

Mechanisms of allergic pulmonary eosinophilia in the mouse

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Although the most observable feature in asthma is the bronchoconstriction associated with an acute "asthmatic attack," this really results from an ongoing inflammatory disorder. As an indication of the chronic nature of asthma, even between attacks, the patient with asthma may have a breathing deficit as indicated by a decreased forced expiratory volume in 1 second (FEV₁). Furthermore, the cardinal hallmark of asthma is ongoing nonspecific hyperreactivity to a variety of stimuli. Some aspects of the chronic pulmonary inflammation^{1, 2} are edema, decreased mucociliary clearance, epithelial damage with the ensuing neuronal hyperresponsiveness, and bronchoalveolar eosinophilia. Therefore when treating asthma, it is important to address the chronic inflammation that underlies the hyperreactivity rather than just the acute bronchospastic episodes. It is only because this inflammation compromises the airways that a challenge that would be irritating to a normal person can become life-threatening to the patient with asthma.

Eosinophils are the major cells infiltrating in the lungs during pulmonary inflammation. They develop from precursors in the bone marrow in response to a variety of cytokines and are stored in the bone marrow for release in response to a stimulus. Once released, they move rapidly through the circulation and become tissue cells. Eosinophils are almost nonexistent in normal lungs, but they accumulate in very large numbers in the lungs of patients with asthma. They have a full complement of lipid-metabolizing pathways, with a host of phospholipases such as phospholipase A₂ and phospholipase D, oxygenating en-

Abbreviations used

BAL: Bronchoalveolar lavage
IFN- γ : Interferon- γ
IL: Interleukin
PBS: Phosphate-buffered saline

zymes that make sulfidopeptide leukotrienes and prostaglandins, and acetyl transferase, which makes platelet activating factor.^{3, 4} Their secretory granules also contain a series of highly cytolytic enzymes and proteins that can destroy the epithelium and are detected in the lungs of patients with asthma.⁵ Furthermore, the role of the eosinophil in immune surveillance is somewhat limited compared with that of neutrophils, macrophages, or lymphocytes, so it should be possible to inhibit the actions of eosinophils without causing severe immunosuppressive consequences.

Using a mouse model, we have investigated the effects of steroids and cytokines on allergic eosinophilia. Eosinophil levels have been measured in the appropriate tissues to establish the mechanism of action of these agents, and mast cell-deficient mice have been studied to determine the endogenous source of the relevant cytokines.

METHODS

Materials

B6D2F1/J, WBB6F1/J-W/W^v and WBB6F1/J-W/W⁺ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Antibodies were from cell lines derived at the DNAX Institute (Palo Alto, Calif.) and were prepared in larger amounts at the Schering-Plough Research Institute (Kenilworth, N.J.). Other reagents were from standard laboratory suppliers.

Sensitization and antigen bronchoprovocation

B6D2F1/J mice were sensitized by an intraperitoneal injection of 0.5 ml of alum-precipitated antigen con-

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taining 8 μ g of ovalbumin adsorbed to 2 mg of aluminum hydroxide (alum) gel in saline solution. A booster injection of this alum-ovalbumin mixture was given 5 days later. Nonsensitized control animals received alum gel only. Twelve days after immunization, the mice were exposed to antigen bronchoprovocation. For the antigen challenge, the mice were placed in a Plexiglas chamber and exposed to aerosolized ovalbumin (0.5%) for 1 hour in both the morning and afternoon of a single day.

Collection and measurement of the specimens

At various times after antigen bronchoprovocation, the animals were killed by CO₂ asphyxiation, then bronchoalveolar lavage (BAL) fluid, blood, bone marrow aspirate, and lung tissue were collected. For the collection of BAL fluid, the tracheas were exposed and cannulated with a ball-tipped 24-gauge needle. The lungs were lavaged with 0.3 ml of phosphate-buffered saline (PBS). Total cell numbers were counted with a standard hemocytometer. Smears of BAL cells were made by cytocentrifuging 100 μ l of BAL fluid at 10 $\times$ g (250 RPM) for 10 minutes. The smears were fixed and stained with Leukostat stain (Fisher Scientific, Pittsburgh, Pa.). The blood samples were obtained by cardiac puncture, and the numbers of leukocytes and differential counts were determined with standard hematologic procedures.⁶ Femoral bone marrow cells were collected and counted according to the procedures of Alexander et al.⁷

For the histologic 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 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 μ m thickness, and stained with hematoxylin and eosin. Histologic evaluation of the bronchial and alveolar regions of the lung was performed with an image analysis system using five randomly selected areas. The number of eosinophils surrounding the bronchi (peribronchial area) were counted at 400 $\times$ magnification. Other histopathologic features studied were the aggregation of eosinophils 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.

Bone marrow reconstitution in W/W^v mice

Bone marrow reconstitution in W/W^v mice was performed by transferring cultured bone marrow mast cells from W/W⁺ mice to W/W^v recipients. Bone marrow cells were collected from femurs and tibias of W/W⁺ mice with Hanks' balanced salt solution containing 10% fetal bovine serum. To obtain mast cells from mice, the bone marrow cells were cultured in RPMI 1640 (Gibco, Grand Island, N.Y.) containing 10% concentrated con-

ditioned medium, 10% fetal calf serum, 2 mmol/L L-glutamine, penicillin and streptomycin. The culture medium was changed every 5 to 7 days. During the third week, 0.1 ng of rmIL-3 (R&D, Minneapolis, Minn.) was added to the medium. After 3 weeks, 90% of the viable cells were mast cells. Mast cells (10⁷) suspended in 0.2 ml of PBS were transferred intravenously into the tail vein of each W/W^v recipient. Animals were sensitized with ovalbumin 42 days after the adoptive transfer with W/W⁺ bone marrow-derived mast cells. The sensitization, the challenge, and the analyses were then performed as described above for the B6D2/F1 mice.

Measurement of serum IgE and IgG₁

Isotype-specific reagents (antibodies and standards) were obtained from Dr. Robert Coffman (DNAX Institute). The antibodies and procedures have been described previously.⁸ Briefly, polyvinyl 96-well plates were coated overnight at 4° C with the appropriate first-step antibody in PBS. Plates were washed, and nonspecific binding was blocked with 10% fetal calf serum for 1 hour at room temperature. Serum samples and standards were added and incubated for 2 hours at room temperature. After washing, appropriate conjugated second-step antibodies were added, and plates were incubated for 1 hour at room temperature. After washing, optimal concentrations of horseradish peroxidase-conjugated monoclonal anti-NIP (nitroiodophenyl acetic acid for IgE) or horseradish peroxidase-conjugated streptavidin (for IgG₁) were added. One hour later, the plates were washed and substrate solution containing 1 mg/ml of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H₂O₂ in 0.1 mol/L Na₂HPO₄, and 0.05 mol/L citric acid were added. The plates were analyzed on a VMAX plate reader (Molecular Devices Corp., Menlo Park, Calif.). Serum immunoglobulin levels were quantified by comparison with purified isotype standards.

Statistical analysis

All data are presented as means $\pm$ SEM. The significance of differences between experimental groups was analyzed with 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 AND DISCUSSION

Tissue distribution of eosinophils in the allergic mouse

During the allergic response in this mouse model, the levels of eosinophils in the various

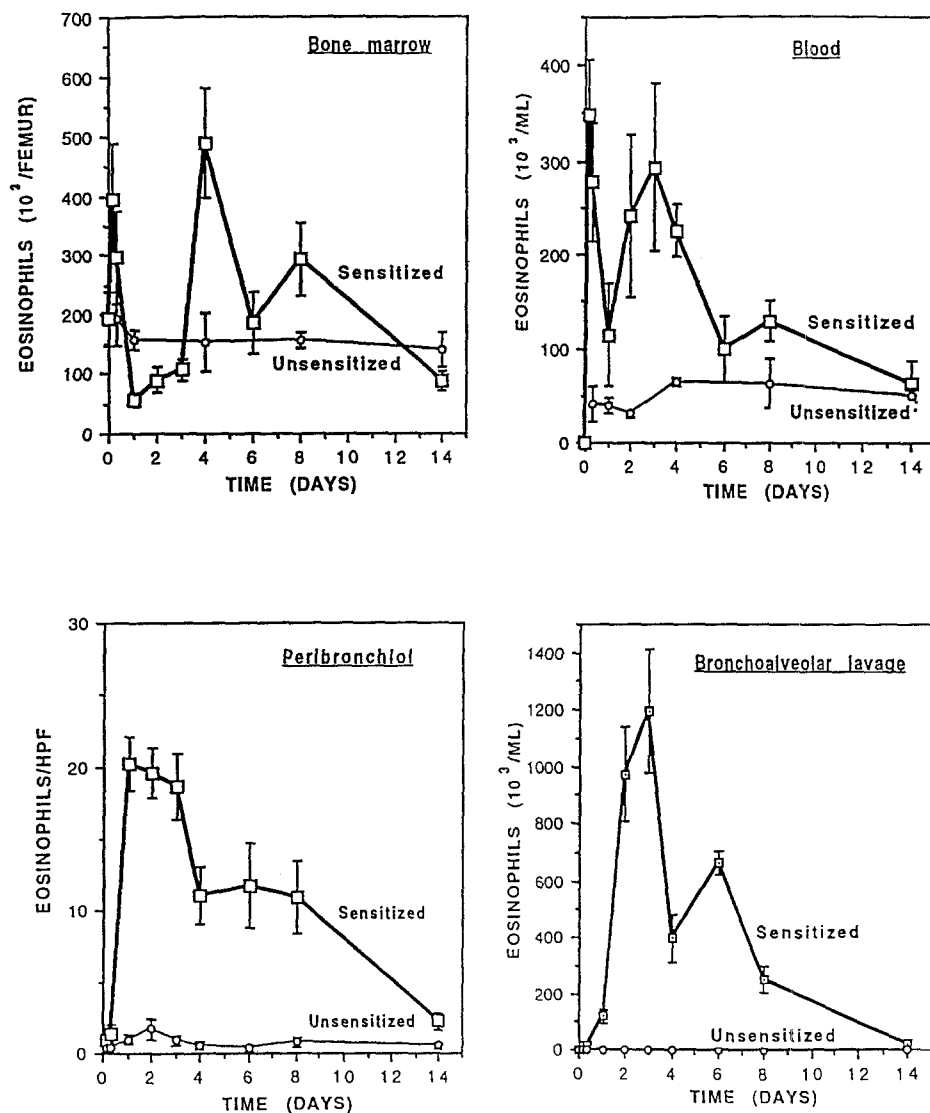


FIG. 1. Eosinophil levels in various tissues after challenge of ovalbumin-sensitized mice.

tissue compartments underwent the anticipated changes (Fig. 1). Bone marrow eosinophils were measured in the femur where they originated and were stored for release into the circulation on demand (Fig. 1, upper left panel). Before challenge, there was a baseline level of approximately 200,000 eosinophils per femur. After stimulation, eosinophils increased somewhat and then plummeted as they were released into the circulation in excess of the ability of the marrow to regenerate new eosinophils. There may have been something of an overshoot on day 4, because resynthesis caught up, then the number of eosinophils largely returned to normal by day 6. There were very few eosinophils in the blood before chal-

lenge (Fig. 1, upper right panel). The level increased dramatically within hours after challenge, dropped off as eosinophils were assimilated by the tissue, and again, resolved itself spontaneously. There were virtually no eosinophils in the lung before challenge (Fig. 1, lower left panel). Accumulation in the lung lagged behind the appearance in the blood by several hours, as expected. Within a day, eosinophils reached a maximum then dropped off gradually. To get into the lavage, eosinophils must travel through the tissue to the luminal surface, so the peak of lavage eosinophils lagged behind the appearance in the tissue, seemingly by about a day (Fig. 1, lower right panel). The peak was transient between days 2 and 3,

TABLE I. Effects of oral steroids on pulmonary eosinophilia in the allergic mouse

Compound	Dose (mg/kg)	% Inhibition		ED ₅₀ (mg/kg)
		Total no. of cells	EOS	
Betamethasone	0.13	24	31	0.2
	0.25	76	78	
	0.50	83	85	
	1.00	95	96	
Prednisolone	1.0	28	28	4.3
	5.0	51	52	
	25.0	69	83	
Hydrocortisone	6.3	37	42	8.5
	12.5	43	62	
	25.0	65	78	
	50.0	80	98	

Six animals were in each group. BAL samples were collected 24 hours after ovalbumin challenge. Drugs were administered 2 hours before ovalbumin challenge.

ED₅₀, Median effective dose.

TABLE II. The effects of cytokine antibodies and IFN- γ on eosinophil accumulation

Cytokine inhibited	Antibody	Protocol	Dose	% Inhibition
IL-5	TRFK-5	-2 hr	0.1 mg/kg	80
IL-5	39D10	-2 hr	1.0 mg/kg	74
IL-4	11B11	3 $\times$ /wk during sensitization	40 mg/kg	95
IL-4	11B11	-2 hr	40 mg/kg	36
IFN- γ	XMG 1.2	3 $\times$ /wk during sensitization	40 mg/kg	0
IFN- γ directly	None	3 $\times$ /wk during sensitization	0.4 mg/kg	74

then the levels declined to normal within 2 weeks, very similar to the tissue itself.

The histology consistently corroborated the findings in the BAL. Under these staining conditions, eosinophils would be reddish bi- or multi-lobular cells, and they were not present in large numbers in unsensitized animals. In contrast, sensitized mice challenged with ovalbumin had large numbers of eosinophils in their lungs. The eosinophils were readily observed in bronchi and bronchioles and to a lesser extent in the alveoli.

Inhibition of BAL eosinophilia in the mouse

Steroids should block pulmonary eosinophilia,⁹ so we examined the effects of betamethasone, prednisolone, and hydrocortisone at appropriate doses on the cell content of BAL from sensitized challenged mice (Table I). These drugs were administered orally 2 hours before the ovalbumin challenge, and the BAL was collected and analyzed 24 hours later. There was a dose-dependent

inhibition of total cell and eosinophil infiltration in each instance. As expected with steroids, there was no specificity for inhibition of eosinophil infiltration. The median effective dose of betamethasone was 0.2 mg/kg; for prednisolone, 4.3 mg/kg; and for hydrocortisone, 8.5 mg/kg. This is roughly in keeping with their rank order of relative potencies in human beings.

The role of IL-5 in murine pulmonary eosinophilia was also studied. IL-5 can enhance eosinophil production and survival,¹⁰ so depletion of IL-5 should selectively suppress eosinophil levels in animal models, and antibodies have been used to accomplish this.^{11, 12} The antibody that was used most extensively is from the TRFK-5 hybridoma line, which secretes a rat IgG₁ isotype that neutralizes recombinant murine IL-5. As an isotype control antibody, we used a rat IgG₁ isotype from GL113 cells. This antibody was raised against bacterial β -galactosidase and should have no mammalian biology. With the standard proto-

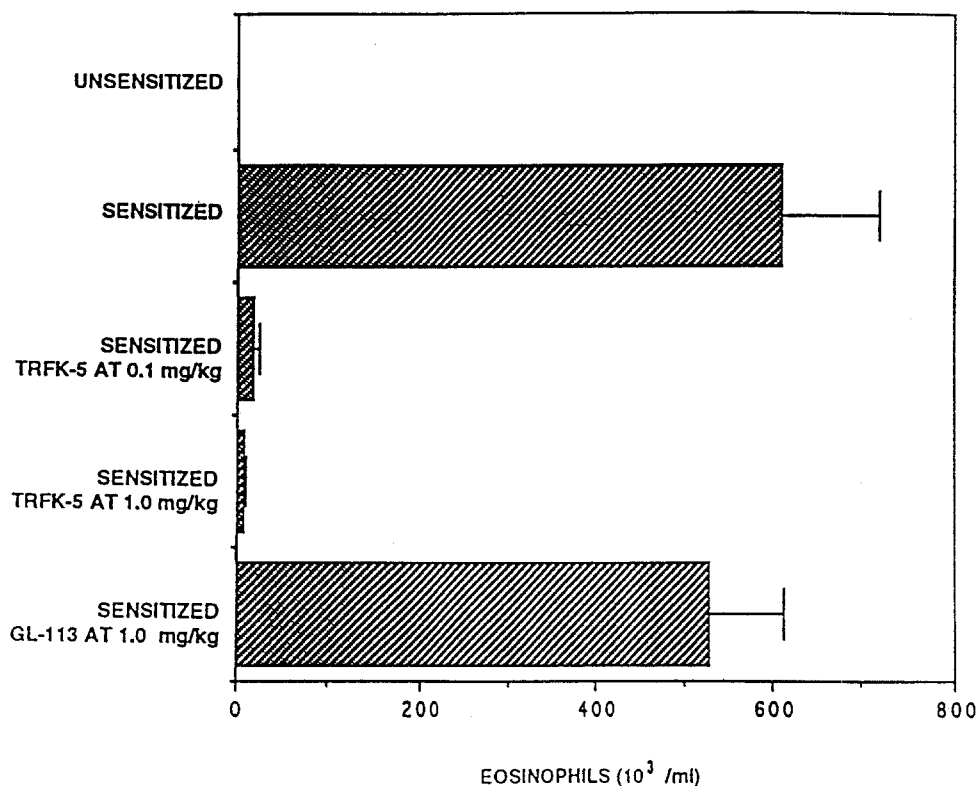


FIG. 2. The effect of anti-IL-5 on mouse BAL eosinophilia after ovalbumin challenge.

col, the antibody was administered by intraperitoneal injection 2 hours before the ovalbumin challenge. In each paradigm the mice were challenged with ovalbumin (Fig. 2). The unsensitized mice did not respond with BAL eosinophilia. In contrast, the sensitized mice produced a BAL eosinophilia of 5×10^5 eosinophils/ml, about 50% of the total cells. The TRFK-5 antibody at 0.1 and 1 mg/kg inhibited the eosinophilia by more than 95%. At 0.01 mg/kg, it had no effect. As a control, the GL113 antibody, which should have had no effect, was inactive at 1 mg/kg. Therefore, specific depletion of IL-5 can almost totally inhibit lavage eosinophilia in the allergic mouse.

The effects of a series of other cytokine-based treatments on eosinophil accumulation in this model have also been assessed (Table II). In addition to the TRFK-5 antibody against murine IL-5, we also evaluated 39D10, a rat monoclonal antibody raised against human IL-5. Administered 2 hours before the ovalbumin challenge, 39D10 inhibited pulmonary eosinophilia to the same extent as the TRFK-5 antibody, but with about 10 times less potency. We evaluated the role of IL-4 in this model by using 11B11, a rat monoclonal antibody against murine IL-4. As op-

posed to IL-5, which crosses species very nicely,¹⁰ IL-4 and interferon- γ (IFN- γ) are very species-specific,^{13, 14} so we used only murine reagents. The monoclonal antibody 11B11 blocked eosinophilia and also the increases in IgE when administered during sensitization at 40 mg/kg. It was as if the animals were not sensitized and therefore did not respond. In contrast, when the 11B11 was administered once 2 hours before the ovalbumin challenge, there was a marginal and not a dose-dependent effect on eosinophil accumulation. IFN- γ itself, administered during sensitization, also blocked eosinophil accumulation, probably by redirecting T-cell maturation away from the Th_2 type and thereby suppressing IL-4 levels. IFN- γ did not inhibit eosinophil accumulation when administered just before the challenge. An antibody to IFN- γ had no effect. Therefore eosinophil accumulation in this mouse model can be inhibited by blocking eosinophil maturation with IL-5-based technology or by inhibiting IgE synthesis with IL-4- or IFN- γ -based technology.

Mechanism of inhibition of BAL eosinophilia

To investigate the mechanisms by which betamethasone and the TRFK-5 antibody inhibited

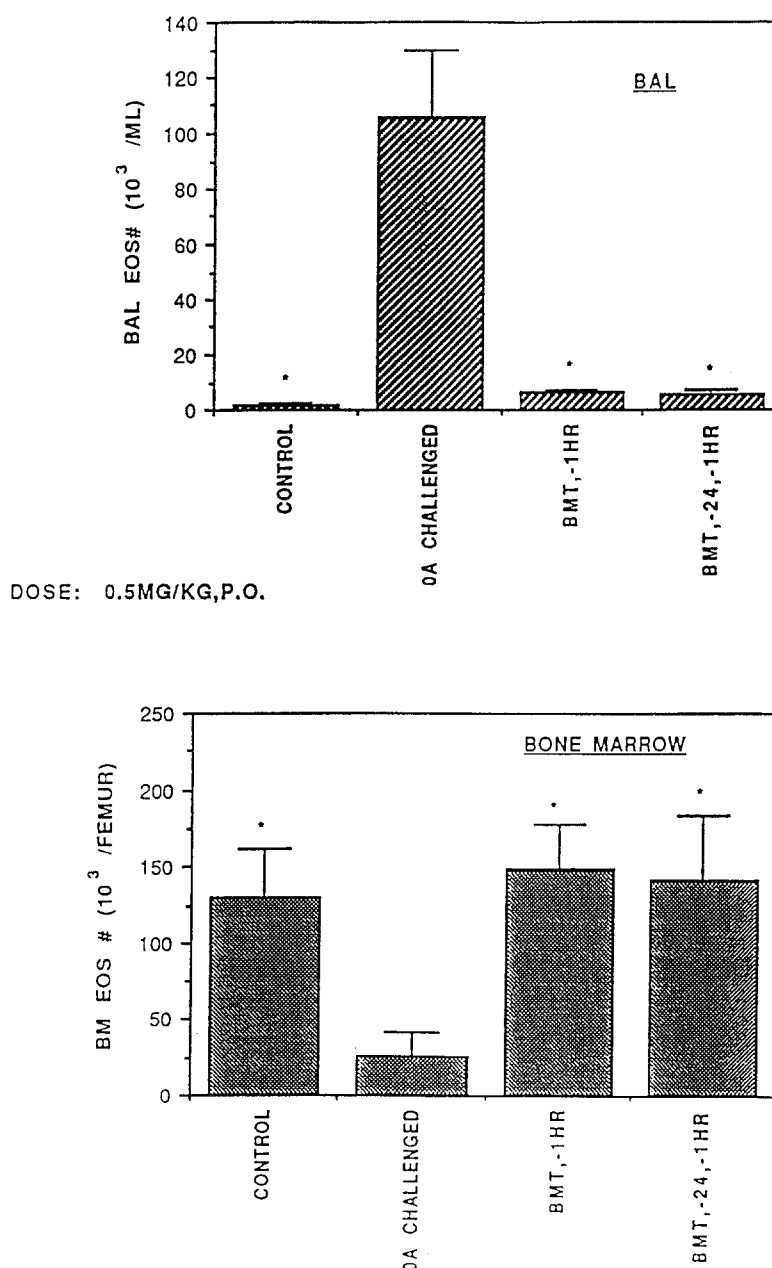


FIG. 3. The mechanism of steroid inhibition of mouse BAL eosinophilia.

pulmonary eosinophilia, we have measured the changes in eosinophil levels in the bone marrow, blood, lung tissue, and BAL in drug-treated mice compared with the changes in untreated, sensitized, challenged mice. Eosinophil measurements were conducted 24 hours after the ovalbumin challenge. The mice were treated orally with 0.5 mg/kg of betamethasone either 1 hour or 1 and 24 hours before the ovalbumin challenge (Fig. 3). There were no noticeable eosinophils in the BAL before challenge; they increased as expected 24

hours after the challenge. That increase was blocked to baseline by both betamethasone treatments. The lower panel in Fig. 3 demonstrates that the steroid is inhibiting at the level of the bone marrow. The decrease in eosinophils, noted in the challenged animals at 24 hours, did not occur when the steroid was present. In other words, the bone marrow did not respond by releasing its store of eosinophils, so they, of course, never accumulated in the lung.

Much as with the steroid, we have also exam-

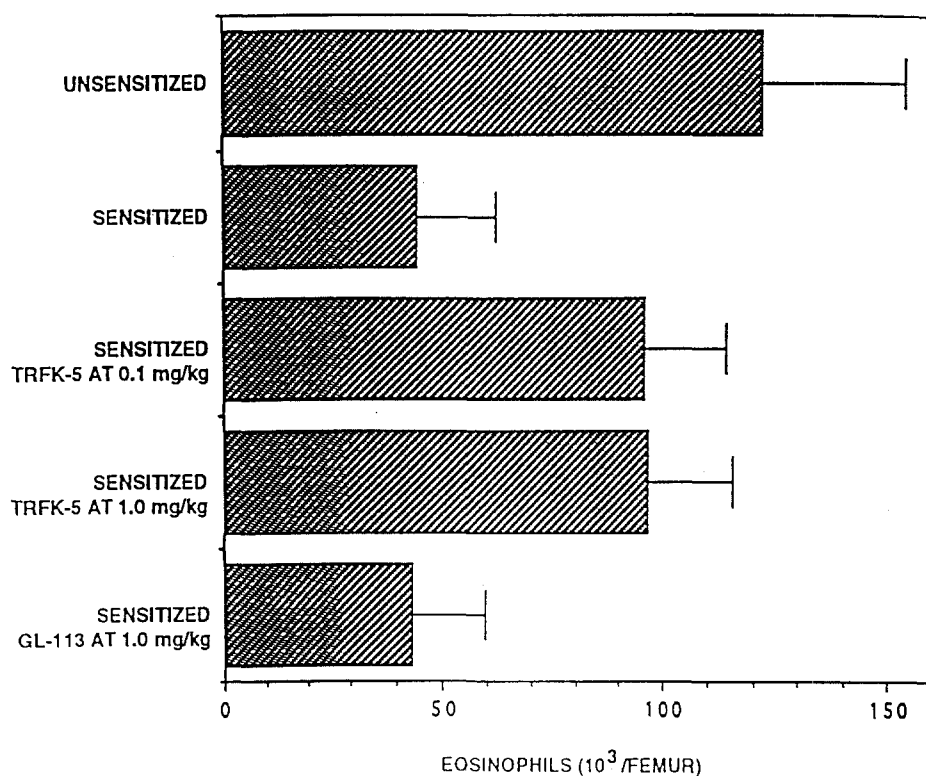


FIG. 4. The effect of anti-IL-5 on mouse bone marrow eosinophil levels after ovalbumin challenge.

ined the effects of the neutralizing anti-IL-5 antibody on marrow eosinophils (Fig. 4). The results are fundamentally the same as those obtained with betamethasone. In the sensitized and challenged mice, there was a decrease in bone marrow eosinophils 24 hours after the ovalbumin challenge. This was largely blocked by the IL-5 antibody, but not by the GL113 antibody. Some studies that we have conducted in blood and tissue suggest that the IL-5 antibody may have an effect on activation of eosinophils, as well as on release of eosinophils.

Mast cell involvement in BAL eosinophilia

It appears that eosinophilia is IL-5-based, but what is the source of the IL-5? TH_2 lymphocytes and mast cells are two reasonable candidates. To address this question, we used a mast cell-deficient mouse, the W/W^V ,¹⁵ and a protocol very similar to the model described above. To make our point conclusively, it was necessary to reconstitute the W/W^V animals with mast cells from the W/W^+ mouse, the normal congenic littermate. When unsensitized W/W^+ mice were challenged with ovalbumin, they did not respond with pulmonary eosinophilia that could be detected in the

BAL (Fig. 5). On the other hand, they did respond when they were both sensitized and challenged. The mast cell-deficient W/W^V mice did respond compared with the unsensitized controls. However, this increase was not statistically significant, although it seemed real and may have been due to lymphocytic sources of IL-5. On the other hand, the mast cell-reconstituted W/W^V mice had a much more profound eosinophilia under the same experimental conditions. Hence, the mast cell appears to be involved in eosinophil accumulation in the W/W^V mouse.

In summary, in many ways this allergic mouse model is consistent with what is seen in human pulmonary inflammation. With so many immunologic reagents available for the mouse, this model allows us to evaluate the roles of a variety of cytokines and to establish mechanisms of inhibition of pulmonary eosinophilia. Nevertheless, the physiologic sequela (airway hyperreactivity) so often observed in the guinea pig, the monkey, and human beings is not consistently observed in the mouse (data not shown), a serious drawback that probably reflects lack of eosinophil degranulation. After allergen challenge, the distribution of eosinophils in the various tissue compartments fol-

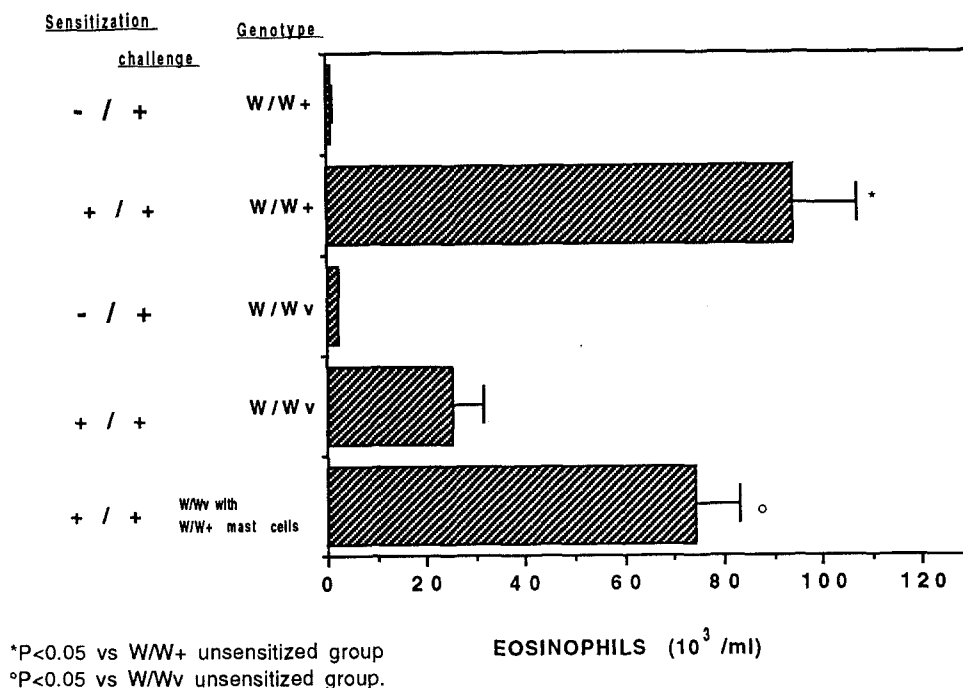


FIG. 5. BAL eosinophilia in W/W^v, W/W⁺, and mast cell-reconstituted W/W^v mice.

lowed the temporal changes that would be expected, culminating in tissue and BAL eosinophilia. Using BAL eosinophilia as a quantitative readout, we found that steroids, anti-IL-5 antibodies, anti-IL-4 antibodies, and IFN- γ block pulmonary eosinophilia by various mechanisms. Lastly, the W/W^v mouse that is mast cell-deficient and possibly immunocompromised in other ways¹⁵ did not respond significantly to antigen challenge with pulmonary eosinophilia until it was reconstituted with purified bone marrow mast cells from its congenic littermate, implicating a role for mast cell products, possibly IL-5, in eosinophil recruitment.

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