

Characterization of a Murine Model of Allergic Pulmonary Inflammation

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Abstract

Pulmonary inflammation with eosinophil (EOs) infiltration is a prominent feature of allergic respiratory diseases such as asthma. In order to study the cellular response during the disease development, an animal model of IgE-mediated pulmonary inflammation with characteristic eosinophilia is needed. We developed a method for inducing severe pulmonary eosinophilia in the mouse and also studied the numbers of EOs in blood and bone marrow and the response to corticosteroid treatment. Animals were sensitized with alum-precipitated ovalbumin (OVA) and challenged with aerosolized OVA 12 days later when serum IgE levels were significantly elevated. Four to eight hours after challenge there were moderate increases in the number of EOs in the bone marrow and peripheral blood, but only a few EOs were observed in the lung tissue and in bronchoalveolar lavage (BAL) fluid. Twenty-four hours after challenge, there was a marked reduction of EOs in bone marrow, while the number of EOs peaked in the perivascular and peribronchial regions of the lung. Forty-eight hours after challenge, the highest number of EOs was found in the BAL fluid, making up >80% of all cells in that compartment. The high levels of EOs in the lung tissue and BAL fluid lasted for 2-3 days and was followed by a more moderate but persistent eosinophilia for another 10 days. Nonsensitized animals showed no significant changes in the number of EOs in BAL fluid, lungs, blood or bone marrow. Histopathological evaluation also revealed epithelial damage, excessive mucus in the lumen and edema in the submucosa of the airways. The pulmonary eosinophilia and decrease in bone marrow EOs induced by OVA challenge responded well to treatment with several standard corticosteroids. The rank order of steroid potency for inhibition of pulmonary eosinophilia was betamethasone > prednisolone > hydrocortisone. Because mice are extremely useful for immunological studies, this model can be invaluable to study the effects of cytokines on pulmonary inflammation.

Key Words

Eosinophils
Pulmonary inflammation
IgE
Allergic mouse
Glucocorticoids

Introduction

Pulmonary inflammation with eosinophil (EOs) infiltration is a prominent feature of allergic respiratory diseases, such as asthma [1-7]. The pathobiology of the pulmonary eosinophil is dependent upon factors which regulate eosi-

nophilopoiesis, cellular viability, adherence, migration, and activation of the EOs. For example, several mediators of allergy and inflammation such as platelet-activating factor, leukotriene C_4 and cytokines such as interleukin 3 (IL-3), interleukin 5 (IL-5) and granulocyte macrophage-colony stimulating factor (GM-CSF), play important roles con-

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trolling eosinophil function [8–17]. The precise mechanism regulating the pulmonary eosinophilia *in vivo* are not clear, but this information could provide a basis for the development of novel and specific therapeutic approaches for the treatment of allergic respiratory diseases. To assist in studying the mechanism underlying pulmonary eosinophilia and consequent inflammation, a relevant animal model is needed that exhibits a similar immunobiology and sensitivity to drugs as found in human asthma.

Several animal models have been developed to study pulmonary inflammation with characteristic manifestations of airways eosinophilia. One of the often-used animal models is the ovalbumin sensitized and challenged guinea pig [18–20], but this model has the disadvantage that the allergic inflammation is mediated predominantly by IgG antibodies, depending upon the sensitization regimen used [21, 22]. Also, guinea pig-specific immunological reagents including various cytokines and cell markers are generally not available which hinders the study of immunological mechanisms that may be relevant to human diseases. Other frequently used animal models include sensitized monkeys, dogs and rabbits [23–26]. However, the limited availability and genetic heterogeneity of these animals, and the unavailability or requirement for large quantities of immunological reagents largely limit the utilization of these model systems.

Pulmonary inflammation has also been induced in the mouse [27, 28]. This species is particularly useful for measuring inflammatory responses because the major homeostatic antibody class in murine pulmonary inflammation is IgE [27, 28], which is similar to that seen in human asthmatics [1, 2]. Furthermore, the mouse has the additional advantage of having many of the immunological reagents and cell markers necessary for studying the inflammatory cascade. The purpose of this study was to develop and characterize a model of pulmonary inflammation in the mouse.

Materials and Methods

Male B6D2F1 J mice, weighing 20–25 g, were obtained from Jackson Laboratory (Bar Harbor, Me., USA). They were housed in a room with a 12-hour light-dark cycle and were allowed food and water *ad libitum*.

Sensitization and Antigen Bronchoprovocation

Animals were sensitized by an intraperitoneal injection of 0.5 ml alum-precipitated antigen containing 8 µg of ovalbumin (OVA) adsorbed to 2 mg of aluminum hydroxide (alum) gel in saline vehicle. A booster injection of this alum-OVA mixture was given 5 days later. Nonsensitized control animals received alum gel only. At selected times after sensitization, the mice were exposed to antigen broncho-

provocation. For the antigen challenge, the mice were placed in a plexiglas chamber (12" × 14" × 10") and exposed to aerosolized OVA (0.5%) for 1 h both in the morning and afternoon of a single day. The aerosolized OVA was produced by an ultrasonic nebulizer (DeVilbiss, Somerset, Pa., USA; Model Ultra-Neb 99) at a flow rate of approximately 5 litres/min.

Collection and Measurement of the Specimens

At various times after antigen bronchoprovocation, the animals were sacrificed by CO₂ asphyxiation then bronchoalveolar lavage (BAL) fluid, blood, bone marrow aspirate and lung tissue were collected.

For the collection of BAL fluid, the trachea was exposed and cannulated with a ball-tipped 24-gauge needle. The lungs were lavaged with 0.3 ml phosphate-buffered saline (PBS). Approximately 0.2 ml of the instilled fluid was consistently retrieved. Total cell numbers were counted with a standard hemocytometer. Smears of BAL cells were made by cytocentrifuging 100 µl of BAL fluid (Shandon Inc., Pittsburgh, Pa., USA) at 250 rpm (~10 g) for 10 min. The smears were fixed and stained with Leukostat stain (Fisher Scientific, Pittsburgh, Pa., USA) or May-Grünwald-Giemsa stain (Diagnostic System, Gibbstown, N.J., USA). Differential cell counts were determined from at least 200 leukocytes. The blood samples were obtained by cardiac puncture and the numbers of leukocytes and differential counts were determined using standard hematological procedures [19]. Femoral bone marrow cells were collected and counted according to the procedures of Alexander *et al.* [29].

For the histological evaluation of pulmonary tissue, the lungs were perfused *in situ* by passing a 24-gauge ball-tipped needle into the pulmonary artery through the right ventricle and flushing the pulmonary vasculature with 2.5 ml of phosphate-buffered saline (PBS). The lungs were then removed and fixed with 10% phosphate-buffered formalin. The left lobe of the lung was embedded in paraffin, sectioned at 5 µm thickness and stained with hematoxylin and eosin. In several experiments, sections were also stained with periodic acid-Schiff reagent (PAS). Histological evaluation of the bronchial and alveolar regions of the lung were determined with an image analysis system (Cambridge Instruments, Deerfield, Ill., USA; Model Quantimet 570) using five randomly selected areas. The number of EOs surrounding the bronchi (peribronchial) were counted at 400 × magnification. Other histopathological features studied were the aggregation of EOs around blood vessels (perivascular), damage to the airway epithelium, mucus formation in the lumen of the airways and infiltration of inflammatory cells into the alveoli.

Measurements of Serum IgE and IgG

Isotope specific reagents (antibodies and standards) were obtained from Dr. R. Coffman (DNAX, Palo Alto, Calif., USA). The antibodies and procedures have been described previously [30]. Briefly, polyvinyl 96-well plates (Dynatech, Alexandria, Va., USA) were coated overnight at 4°C with the appropriate first-step antibody in PBS. Plates were washed with PBS plus 0.04% Tween 20. Nonspecific binding was blocked with PBS plus 10% fetal calf serum (FCS) for 1 h at room temperature. Serum samples and standards were added and incubated for 2 h at room temperature. After washing, appropriate conjugated second-step antibodies were added and plates were incubated for 1 h at room temperature. After washing, optimal concentrations of horseradish peroxidase-conjugated monoclonal anti-NIP (nitroiodophenyl) acetate acid, for IgE (1:10,000) or horseradish peroxidase-conjugated streptavidin (for IgG) (Jackson ImmunoResearch, West Grove, Pa., USA) were

added. One hour later, the plates were washed and substrate solution containing 1 mg/ml azinobis 3-ethylbenz-thiazoline sulfonic acid (ABTS), 0.003% H_2O_2 in 0.1 M Na_2HPO_4 and 0.05 M citric acid was added. The plates were read on a VMAX plate reader (Molecular Devices, Palo Alto, Calif., USA). Serum immunoglobulin levels were quantified by comparison to purified isotype standards. Specificities and sensitivities of these ELISAs have been described elsewhere [30].

Compounds

Betamethasone, prednisolone and hydrocortisone were synthesized at the Schering-Plough Research Institute (Kenilworth, N.J., USA). They were prepared in 0.4% methylcellulose in isotonic saline and administered orally 2 h before OVA challenge. Control animals received vehicle only.

Statistical Analysis

All data are presented as the mean $\pm$ SEM. The significance of differences between experimental groups was analyzed with the unpaired Student's *t* test or Dunnett's multiple range analysis.

Animal Care and Use

This study was done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in a program approved by the American Association for the Accreditation of Laboratory Animal Care.

Results

The Course for Development of Pulmonary Eosinophilia after Sensitization

Animals were sensitized with OVA and challenged with OVA aerosol at various times after the sensitization to assess the appearance of EOs in the BAL fluid. Aerosolized OVA (0.5%) was given for 1 h both in the morning and afternoon of a single day and BAL fluid was obtained 24 h after the morning exposure. This concentration and exposure time for OVA was found to induce a consistent level of pulmonary eosinophilia (see below). A lower concentration of OVA or shorter exposure gave inconsistent levels of pulmonary eosinophilia.

OVA challenge produced little change in BAL EOs up to 8 days after sensitization. However, at 10 days after sensitization, there was a statistically significant increase in BAL EOs after OVA challenge (fig. 1). The eosinophil response in BAL peaked after 12 days and then gradually returned toward baseline between days 17 and 20. The increase in total cell numbers in the BAL correlated with the increase in BAL eosinophils and on day 12 the EOs constituted greater than 50% of the total cells in the BAL. Nonsensitized mice challenged with OVA or sensitized, unchallenged mice showed only slight and insignificant changes in the number of EOs in the BAL fluid (data not shown). There was also no significant change in the number of eosinophils in the BAL

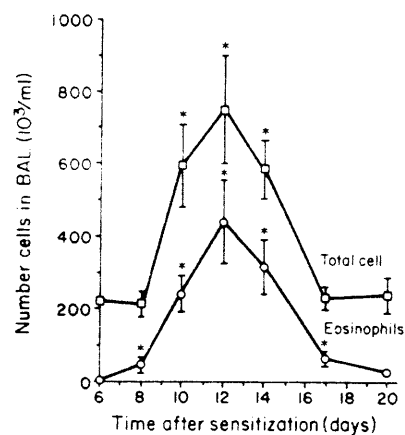


Fig. 1. Total cells and EOs in the BAL from allergic mice challenged with aerosolized OVA (0.5%) at various days after sensitization. BAL samples were collected 24 h after OVA challenge. Data represents the mean $\pm$ SEM (*n* = 5 per group). **p* < 0.05 compared to day 0 after sensitization.

fluid of sensitized mice challenged with aerosolized bovine serum albumin (data not shown). At the peak of eosinophilia in the BAL, 12 days after sensitization, total serum IgE levels were significantly elevated with no significant change in IgG₁ (fig. 2). In subsequent experiments, we challenged the animals with OVA 12 days after sensitization.

Development of Pulmonary Eosinophilia and Inflammation after Antigen Challenge

In sensitized mice exposed to OVA on day 12, only small numbers of EOs (5–6% of total cells) were found in the BAL 4–8 h after OVA challenge (table 1). However, after 24 h there was a large increase in total cells and EOs (29% of total) in the BAL. The peak increase in total cells and EOs in BAL occurred 2–3 days after OVA challenge when more than 80% of the total cells were EOs. By 4 days after OVA, the number of total cells and EOs in BAL were reduced from peak values. There was still an elevated number of EOs in the BAL (44% of total cells) 8 days after OVA challenge but by 14 days after OVA challenge, the total cells and EOs in BAL had substantially returned toward control values (table 1). In nonsensitized mice exposed to OVA aerosol, there was no significant increase in total cells or EOs in the BAL up to 14 days after OVA challenge.

There were also sporadic increases in the number of neutrophils in the BAL of sensitized animals after OVA challenge (table 1). However, in nonsensitized animals exposed

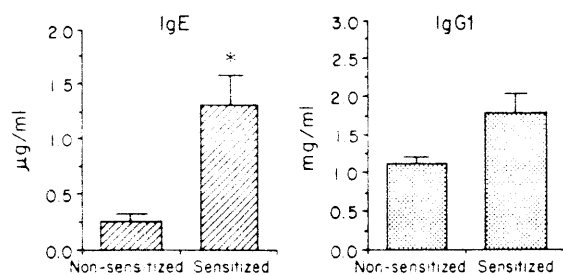


Fig. 2. Serum levels of IgE and IgG in nonsensitized and sensitized mice 12 days after sensitization with OVA. Data represent the mean $\pm$ SEM ($n = 5$ per group). * $p < 0.05$ compared to nonsensitized animals.

Table 1. Bronchoalveolar lavage cell count at various time intervals after aerosolized OVA challenge in mice

Time after challenge with OVA ^a	BAL cells (mean $\pm$ SEM)			
	Total cells (10 ⁶ /ml)	% eosinophils	% neutrophils	% monocytes-macrophages
0 h	193 $\pm$ 17	0	0	98 $\pm$ 2
1 h	206 $\pm$ 20	5 $\pm$ 0*	35 $\pm$ 16	57 $\pm$ 5*
2 h	176 $\pm$ 21	6 $\pm$ 2	25 $\pm$ 4	65 $\pm$ 5
4 h	556 $\pm$ 48*	42 $\pm$ 4*	9 $\pm$ 4	49 $\pm$ 5*
8 days	1,116 $\pm$ 32*	87 $\pm$ 2*	0	13 $\pm$ 2*
12 days	1,346 $\pm$ 40*	87 $\pm$ 2*	2 $\pm$ 0	11 $\pm$ 3*
16 days	643 $\pm$ 54*	58 $\pm$ 9*	11 $\pm$ 3*	29 $\pm$ 8*
20 days	953 $\pm$ 49*	62 $\pm$ 8**	4 $\pm$ 0	32 $\pm$ 8*
24 days	498 $\pm$ 72	44 $\pm$ 9*	12 $\pm$ 3	42 $\pm$ 9*
28 days	278 $\pm$ 31	8 $\pm$ 2*	14 $\pm$ 4*	68 $\pm$ 2*

* $p < 0.05$ compared to nonsensitized control animals at the same time point.

^aAnimals were challenged with aerosolized OVA (0.5%) for 1 h both in the morning and afternoon on day 12 after sensitization. BAL samples were collected at the time intervals indicated.

to OVA, there were the same sporadic increases in the number of neutrophils in the BAL. There were minimal changes in the number of lymphocytes of sensitized mice in the BAL after OVA challenge (data not shown).

As with the BAL, lung biopsies of sensitized mice showed that only a few EOs were found in the lungs 4–8 h after OVA challenge. However, by 24 h after OVA, there were large numbers of EOs surrounding the bronchial air-

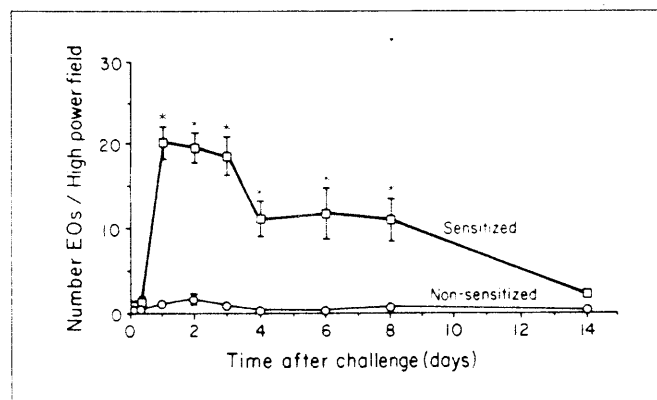


Fig. 3. Changes in EO numbers in the peribronchial region of the lung after challenging nonsensitized and sensitized mice with aerosolized OVA (0.5%). Biopsies were collected 4, 8 and 24 h and up to 14 days after OVA challenge. Data represent the mean $\pm$ SEM ($n = 5$ per group). * $p < 0.05$ compared to nonsensitized animals.

way (fig. 3). This high level of peribronchial eosinophilia persisted for 3 days. The EO infiltration into the peribronchi was still elevated after 8 days and subsided to normal levels 14 days after OVA challenge. There was no evidence of EO migration into the bronchial region of nonsensitized mice after OVA (fig. 3).

Histological evaluation of the lungs of sensitized mice revealed that EOs had infiltrated the tissues surrounding the bronchial airways and blood vessels 24–48 h after OVA challenge (fig. 4a, b). In some sections, the blood vessels were encased by layers of EOs up to 5–10 cells deep. Around the bronchi, the EOs appeared as patchy cuffs. Only a small number of EOs were observed in the mucosal layer or the lumen of the airways. There was also evidence of a damaged airway epithelium and interstitial airway edema in the bronchi 48 h after OVA challenge (fig. 4b). Increased PAS-positive mucus secretions were also found in the bronchi 48 h after OVA challenge in sensitized mice (fig. 4c). A few EOs were occasionally observed in the alveoli. In nonsensitized mice challenged with OVA, there was no evidence of EO infiltration into the tissues surrounding the bronchi or blood vessels (fig. 4d). Furthermore, there was no indication of a damaged airway epithelium, airway edema or PAS-positive mucus secretion (fig. 4e) after OVA challenge to nonsensitized mice.

Changes in Eosinophil Numbers in Bone Marrow and Blood after Antigen Challenge

In sensitized mice exposed to OVA on day 12, there was a moderate increase in the number of EOs in bone marrow 4 h after OVA challenge (fig. 5a). This was followed by a reduc-

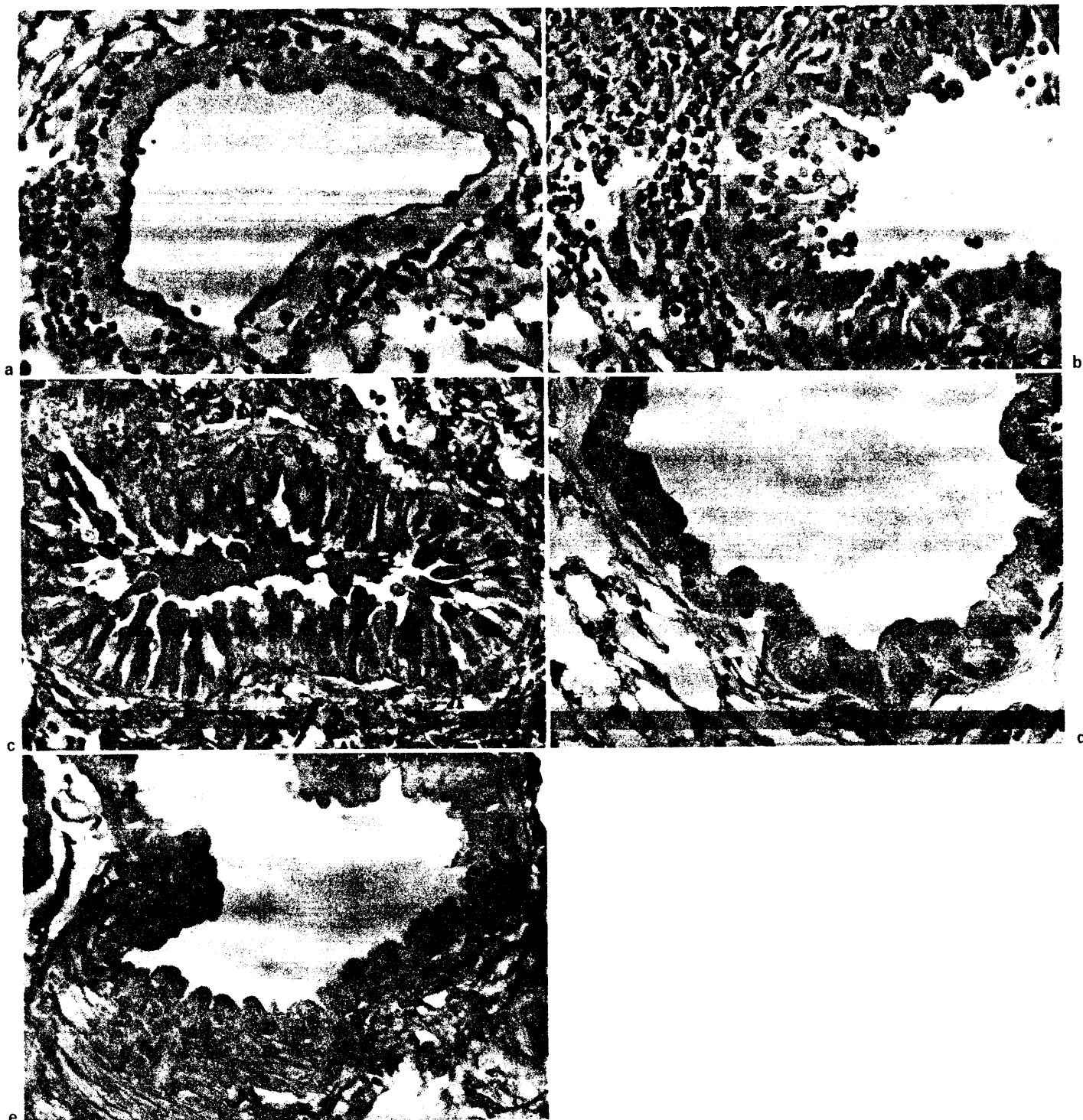


Fig. 4. Light photomicrographs of paraffin sections of lung tissues from sensitized and nonsensitized mice challenged with aerosolized OVA (0.5%). **a** Sensitized mouse 24 h after challenge with aerosolized OVA. There is marked margination and aggregation of EOs around the blood vessels. HE, $\times 125$. **b** Sensitized mouse 48 h after aerosolized OVA challenge. There is aggregation of EOs in the peribronchial area, edema in the lamina and damage of epithelial cells of a bronchus.

HE, $\times 250$. **c** Sensitized mouse 48 h after aerosolized OVA challenge. There is excessive PAS-positive material (mucus) and debris in the lumen. PAS stain, $\times 125$. **d** Nonsensitized mouse 48 h after challenge with aerosolized OVA. No EO infiltration in the peribronchial area is found. HE, $\times 250$. **e** Nonsensitized mouse 48 h after challenge with OVA. There is no EO infiltration and no appearance of PAS-positive material in the airway lumen. PAS, $\times 125$.

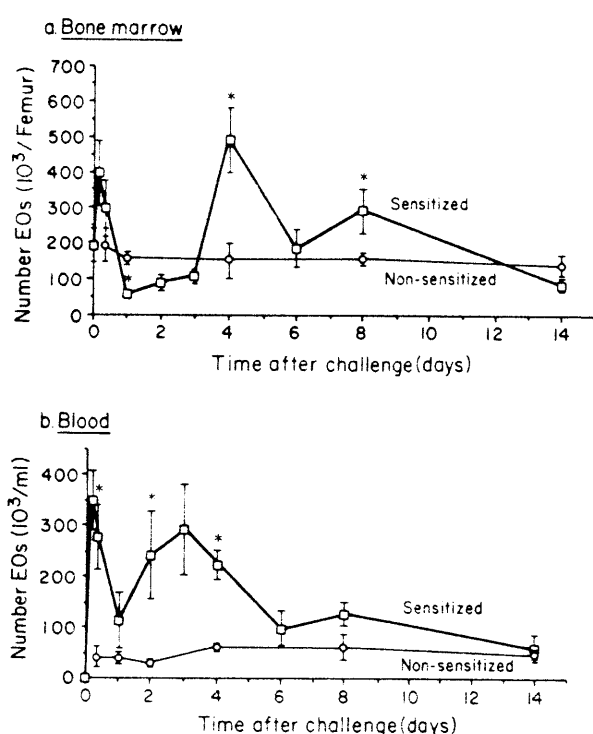


Fig. 5. Changes in EO numbers in the bone marrow (a) and blood (b) after challenging nonsensitized and sensitized mice with aerosolized OVA (0.5%). Samples were collected 4, 8 and 24 h and up to 14 days after OVA challenge. Data represent the mean $\pm$ SEM ($n = 5$ per group). * $p < 0.05$ compared to nonsensitized animals.

Table 2. Effect of betamethasone, prednisolone and hydrocortisone on the eosinophil numbers in the BAL of allergic mice

Compounds ^a	Oral dose mg/kg	% inhibition ^b EOs	ED ₅₀ , mg/kg (confidence limits at 95%)
Betamethasone	0.125	31 $\pm$ 11	0.2 (0.1–0.3)
	0.25	78 $\pm$ 2*	
	0.5	85 $\pm$ 9*	
	1	96 $\pm$ 1*	
Prednisolone	1	28 $\pm$ 11	4.3 (0.9–18.0)
	5	52 $\pm$ 10*	
	25	83 $\pm$ 2*	
Hydrocortisone	6.25	42 $\pm$ 15	8.5 (1.9–20.4)
	12.5	62 $\pm$ 14*	
	25	78 $\pm$ 7*	
	50	98 $\pm$ 4*	

* $p < 0.05$ compared to vehicle.

^a Corticosteroids were administered 2 h before OVA challenge. BAL fluid was obtained 24 h after OVA challenge. Mean $\pm$ SEM ($n = 6$ per dose).

tion of EOs in bone marrow for 3 days. The number of bone marrow EOs gradually returned to normal levels on day 14. There was no change in bone marrow EOs of nonsensitized mice after OVA challenge.

In the blood, there was an increase in EOs that peaked 4–8 h after OVA challenge in sensitized mice (fig. 5b). Blood EOs in sensitized challenged mice returned to normal levels 6 days after challenge. There was no change in blood EOs of nonsensitized mice after OVA challenge.

Effect of Corticosteroids on Eosinophils in BAL and Tissues

Betamethasone was compared to prednisolone and hydrocortisone for their ability to inhibit the accumulation of EOs in the BAL after OVA challenge. Treatment of sensitized mice with betamethasone, prednisolone or hydrocortisone dose-dependently inhibited the infiltration of EOs into the BAL 24 h after OVA challenge (table 2). Betamethasone (ED₅₀ = 0.2 mg/kg) was more potent than prednisolone (ED₅₀ = 4.3 mg/kg) and hydrocortisone (ED₅₀ = 8.5 mg/kg) for inhibiting the BAL eosinophilia after OVA challenge (table 2). Treatment of sensitized mice with betamethasone (0.5 mg/kg p.o.) also reduced the accumulation of EOs in the peribronchial region of the lung.

Treatment of sensitized mice with betamethasone also prevented the decrease in bone marrow EOs 24 h after OVA challenge. In these studies, bone marrow EOs averaged $26 \pm 15 \times 10^3$ EOs/femur in sensitized, challenged animals. When sensitized, challenged animals were treated with betamethasone (0.5 mg/kg p.o.) bone marrow EOs averaged $149 \pm 29 \times 10^3$ EOs/femur. This was not significantly different from values ($131 \pm 31 \times 10^3$ EOs/femur) seen in OVA-challenged nonsensitized mice. Betamethasone (0.5 mg/kg p.o.) had no significant effect on the increase in blood EOs 24 h after OVA challenge (data not shown).

Discussion

The mouse is a particularly useful model for studying allergic responses in the lungs, because the major antibody class in murine pulmonary inflammation is IgE [27, 28] and because many of the immunological reagents and cell markers for inflammation are available for studies in mice. The results of this study show that sensitized mice challenged with aerosolized OVA can mount a pulmonary inflammation with a predominance of eosinophils. The eosinophil response is specific to antigen challenge because exposure to aerosolized bovine serum albumin had no effect. The time between sensitization and challenge and the duration of

challenge with OVA aerosol are important for inducing the maximal lung eosinophilia. The optimum challenge time was 12 days after sensitization. At this time, the serum IgE level was significantly elevated indicating that this inflammatory is IgE-related. We also found that a prolonged exposure to OVA aerosol was necessary to ensure the development of consistent and severe pulmonary eosinophilia.

Eosinophils first appear in large numbers in the pulmonary interstitium and BAL fluid 24 h after OVA challenge. By 48 h, there was a larger increase in BAL EOs. Furthermore, antigen challenge produced damage to the bronchial epithelium, interstitial airway edema and increased mucus secretion. The accumulation of EOs in the lung after antigen challenge was localized to regions surrounding pulmonary blood vessels and bronchial airway, although very few EOs were found in the airway mucosa. This is quite different to the findings in guinea pigs where large numbers of EOs are found in the airway mucosa after antigen challenge [18, 19, 31]. It is noteworthy that murine airways have a very thin mucosal layer that may fail to entrap large numbers of EOs during their migration into the airways. Interestingly, bronchial biopsies taken from human asthmatics also show large numbers of EOs in clusters around blood vessels and very few EOs are distributed in the airway submucosa [32]. Therefore, the distribution of EOs in murine airways after antigen challenge is similar to the EO distribution observed in human asthmatic airways.

Antigen provocation to sensitized mice produced an early and transient increase of EOs in the bone marrow and blood. These changes probably reflect eosinophilopoiesis at the level of the bone marrow and migration of bone marrow EOs into blood. The early increase in blood EOs may also reflect the release of a resident marginated population into the circulation. The apparent rapid drop in blood EOs 24 h after OVA occurred at a time when EOs in the lung were increasing. This may represent the migration of EOs from the blood into the lungs. By 48 h after OVA, there was a second peak of EOs in the blood. This may reflect the continued output of EOs from bone marrow.

Corticosteroids are effective drugs for the treatment of the inflammatory component of asthma [1] and are known to reduce pulmonary eosinophilia associated with inflammation [1, 19]. In this study, the increase in BAL EOs 24 h after OVA challenge was reduced by treatment with betamethasone, prednisolone and hydrocortisone. The rank order potency of these corticosteroids is similar to that reported for their anti-inflammatory activity in humans [33]. Betamethasone, which was the most potent of the three corticosteroids used, also prevented the reduction of EOs in bone marrow 24 h after OVA challenge. Therefore, corticosteroids

may reduce pulmonary inflammation by blocking the migration of EOs from bone marrow into blood or by inhibiting the activity of inflammatory mediators that recruit EOs into the lung [34, 41].

In conclusion, the pulmonary inflammation model in the allergic mouse reported here is characterized by elevated levels of IgE in the serum and an intense pulmonary eosinophilia. The temporal profile for EO migration into the lungs and the histological changes seen in the airways after antigen challenge are characteristic of a late-phase inflammatory response. The pulmonary inflammation responds to treatment with corticosteroids. This model should be very useful to study the effect of specific antibodies to IL-4, IL-5 and GM-CSF on pulmonary eosinophilia and to evaluate the role of these cytokines on the inflammatory cascade.

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