

# A new murine model of pulmonary eosinophilic hypersensitivity: Contribution to experimental asthma

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**Background:** We have recently described a model of hypersensitivity reaction in the mouse paw, which induces a typical late-phase reaction with a marked eosinophilic infiltrate.

**Objective:** In the search for a murine model of asthma, this model was adapted to the lungs and compared with other models of pulmonary hypersensitivity.

**Methods:** A fragment of heat-coagulated hen's egg white was implanted subcutaneously, and 14 days later, the mice were challenged intratracheally with aggregated ovalbumin. Comparison was made with a group that received subcutaneous injection of soluble ovalbumin in alumen, challenged as described above and with four additional protocols of immunization and challenge.

**Results:** Forty-eight hours after challenge, the percentage of eosinophils was higher in the egg white implant group (35%) than in the group immunized with ovalbumin in alumen (10.4%). The eosinophil peroxidase activity in lung homogenates of the first group was also significantly higher (529 ng/ml) than that of the second group (43 ng/ml). These results were reproduced in five different mouse strains. Compared with five different models of lung hypersensitivity, the egg white implant model was unique in terms of persistence of the pulmonary eosinophilia. Histopathologic analysis of the lungs of mice immunized with egg white implant showed peribronchial, perivascular, and intraepithelial eosinophil infiltration; morphologic characteristics of bronchoconstriction; and patchy epithelial shedding. At 21 days, in addition to persistence of eosinophil infiltrate, enlarged alveoli, reflecting air trapping, were observed.

**Conclusion:** On the basis of the characteristics of the model described here, we propose it as a suitable murine model of asthma. (*J Allergy Clin Immunol* 1997;100:383-8.)

**Key words:** Asthma, late-phase reaction, eosinophil, mice, eosinophil peroxidase

## Abbreviations used

|        |                           |
|--------|---------------------------|
| BAL:   | Bronchoalveolar lavage    |
| EPO:   | Eosinophil peroxidase     |
| EWI:   | Egg white implant         |
| LPR:   | Late-phase reaction       |
| OA-AL: | Ovalbumin in alumen       |
| PBS:   | Phosphate-buffered saline |

Asthma is a chronic inflammatory disease of the airways. It is characterized by intermittent, reversible airway obstruction and by bronchial hyperreactivity. It appears that the airway inflammatory reaction is mediated by T-helper type 2 lymphocytes ( $T_{H2}$ ), eosinophils, and mast cells.<sup>1-4</sup> Lung eosinophilia correlates with T-lymphocyte infiltration.<sup>5,6</sup> A large number of eosinophils is found during the late-phase reaction (LPR) in the lungs.<sup>7</sup> Blocking the  $T_{H2}$  development by IL-12 administration in vivo inhibits both the allergen-induced eosinophilia and bronchial hyperreactivity.<sup>6</sup> The pattern of cytokines produced by  $T_{H2}$  cells promotes proliferation of mast cells, stimulates IgE and IgG<sub>1</sub> production, and facilitates eosinophil migration, activation, and survival.<sup>7,8</sup> Indeed, it was shown that airway eosinophilia and hyperreactivity did not occur in IL-5-deficient mice.<sup>9</sup> Furthermore, the severity of bronchial hyperreactivity, which is one of the major characteristics of asthma, parallels the intensity of pulmonary eosinophil infiltration.<sup>10-12</sup> However, Pretolani et al.<sup>13</sup> showed that the sole presence of eosinophils in the airways is not sufficient to induce bronchial hyperreactivity. They suggested that eosinophil activation correlates better with hyperreactivity than eosinophil number per se. Indeed, products of activated eosinophils, such as cytotoxic proteins, were shown to damage the epithelium of the airways. It was shown that injection of eosinophil-derived cationic proteins into the airways causes desquamation of the epithelium and bronchial hyperreactivity.<sup>14-17</sup> These cells can also produce leukotrienes and platelet activating factor.<sup>18,19</sup> Leukotrienes induce bronchoconstriction and mucus secretion; platelet activating factor increases eosinophil infiltration into the airways.<sup>20,21</sup>

The advances in the understanding of the mechanisms involved in the pathogenesis of asthma came from studies with animal models. A great variety of experi-

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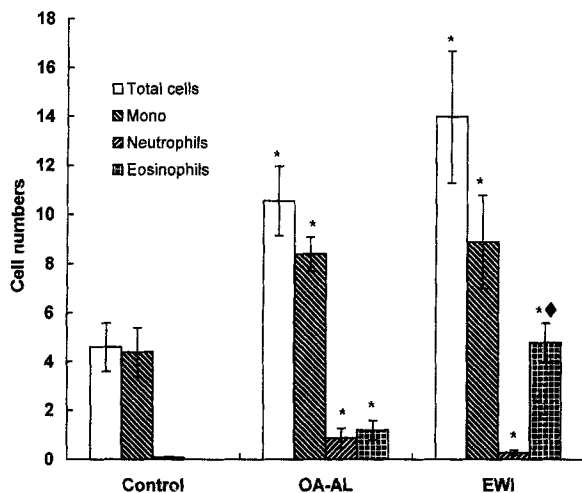


FIG. 1. Cell infiltration into BAL of mice after stimulation with antigen. Groups of mice were immunized with OA-AL or with EWI or were nonimmunized (control). After 14 days, all groups received an intratracheal instillation of aggregated ovalbumin (600  $\mu$ g), and 48 hours later, BAL was performed, and cells were counted. Data represent means  $\pm$  SEM of four to nine animals per group. \* $p < 0.05$  compared with control group.  $p < 0.05$  compared with OA-AL group.

mental models has been used; most of them consisted of immunization with adjuvants and multiple boosters.

We have recently described that immunization of mice with a subcutaneous implant of fragments of heat-coagulated hen's egg white induces a typical LPR with a prominent eosinophilic infiltrate in the mouse paw.<sup>22</sup> In the search for an experimental model of asthma, we adapted this model to induce an LPR in mouse lungs. This study compared the kinetics of eosinophil infiltration, the eosinophil peroxidase (EPO) activity in bronchoalveolar lavage (BAL) and in pulmonary tissue, and the histopathologic alterations of the lungs obtained with this new protocol with that obtained with classical protocols of immunization in which alumen was used as adjuvant.

## METHODS

### Animals

F1(BALB/c  $\times$  A/J), BALB/c, A/J, C57Bl/6J, and CH3/HePas mice (6 to 8 weeks of age) were obtained from our own animal facilities.

### Preparation of the antigens

**Heat-coagulated hen's egg white.** Separated hen's egg white was placed in a water bath at 100°C for 30 minutes. The solidified egg white was washed in distilled water, dehydrated in 100% ethanol for 48 hours, and cut into small fragments of 4  $\times$  2  $\times$  2 mm, weighing about 40 mg each. The egg white fragments were rehydrated in Dulbecco's phosphate-buffered saline (PBS) for 2 hours before implantation.

**Heat-aggregated ovalbumin.** Heat-aggregated ovalbumin was prepared according to the method of Titus and Chiller.<sup>23</sup> Type II ovalbumin was diluted in saline solution, pH 7.4, and heated for 60 minutes at 80°C. After centrifugation at 1900 g for 10

minutes, the supernatant was discharged, and the pellet was resuspended in sterile PBS solution.

### Experimental models

**Egg white implant (EWI).** A fragment of about 40 mg of heat-coagulated hen's egg white was implanted into the subcutaneous tissue of the dorsal region of mice. After 14 days, the animals were challenged with 600  $\mu$ g of heat-aggregated ovalbumin instilled into the trachea.

**Ovalbumin in alumen (OA-AL) immunization 1.** A mixture containing 100  $\mu$ g of grade II ovalbumin and 4 mg of Al(OH)<sub>3</sub> in gel suspension (Aldrex; Wyeth Whitehall Ltd., Brazil) was injected into the subcutaneous tissue of the dorsal region of mice. After 14 days, the animals were challenged with 600  $\mu$ g of heat-aggregated ovalbumin instilled into the trachea.

**OA-AL immunization 2.** A mixture containing 100  $\mu$ g of grade II ovalbumin and 1.6 mg Al(OH)<sub>3</sub> in gel suspension (Aldrex) was injected into the subcutaneous tissue. After 7 days, the animals received the same dose of ovalbumin with Al(OH)<sub>3</sub> subcutaneously and were challenged 7 days later with 600  $\mu$ g of heat-aggregated ovalbumin instilled into the trachea.

**OA-AL immunization 3.** A mixture containing 10  $\mu$ g of ovalbumin and 1.6 mg of Al(OH)<sub>3</sub> was injected intraperitoneally. After 14 days, the animals were exposed for 20 minutes to an aerosol of ovalbumin at a concentration of 20 mg/ml in 0.9% saline solution generated by an ultrasonic nebulizer (ICEL US-800, São Paulo, Brazil).

**OA-AL immunization 4.** A mixture containing 10  $\mu$ g of ovalbumin and 1.6 mg of Al(OH)<sub>3</sub> was injected intraperitoneally. Seven days later, the animals received the same dose of ovalbumin with Al(OH)<sub>3</sub> by the same route and were challenged 7 days later with 600  $\mu$ g of heat-aggregated ovalbumin instilled into the trachea.

**Ovalbumin aerosol.** Mice were exposed for 20 minutes to an aerosol of grade II ovalbumin at a concentration of 20 mg/ml in 0.9% saline solution as described for the OA-AL 3 model. The animals received the ovalbumin in aerosol form on alternate days for 10 days, and the lung EPO activity was measured 7 days later.

**Control group.** Mice that were not immunized received 600  $\mu$ g of heat-aggregated ovalbumin by intratracheal instillation.

### BAL

The animals were killed by an overdose of chloral hydrate. The tracheae were cannulated, and the airway lumina were washed with 6  $\times$  0.5 ml of PBS at 4°C.

### Total and differential cell counts

The BAL fluid was centrifuged at 170 g for 10 minutes, the supernatant was removed, and the cell pellet was resuspended in 0.5 ml of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hemacytometer. Differential cell counts were performed in cytocentrifuge preparations of BAL stained with Hema 3 (Biochemical Sciences Inc., Swedesboro, N.J.).

### Assay of EPO activity

The EPO activity in the BAL was measured according to the method of Strath et al.<sup>24</sup> Briefly, BAL was obtained after 4  $\times$  0.5 ml washes with Tris-HCl buffer (pH 8.0) and sonicated for 1 minute. The samples were then centrifuged at 40,000 g for 5 minutes. The supernatant was further centrifuged at 48,000 g for 60 minutes. The resulting pellet was resuspended in 0.6 ml of

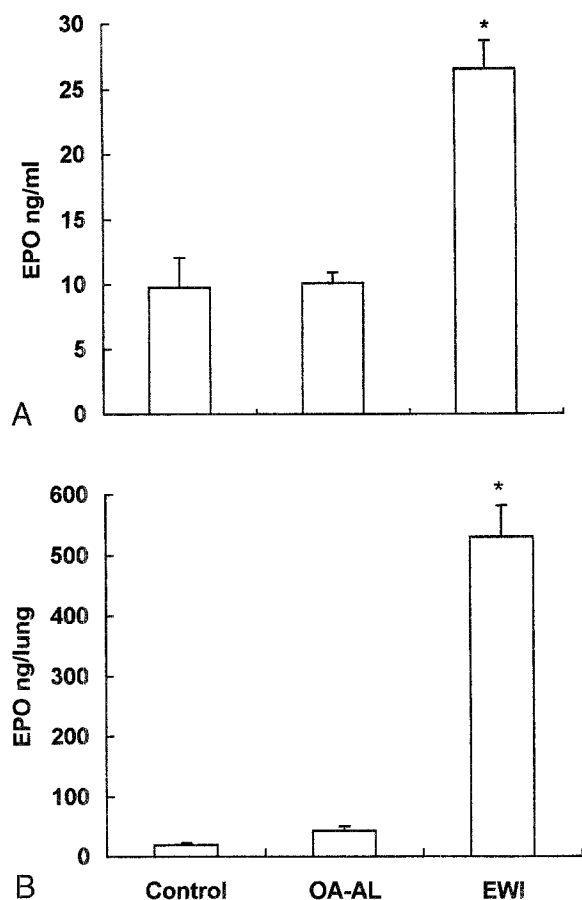


**FIG. 2.** EPO in BAL (A) and lung tissue (B) of mice after antigen stimulation. Groups of mice were immunized with OA-AL or with EWI or were nonimmunized (control). After 14 days, all groups received an intratracheal instillation of aggregated ovalbumin (600  $\mu$ g), and 48 hours later, EPO activity was determined. Data represent means  $\pm$  SEM of four to six animals per group. \* $p$  < 0.05 compared with control and OA-AL groups.

Tris-HCl buffer and centrifuged at 12,000  $g$  for 5 minutes. One hundred microliters of each supernatant was incubated with an equal volume of 1.5 mmol/L *o*-phenylenediamine dihydrochloride in 0.05 mmol/L Tris-HCl buffer and 6.6 mmol/L  $H_2O_2$  for 30 minutes at 37°C. The reaction was stopped by addition of 50  $\mu$ l of 4 mol/L  $H_2SO_4$ , and the absorbance of the samples was determined at 492 nm. The concentration of EPO was estimated from a standard curve by using 1 to 125 ng/ml of horseradish peroxidase. The results were expressed as nanogram equivalents of horseradish peroxidase per milliliter of BAL. To determine the EPO activity in the pulmonary tissue, lungs were perfused with saline-heparin solution through the pulmonary artery to remove residual blood and homogenized with a Polytron homogenizer (Brinkmann Instruments) for 30 seconds in 2 ml of 0.05 mmol/L Tris-HCl containing 0.1% Triton X-100, and the resultant homogenate was sonicated for 1 minute. The samples were then sequentially centrifuged as described above, and the final supernatants were serially diluted and assayed for peroxidase activity.

#### Histopathologic analysis

Animals used for histologic analysis were challenged with a solution of aggregated ovalbumin resuspended in 0.01% Evans

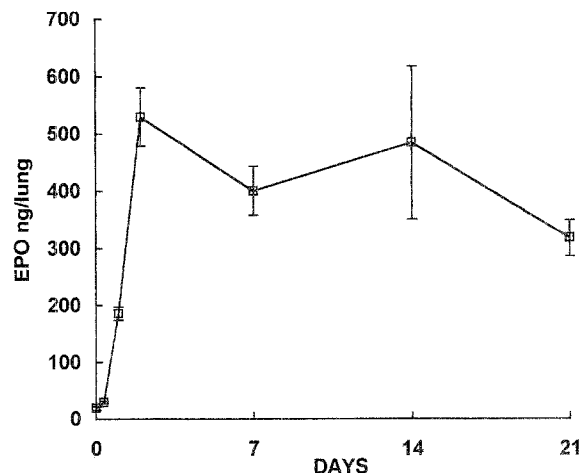


**FIG. 3.** Time course of EPO activity in lung tissue. Mice were immunized with EWI and 14 days later received an intratracheal instillation of ovalbumin (600  $\mu$ g). Animals were killed on indicated days after antigen challenge. Data represent means  $\pm$  SEM of three to five animals per group.

blue dye to localize the site of inoculation. Lungs of experimental and control groups were removed 48 hours after challenge and fixed for 24 hours in a solution of buffered formalin. Fragments of lung tissue were embedded in paraffin and processed for routine histologic examination. Sections were stained by hematoxylin-eosin for eosinophil characterization.

#### Drugs and reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.): ovalbumin grade II, Tris (hydroxymethylaminomethane), ortho-phenylenediamine dihydrochloride, and horseradish peroxidase (type II). Triton X100 was purchased from LKB Products AB (Bromma, Germany).

#### Statistics

The data were analyzed by one-way analysis of variance. A  $p$  value equal to or lower than 0.05 was considered significant.

### RESULTS

#### Cells in the BAL

Groups of F1 (BALB/c X A/J) mice were immunized with OA-AL or with EWI and 14 days later challenged intratracheally with aggregated ovalbumin. Fig. 1 shows that 48 hours after antigen challenge, the total number of cells in the BAL increased significantly in OA-AL and EWI-immunized groups in comparison with nonimmunized mice. Both immunized groups had an increased influx of mononuclear cells, neutrophils, and eosinophils. However, the EWI group exhibited a significantly higher mobilization of eosinophils (35%) than the OA-AL group (10%) (Fig. 1).

#### EPO activity in BAL and lung tissue

Analysis of the EPO activity in the BAL at 48 hours after intratracheal antigen challenge showed that immunization with EWI induced significantly higher levels of EPO activity than immunization with OA-AL (Fig. 2, A),

TABLE I. EPO activity in different experimental models

| Experimental model | Days after challenge |                     |                    |
|--------------------|----------------------|---------------------|--------------------|
|                    | 2                    | 7                   | 30                 |
| EWI                | 529.98 $\pm$ 51.50   | 496.48 $\pm$ 105.60 | 145.90 $\pm$ 11.95 |
| OA-AL 1            | 43.08 $\pm$ 7.20*    | 36.60 $\pm$ 4.20*   | ND                 |
| OA-AL 2            | 32.81 $\pm$ 3.45*    | ND                  | ND                 |
| OA-AL 3            | 101.50 $\pm$ 57.50*  | 68.45 $\pm$ 5.70*   | ND                 |
| OA-AL 4            | 529.00 $\pm$ 79.10   | 362.13 $\pm$ 77.23  | 58.70 $\pm$ 13.28* |
| Ovalbumin aerosol  | ND                   | 54.13 $\pm$ 17.36*  | ND                 |
| Control lungs      | 19.33 $\pm$ 3.20*    | 39.50 $\pm$ 18.25*  | ND                 |

EPO activity (expressed as nanograms per lung) was measured in lung homogenates, 48 hours after antigen challenge. Data represent mean(s)  $\pm$  SEM of three to six animals per group. For description of experimental models, see Methods.

ND, Not determined.

\*  $p < 0.05$  compared with EWI group.

TABLE II. EPO activity in mouse strains

| Mouse strain     | EPO activity*      |
|------------------|--------------------|
| F1(BALB/c X A/J) | 529.98 $\pm$ 51.50 |
| A/J              | 336.60 $\pm$ 48.06 |
| BALB/c           | 291.58 $\pm$ 43.20 |
| C3H/HePas        | 354.11 $\pm$ 69.05 |
| C57Bl/6J         | 369.36 $\pm$ 35.15 |

EPO activity (expressed as nanograms per lung) was measured in lung homogenates 48 hours after antigen challenge of mice immunized with EWI. Data represent mean(s)  $\pm$  SEM of three to five animals per group.

thus confirming the data obtained with differential cell counts. Likewise, the EPO activity in the lung tissue was also significantly higher in the EWI group. It should be mentioned that EPO activity in lung homogenates of mice that were immunized with OA-AL and challenged 7 days later with the antigen was not significantly different from that found in the group without the booster (data not shown).

The temporal profile of the EPO activity in lung homogenates of the group that received EWI is shown in Fig. 3. As can be seen, there is a sharp increase in EPO activity 24 hours after ovalbumin challenge compared with the control group. Interestingly, these high levels of EPO activity persisted for 21 days.

The EWI model was also compared with models in which different protocols of sensitization and challenge were used. Table I shows that immunization with OA-AL, given subcutaneously with or without a booster injection, induced very low levels of EPO activity in lung homogenates obtained 2 or 7 days after antigen challenge (OA-AL 1 and OA-AL 2 models). Sensitization by repeated doses of ovalbumin in aerosol (ovalbumin aerosol) induced similarly low levels of EPO. Immunization with ovalbumin in alumen (OA-AL 3) given intraperitoneally also induced low levels of EPO. When a booster injection of OA-AL (administered intraperitoneally) was introduced (OA-AL 4), the levels of EPO markedly increased and were similar to those found in

the EWI group. However, 30 days after challenge, the levels of EPO were significantly higher in the EWI group than in the OA-AL 4 group.

Next, we analyzed the EPO activity in lung homogenates of other mouse strains that were immunized with EWI. As shown in Table II, all mouse strains tested displayed high levels of EPO activity in their lung homogenates 48 hours after ovalbumin challenge. The EPO activity was roughly similar among them.

### Histopathologic examination of inflamed lungs

The instillation of antigen into the trachea of mice immunized with EWI resulted in a marked inflammatory response. The main histologic lesion 48 hours after challenge was an intense bronchitis-bronchiolitis along the different segments of the intrapulmonary airway—from the axial bronchus up to the bronchioli. The alveolar parenchyma around the airways also showed an interstitial inflammatory process characterized by recruitment of effector cells. Mononuclear cells and eosinophils were prominent among the infiltrating elements (Fig. 4, A). The peribronchial inflammation was intense (Fig. 4, A and B), and areas of intraluminal epithelial shedding and degeneration of the epithelial cell layer (Fig. 4, C) were also present. The architecture of the airways was altered, demonstrating mucosal folding, characteristic of bronchoconstriction (Fig. 4, A and B). A large mucus plug is seen in Fig. 4, B. Infiltration of eosinophils and mononuclear cells around the venules and arterioles, as well as in the lumen of the airways, was frequently found. On day 21 after challenge, the cellular infiltrate still showed eosinophils but with a predominance of mononuclear cells. Alveoli with increased airway space and alveolar wall destruction characteristic of emphysema were observed (Fig. 4, D).

### DISCUSSION

Asthma is a chronic lung disease that is increasing in industrialized countries. A prominent eosinophil infiltrate in the airway tissue is one of the characteristic features of asthma.<sup>25</sup> Thus it is of interest to develop experimental models that mimic this disease. Although

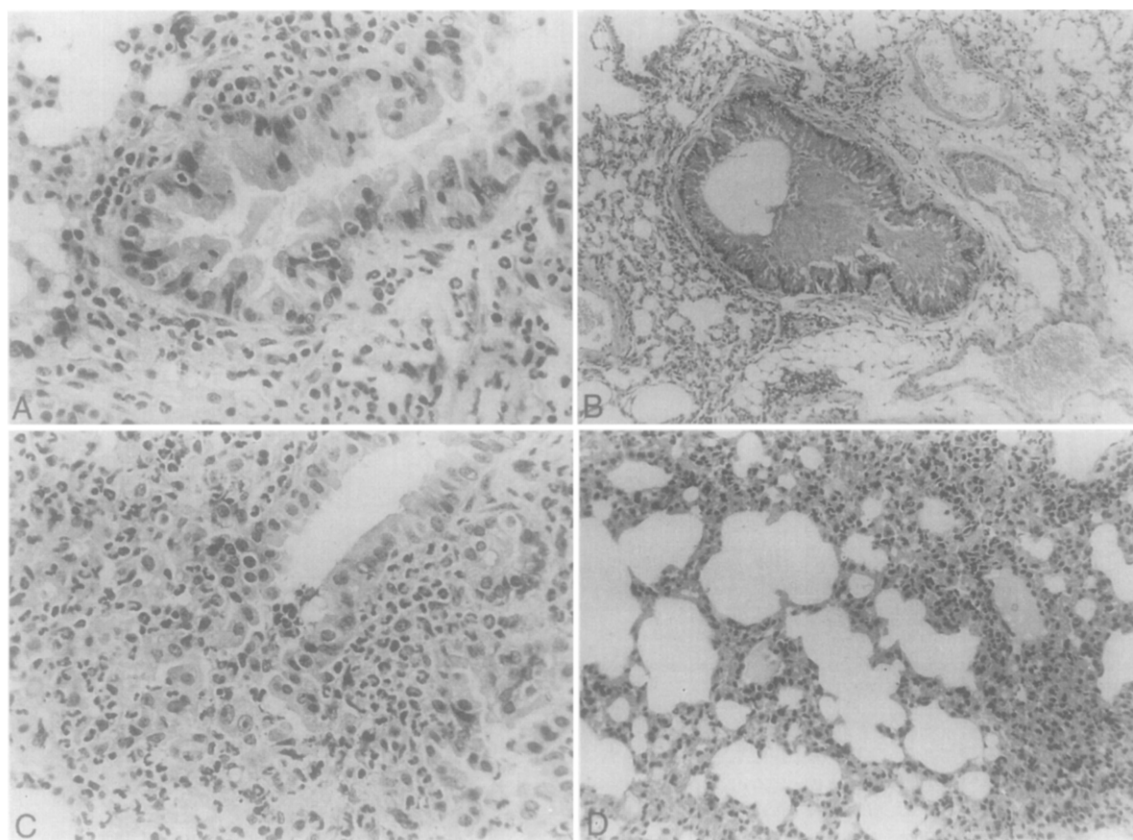


**FIG. 4.** Histopathologic analysis of mouse lungs immunized with EWI 48 hours (**A, B, C**) or 21 days (**D**) after intratracheal challenge with ovalbumin. **A**, Small bronchus showing typical morphologic features of bronchoconstriction and peribronchial infiltration of eosinophils and mononuclear cells with eosinophils infiltrating the epithelial layer (original magnification,  $\times 40$ ). **B**, Small bronchus with mucus plug, mucosal folding, and inflammatory cell infiltration (original magnification,  $\times 25$ ). **C**, Small bronchus showing degeneration of epithelial cell layer and shedding (original magnification,  $\times 40$ ). **D**, Lung parenchyma with infiltration of inflammatory cells and alveoli with increased air space characteristic of emphysema (original magnification,  $\times 25$ ).

several animal models have been described in the literature,<sup>21</sup> all of them require adjuvants in the immunization protocols, and some of them do not present a typical allergic LPR as it occurs in human beings. We have recently shown<sup>22</sup> that an exuberant eosinophilic LPR can be induced in the footpads of mice after immunization with EWI.

In this study we observed that mice immunized with EWI and intratracheally challenged with ovalbumin demonstrated fourfold more eosinophils than the group immunized with OA-AL. Analysis of EPO activity in the BAL confirmed these results. When EPO activity was measured in homogenates of lung tissue, the difference between the two groups was even more pronounced, being roughly tenfold higher in the EWI group. This result indicated that a large number of eosinophils was infiltrating the pulmonary parenchyma. Interestingly, this strong eosinophil infiltration persisted for 21 days as assessed by EPO activity in the lung tissue. Histopathologic analysis of the lungs showed that this cell type is localized in the parenchyma around the small bronchi, as well as in the alveoli.

Desquamation of bronchial epithelium is one charac-

teristic of chronic pulmonary inflammation, and in patients with asthma different degrees of destruction of the epithelium were reported.<sup>26, 27</sup> In the EWI model, we found shedding of airway epithelial cells, epithelial cell fusion, intraepithelial infiltration of eosinophils, and morphologic characteristics of bronchoconstriction. These features were not observed in the OA-AL group. The most striking finding in the EWI group at 21 days after challenge was the existence of large areas of lung tissue with unequivocal characteristics of emphysema. The development of this disease is indirect evidence of long-standing bronchoconstriction, morphologically characterized in this model.

The mechanism by which EWI induces persistent eosinophilia is not easy to understand but is probably related to stimulation of  $T_{H2}$ -like cell activity. It has been shown recently<sup>28</sup> that the delivery of low doses of soluble antigens by mini osmotic pumps implanted in the subcutaneous tissue leads to a selective development of  $T_{H2}$  cells. Thus by analogy it is possible that in the EWI model, small doses of soluble ovalbumin are released continuously.

A relevant finding of our study is that except for EWI,

none of the protocols of immunization tested here were able to induce significant EPO activity 30 days after challenge. Immunization with subcutaneous OA-AL, even with one booster injection, was unable to induce a significant eosinophilic infiltration into the lungs. Exposure of mice to ovalbumin aerosol was also ineffective. Intraperitoneal immunization with OA-AL induced a strong infiltration but only when a booster injection was given. In this situation the EPO activity was similar to that found in the EWI group on day 2 after challenge but declined thereafter, and on day 30 it reached almost basal levels. Thus persistence of lung eosinophilia is characteristic of the EWI model.

The model proposed in this study has several advantages. First, it is simple to perform and is reproducible in different mouse strains and different species, such as rats in which it induces bronchial hyperreactivity (Carvalho et al. Unpublished data). Second, this model does not require adjuvant or immunization with multiple boosters. Third, the allergen persists in the animal, which resembles the continuous exposure to allergen(s) present in the environment. Fourth, it induces long-lasting lung lesions that resemble those found in human asthma.

Further basic investigations on lymphocytes, cytokines, and other mediators that may participate in the induction of cellular and humoral events in this model, as compared with others induced by adjuvants, are under investigation.

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