflammatory changes (Fig 4, *D*).

Areas of inflammation were noted in the lower airways of all OVA-treated groups, with lung sections showing eosinophil and lymphocytic peribronchial infiltration (Fig 5). We noted that the route of sensitization did not appear to affect the nature of the inflammatory response, whereas antigenic dose, specifically low-dose sensitization (Fig 5, *B*), affected the degree of cellular infiltrate, particularly eosinophils, when compared with that seen in the high-dose (Fig 5, *C*) and systemic (Fig 5, *D*) groups. Thus immune responses vary depending on antigen stimulus, site of initial stimulation, and initial concentration of antigen. Finally, eosinophilic inflammatory changes occurred in the lungs despite a lack of significant direct allergen deposition in the lower airways.

Analysis of BAL fluid

Fig 6 shows the expression of IL-4, IL-5, and IFN- γ from BAL fluid. In all groups IL-4 and IFN- γ were not detected at greater than background levels. By using the A/J strain of animals, IFN- γ and IL-4 were detectable at greater than control levels in treated animals (data not

shown), indicating that although these cytokines are detectable with our ELISA assay system, the levels of expression were less than the detection limits in these BALB/c mice. It is possible that IL-4 expression occurs earlier in the challenge phase and is downregulated by day 5 in BALB/c mice. Also, IL-4 might be more rapidly degraded and therefore not detectable in BAL fluid 24 hours after challenge. We are currently investigating the kinetics of IL-4 and IL-5 expression over the 5-day challenge period. In contrast, the IL-5 level was significantly increased in all treatment groups compared with in control animals. Intranasal sensitization leads to similar if not greater increases in IL-5 compared with that seen in intraperitoneally sensitized animals (Fig 6).

Differential leukocyte cell counts from BAL fluid were determined by means of light microscopy. One hundred leukocytes were counted per high-powered field and categorized as lymphocytes or PMNs. Animals with inflammatory changes in their lungs showed increased accumulation of PMNs in their BAL fluid (Fig 7). The total number of PMNs was greatest ($P = .005$) in the systemically sensitized group at 40.6 ± 11.8 PMNs per 100 cells counted. The high- and low-dose groups also showed increased PMNs relative to that seen in control animals, but this did not achieve statistical significance.

These data show immunologic changes in both the upper and lower airways of sensitized animals of the T_H2 or allergic phenotype. We have demonstrated eosinophilia on histologic sections and increases in IL-5 in BAL fluid from these animals, as well as OVA-specific IgE production. Exclusive sensitization and challenge of the upper airways in this animal model results in the inflam-

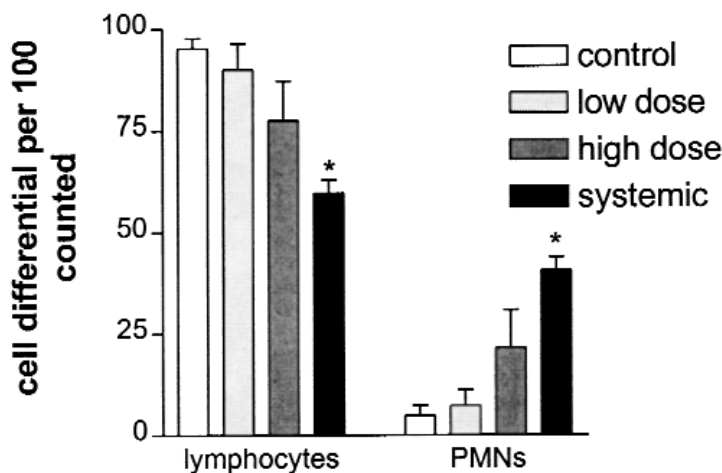


FIG 7. Differential cell counts from BAL fluid of animals 24 hours after final challenge. Lymphocytes and PMNs were enumerated by using light microscopy, as described in the "Methods" section. Four to six animals were used per group, and means $\pm$ SEM were presented. * $P \leq .005$.

matory changes of the lower airways seen in both human and mouse models of AA, further emphasizing the importance of the nares in allergic sensitization, AR, and AA development.

DISCUSSION

This model of AR has allowed us to study the nasal mucosa and systemic responses to allergens and to demonstrate the pathologic changes that occur in both the nose and lung after allergen challenge.

There is a close relationship between upper and lower airway allergic diseases. The primary site for de novo immune responses in human patients is the nose and its draining lymphoid system,⁹ and evidence suggests that little allergen is deposited directly in the lungs. In our study we have shown that deposition of intranasal allergen is almost exclusively in the nares of mice (Fig 2). We were able to show allergic inflammation in both the upper and lower airways of these animals, despite little or no allergen deposition in the lungs (Fig 5). In addition, the BAL fluid from these animals showed increases in IL-5 levels (Fig 6) and numbers of PMNs (Fig 7) after local intranasal challenge, suggesting that allergic inflammation is occurring distal to the site of direct antigen deposition. These data demonstrate that the upper airway is the first site of response to allergic stimulation and mediates effects along the airway and respiratory tree.

Studies in human patients support these findings.^{4,12,13,34} In one study³⁵ segmental bronchial provocation was performed in individuals with AR but not in individuals with AA. Upper airway inflammation and symptoms of AR occurred in the absence of direct application of allergen to the nose, suggesting that the regula-

tion of the allergic response in both the upper and lower airways are linked and that local challenge plays a significant role in distal allergic responses.

Murine models of AA rely primarily on systemic intraperitoneal priming and local challenge.^{26,34-49} Hellings et al⁴⁶ have examined the effect of intraperitoneal sensitization local challenge with nebulized OVA on the upper airway. They report both upper and lower airway eosinophilic inflammation, providing evidence of global airway allergic responses. Tsitoura et al⁴⁷ examined the effects of local sensitization on subsequent systemic responses in a similar model. Mice received intranasal OVA or PBS for 3 days before the routine intraperitoneal sensitization protocol. They noted a significant blunting of the systemic response, as measured by serum immunoglobulins and, in specific, T-cell proliferative responses. In a follow-up study⁴⁸ the authors showed decreased airway responsiveness in these animals. The present study compares local versus systemic sensitization. We show that exclusive local sensitization results in an increase in serum IgE and IgG levels, although the time course of the immune response appears to be slower than in systemically sensitized animals (Fig 3). We have demonstrated eosinophilic infiltrates in the nose and lungs of all challenge groups (Figs 4 and 5). Thus exclusive local sensitization and challenge is not tolerizing in our model. Taken together, these studies suggest that although local application of allergen results in a robust T_H2 -type response, subsequent systemic exposure might result in desensitization or tolerance, highlighting the importance of local immune responses in the regulation of allergic disease.

We have shown that local application of allergen results in immune responses that have an allergic phe-

notype of increased IgE and eosinophilia but that differ in kinetics and severity when compared with those seen in systemically sensitized animals. Analysis of the draining lymphoid system of the nares shows marked differences in cell populations after challenge in locally versus systemically sensitized animals (data not shown). Thus the nasal environment at the time of de novo allergen introduction is an essential regulator of subsequent responses in both the upper and lower airways. Using this model of AR, we can examine induction and regulation of the allergic response in vivo and also explore the efficacy of therapeutic interventions in abrogating both AA and AR.

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