

Effect of antigen provocation of IL-5 transgenic mice on eosinophil mobilization and bronchial hyperresponsiveness

Jean Lefort, Claude-Marie Bachelet, PhD, Dominique Leduc, and
B. Boris Vargaftig, MD, PhD Paris, France

We investigated whether allergen-induced eosinophil recruitment into mouse airways modifies the in vivo bronchopulmonary responses to standard agonists, an adaptation of a technique described for larger animals. Swiss, CBA, and IL-5 transgenic mice were immunized with ovalbumin and challenged intranasally after 14 days. Immunization alone was followed by increased eosinophil counts in bone marrow and blood, whereas antigenic challenge induced eosinophil infiltration in lungs and bronchoalveolar lavage fluid, which was suppressed by dexamethasone. Despite the high eosinophil counts, no bronchopulmonary hyperreactivity to methacholine or serotonin was detected 3 to 96 hours after antigenic provocation. Our results demonstrate that immunization augments the production of eosinophils by mice, which is further increased by antigenic challenge, but that eosinophil overproduction and lung infiltration, per se, are not sufficient to induce bronchopulmonary hyperreactivity, even in constitutively hypereosinophilic IL-5 transgenic mice. (J ALLERGY CLIN IMMUNOL 1996;97:788-99.)

Key words: Asthma, bone marrow, bronchoalveolar lavage, corticosteroids, lungs, IL-5, mice

Eosinophils are claimed to account for tissue damage and the consequent bronchopulmonary hyperresponsiveness. They are indeed found in increased numbers in the bronchoalveolar lavage fluid (BALF) and in the bronchial mucosa of human beings with allergy¹⁻⁴ and of immunized and challenged guinea pigs⁵ and mice.⁶ Nevertheless, no evidence of a causal relationship between eosinophil recruitment and bronchopulmonary hyperresponsiveness is available; on the contrary, it has been shown⁷ that the BALF of allergen-challenged guinea pigs can be markedly enriched in eosinophils in the absence of bronchopulmonary hyperresponsiveness. Because of limitations in the use of guinea pigs to study experimental allergy, particularly the unavailability of immunologic tools and syngeneic strains, we developed a model in which the allergen-induced eosinophil recruitment

Abbreviations used

BALF: Bronchoalveolar lavage fluid
5-HT: 5-Hydroxytryptamine, serotonin

to blood, bone marrow, and lungs was studied together with bronchial responsiveness in two strains of mice and in IL-5 transgenic mice.⁸ Finally, because glucocorticosteroids are widely used in the treatment of asthma, dexamethasone was applied in some experiments. Our results indicate that the presence of eosinophils in the lungs and airways of mice is not necessarily accompanied by nonspecific bronchopulmonary hyperresponsiveness.

METHODS

Immunization

Male Swiss and CBA mice, weighing 20 to 22 gm, (Iffa Credo, France) and IL-5 transgenic mice on a CBA background⁸ courtesy of Dr. C. Sanderson, Western Australian Research Institute, Perth, Australia, and Mr. M. Strath, National Institute for Medical Research, Mill Hill, U.K.) were injected twice at 7-day intervals with 100 µg ovalbumin in 0.4 ml of a 4 mg/ml suspension of Al(OH)₃ in saline solution. Sham-immunized mice were injected with Al(OH)₃ alone; and in one series of experiments the first injection contained ovalbumin and

From Unité de Pharmacologie cellulaire, Unité Associée Institut Pasteur, Paris.

Received for publication Jan. 5, 1995; revised May 11, 1995; accepted for publication May 17, 1995.

Reprint requests: Boris Vargaftig, Unité de Pharmacologie cellulaire, Unité Associée Institut Pasteur-INSERM no. 285, 25, Rue du Dr. Roux, 75724 Paris Cedex 15, France.

Copyright © 1996 by Mosby-Year Book, Inc.

0091-6749/96 \$5.00 + 0 1/166383

Al(OH)₃, and the second, Al(OH)₃ alone. One week after the second injection, mice were lightly anesthetized with diethyl ether and challenged with 10 µg ovalbumin in 50 µl 0.9% NaCl instilled into the nostrils with a micropipette. Care was taken to ensure that the solution was fully administered. Mice were killed at different intervals after challenge. Controls were immunized and either unchallenged or challenged with saline solution and killed after 24 hours.

Determination of the respiratory functions

Mice were anesthetized with methyl carbamate (intraperitoneally, 45 mg/10 gm) and paralyzed with 10 mg of Pavulon (Organon Teknika BV, Turnhout, Belgium). The trachea of each mouse was cannulated and connected to a respiratory pump (BioSciences, Sheerness, Kent, U.K.), delivering a tidal volume of 0.2 ml/10 gm at 100 breaths/min. Airway resistance and dynamic lung compliance were calculated from the differential pressure between the airways and the pleural cavity, and the airflow was evaluated during the breath cycle with a computerized pulmonary mechanical analyzer (Mumed PR800 system; Mumed Ltd., London, U.K.). The jugular vein was cannulated to inject 10 to 320 µg/kg of 5-hydroxytryptamine (5-HT) or methacholine at 5-minute intervals. These assays were performed 3, 24, or 96 hours after challenge. The doses of 5-HT or methacholine that augmented airway resistance by 30%, 60%, and 90% were called PD30, PD60, and PD90, respectively.

Cell counts in blood, BALF, and bone marrow preparations

Blood smears prepared from tail blood were stained with May-Grunwald-Giemsa stain for differential counts. To collect BALF, animals were killed with sodium pentobarbital, and the lungs of each mouse were washed, through the cannulated trachea, with 8 volumes of 0.5 ml saline solution, which were pooled. Total cell numbers were counted in a Mallassez hemocytometer (Polylab, Strasbourg, France), and cytocentrifuge preparations were stained with Diff-Quik (Baxter Diagnostics AG, Duedingen, Switzerland) for differential cell counts. Finally, one femur was removed, the bone marrow was harvested in phosphate buffer, and cytocentrifuged preparations were stained with May-Grunwald-Giemsa stain to determine the percent of eosinophil line cells within the total myeloid population.

Histology

Mice were anesthetized with sodium pentobarbital (intraperitoneally, 3 mg/10 gm) and exsanguinated. The lungs were fixed with phosphate-buffered formalin (10%), through the trachea, at a pressure of 20 cm H₂O, then immersed in the fixative solution and embedded in paraffin. Longitudinal sections of 5 µm were stained with hematoxylin and eosin according to a method that identifies cyanide-resistant eosinophil peroxidase activity in human cells,^{9, 10} which also distinguishes murine

eosinophils from neutrophils. Sections were covered for 10 minutes with 13.3 ml of phosphate buffer with 10 mg diaminobenzidine, 4 µl 30% H₂O₂, and 6 mmol/L KCN and counterstained with Harris hematoxylin. Whole sections of the five lobes of each lung were examined, and the intensity of inflammation was graded in blinded fashion with the use of an arbitrary scale: 0, normal lung; 1, a few disseminated inflammatory cells; 2, moderate inflammation (one or two cell layers around some bronchi or blood vessels); 3, strong inflammation (several cell layers around several bronchi or blood vessels); 4, severe inflammation (numerous cells around most bronchi and blood vessels). Other features were noted, such as eosinophil adhesion to the endothelium, alveolitis, pleural inflammation, presence of inflammatory cells in the parenchyma (alveolar septa), and the condition of the epithelium.

Dexamethasone treatment

Groups of six to 20 immunized Swiss mice received a subcutaneous injection of 1.25 to 5 mg/kg dexamethasone phosphate in saline solution just before the intranasal challenge with ovalbumin, and BALF was collected after 24 or 96 hours. Naive, immunized, and immunized and challenged mice were treated with 2.5 mg/kg dexamethasone and killed 2, 6, 24, and 96 hours after the challenge or 1 hour after treatment in the case of naive mice; and blood, bone marrow, and histologic analyses were performed. Results in control animals receiving the same volume of 0.9% NaCl were similar to those in untreated mice.

Materials

Unless otherwise stated, all reagents were from Sigma Chemical Company (St. Louis, Mo.).

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed with the unpaired Student's *t* test for BALF, blood, and bone marrow cell content and the Mann-Whitney U test for the score values in histology. Differences between results were considered significant when the *p* value was less than or equal to 0.05.

RESULTS

Modulation of effects of 5-HT and methacholine on mouse airway resistance and compliance

A typical tracing of the dose-dependent increase in airway resistance and decrease in compliance in response to 5-HT is shown in Fig. 1. The PD30, PD60, and PD90 values for 5-HT in CBA and IL-5 transgenic mice, tested 24 hours after challenge with saline solution or antigen, were similar (Fig. 2). Antigen challenge also failed to induce bronchial hyperresponsiveness to 5-HT after 3 hours or when methacholine was used in Swiss, CBA, and

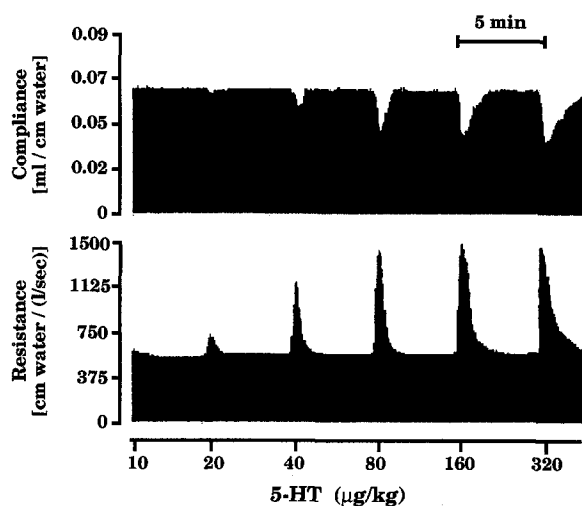


FIG. 1. Decreased airway compliance and increased resistance after intravenous administration of 5-HT to an anesthetized Swiss mouse.

IL-5 transgenic mice. In addition, the responses to 5-HT were not modified 96 hours after challenge of IL-5 transgenic mice. Indeed, the PD30, PD60, and PD90 values (in micrograms per kilogram) were 67.4 ± 18 , 52.5 ± 11 , and 28 ± 6 in immunized saline-challenged mice, respectively, and 53.8 ± 12 , 42 ± 10 , and 21 ± 5 in immunized antigen-challenged mice, respectively.

Eosinophil content in blood, bone marrow, and BALF of Swiss, CBA, and IL-5 transgenic mice after immunization

As seen in Fig. 3, the percent of eosinophils in blood of naive or sham-immunized and ovalbumin-challenged Swiss mice (AA+O) was not significantly different ($2.3\% \pm 0.6\%$ vs $2.5\% \pm 0.4\%$). The percent of eosinophils in bone marrow was also not significantly different ($3.0\% \pm 0.6\%$ for naive vs $4.8\% \pm 1.1\%$ for sham-immunized and ovalbumin-challenged mice). In contrast, the percents of eosinophils in blood and bone marrow were significantly increased in ovalbumin-immunized and saline-challenged mice, as compared with naive, Swiss mice (blood: $5.2\% \pm 0.8\%$ vs $2.3\% \pm 0.6\%$ and bone marrow: $5.2\% \pm 0.5\%$ vs $3.0\% \pm 0.6\%$). Immunization thus augmented both the blood and bone marrow proportions of eosinophils.

As seen in Figs. 3 and 4, the bone marrow of Swiss mice contained roughly double the number of eosinophils in terms of percent, as compared with CBA mice. These figures were not modified by sham immunization, but immunization with ovalbumin and challenge with saline solution raised them signifi-

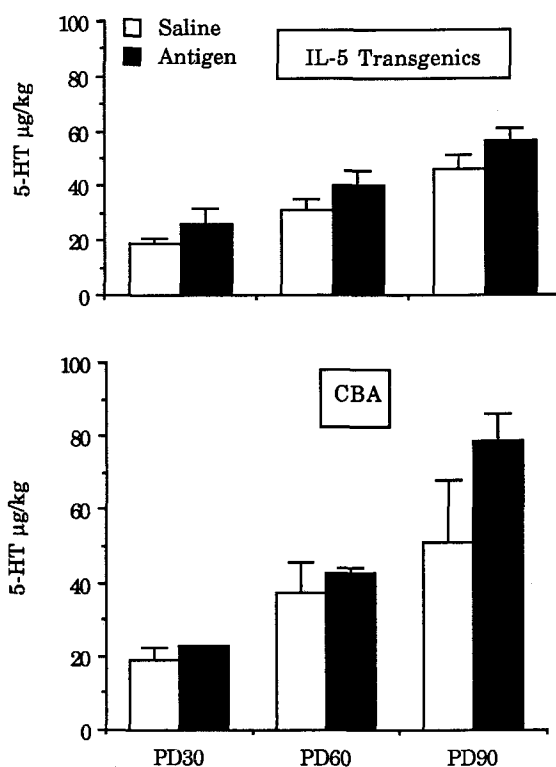


FIG. 2. Comparison between responsiveness to 5-HT of immunized IL-5 transgenic and CBA mice 24 hours after intranasal challenge with saline solution or ovalbumin. Bronchoconstriction in groups of four to five immunized mice was measured. Doses (in micrograms per kilogram) of 5-HT that induced 30%, 60%, and 90% increase in airway resistance are expressed as PD30, PD60, and PD90.

cantly. In contrast to CBA mice, naive IL-5 transgenic mice had approximately 35% eosinophils in blood and bone marrow (Fig. 5). Sham immunization, followed by challenge with ovalbumin (AA+O) or immunization with ovalbumin followed by challenge with saline solution (OO+S), led to a similar increase in the percent of eosinophils in blood and bone marrow, up to 50% to 60%.

The total number of cells in the BALF of Swiss, CBA, and IL-5 transgenic mice (the majority of which were alveolar macrophages) varied from 90,000 to 150,000 per animal. These values were not affected by sham immunization and challenge with ovalbumin in Swiss and CBA mice or by sensitization with ovalbumin and challenge with saline solution (AA+O and OO+S, respectively, in Figs. 3 and 4). In contrast, the BALF of IL-5 transgenic mice of the groups AA+O and OO+S contained 7% to 16% and 3.5% to 16% eosinophils 24 hours after the intranasal instillations, respectively (Fig. 5).

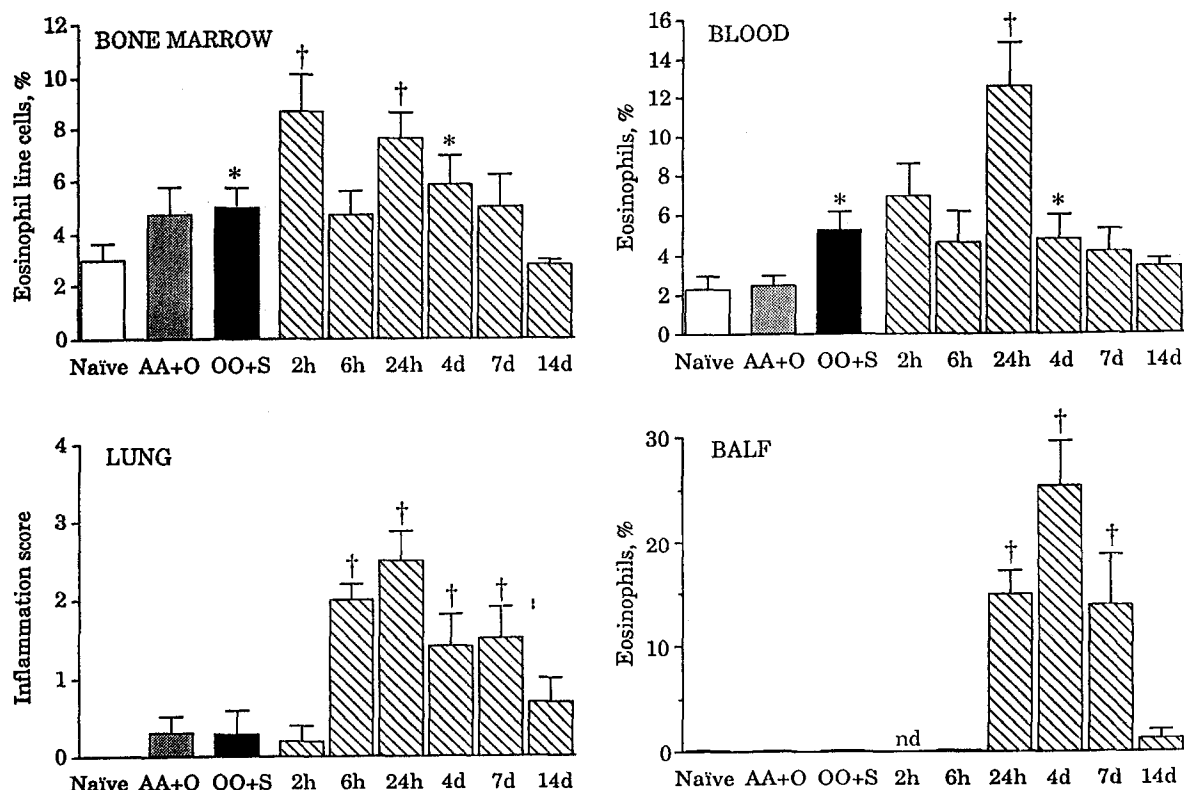


FIG. 3. Eosinophils in bone marrow, blood, and BALF as compared with intensity of lung inflammation in Swiss mice. AA+O, Sham-immunized and ovalbumin-challenged mice; OO+S, ovalbumin-immunized and saline-challenged mice. Hatched columns correspond to values obtained at indicated time points after challenge of immunized mice with ovalbumin. * $p \leq 0.05$ as compared with values for naive mice. † $p \leq 0.05$ as compared with immunized and saline-challenged mice.

Modifications of eosinophil content in bone marrow, blood, and BALF after antigenic challenge of immunized Swiss, CBA, and IL-5 transgenic mice

As seen in Fig. 3, blood counts were not further increased in the immunized Swiss mice 2 and 6 hours after antigen challenge, but at 24 hours a marked blood eosinophilia of $12.6\% \pm 2.2\%$ was observed, which was normalized on day 7. The eosinophil counts in the bone marrow were increased up to $8.1\% \pm 0.9\%$ 2 hours after challenge, returned at 6 hours to values equivalent to those of immunized unchallenged mice (OO+S, $4.7\% \pm 0.9\%$) and were increased at 24 hours to $7.6\% \pm 1.0\%$. Those counts returned to baseline levels 7 days after challenge.

The booster injection of ovalbumin on day 7 after immunization was not required to support the antigen-induced increase in the eosinophil blood content, because Swiss mice immunized with ovalbumin only in the first injection had

$11.7\% \pm 2.1\%$ eosinophils in blood 24 hours after challenge, as compared with $12.6\% \pm 2.2\%$ in mice immunized with two injections of ovalbumin. In contrast, the booster was required to augment the eosinophil content in the bone marrow. In addition, $6.8\% \pm 2.2\%$ eosinophils were found in the BALF 24 hours after the challenge of mice immunized with ovalbumin in the first injection only, as compared with $13.5\% \pm 2.5\%$ for mice immunized with two injections of ovalbumin.

Eosinophil counts in blood and bone marrow of immunized CBA or IL-5 transgenic mice were not augmented by the antigenic challenge, as compared with the values of the ovalbumin-immunized and saline-challenged animals (OO+S) (Figs. 4 and 5).

Eosinophil numbers in the BALF of Swiss mice increased to 10% to 15% and 20% to 30% of the total cell numbers 24 and 96 hours after antigenic challenge, respectively. Their content returned to baseline values on day 14 (Fig. 3).

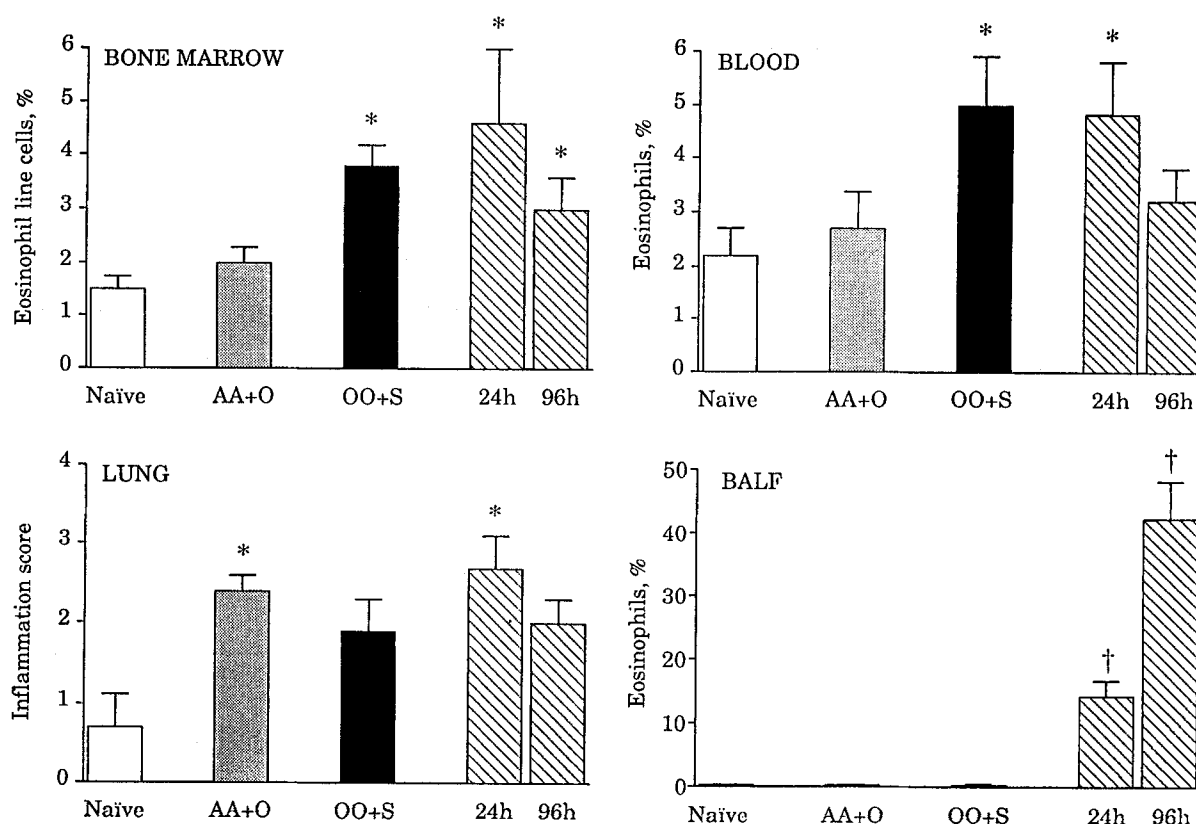


FIG. 4. Eosinophils in bone marrow, blood, and BALF as compared with intensity of lung inflammation in CBA mice. AA+O, Sham-immunized and ovalbumin-challenged mice; OO+S, ovalbumin-immunized and saline-challenged mice. Hatched columns correspond to values obtained at indicated time points after challenge of immunized mice with ovalbumin. * $p \leq 0.05$ as compared with values for naive mice. † $p \leq 0.05$ as compared with immunized and saline-challenged mice.

Antigen-challenged CBA mice seemed slightly more responsive in terms of percent of eosinophils in the BALF as compared with Swiss mice at 96 hours, but not at 24 hours (Fig. 4). In contrast, 24 hours after challenge with ovalbumin, the percent of eosinophils in the BALF of IL-5 transgenic mice was elevated and persisted at 96 hours. As stated, 3.5% to 16% eosinophils were present in the BALF of sham-immunized and ovalbumin-challenged mice and in the BALF of ovalbumin-immunized and saline-challenged IL-5 transgenic mice, whereas neither CBA nor Swiss mice showed this background cell recruitment (Figs. 3, 4, and 5).

Modifications of the tissue content in eosinophils after immunization and antigenic challenge

No inflammation was noted in lungs from naive Swiss mice, and only very mild inflammation was seen in sham-immunized and ovalbumin-

challenged or ovalbumin-immunized and sham-challenged animals (Fig. 3). Fig. 6 shows the microscopic appearance of lungs from immunized and challenged Swiss mice. After 2 hours, lungs showed a normal aspect. In contrast, at 6 hours and particularly after 24 hours, lungs were strongly inflamed, with alveolitis and occasional granulomatous lesions. Eosinophils, identified as cyanide-resistant peroxidase-positive cells, were located mostly around the central airways and blood vessels. At 24 hours, their adhesion to the endothelium was marked in nine of 11 animals, alveolitis and parenchymal inflammation in eight of 11 animals, and pleural inflammation in six of 11 animals. Eosinophil adhesion to the endothelium was the predominant feature 6 hours after challenge (9 of 12 mice) with parenchymal inflammation (7 of 12 mice), and both were still present in half of the mice 4 days after challenge. At no time was epithelial damage (i.e., shedding of cells with naked basal lamina or inflammatory cells within the

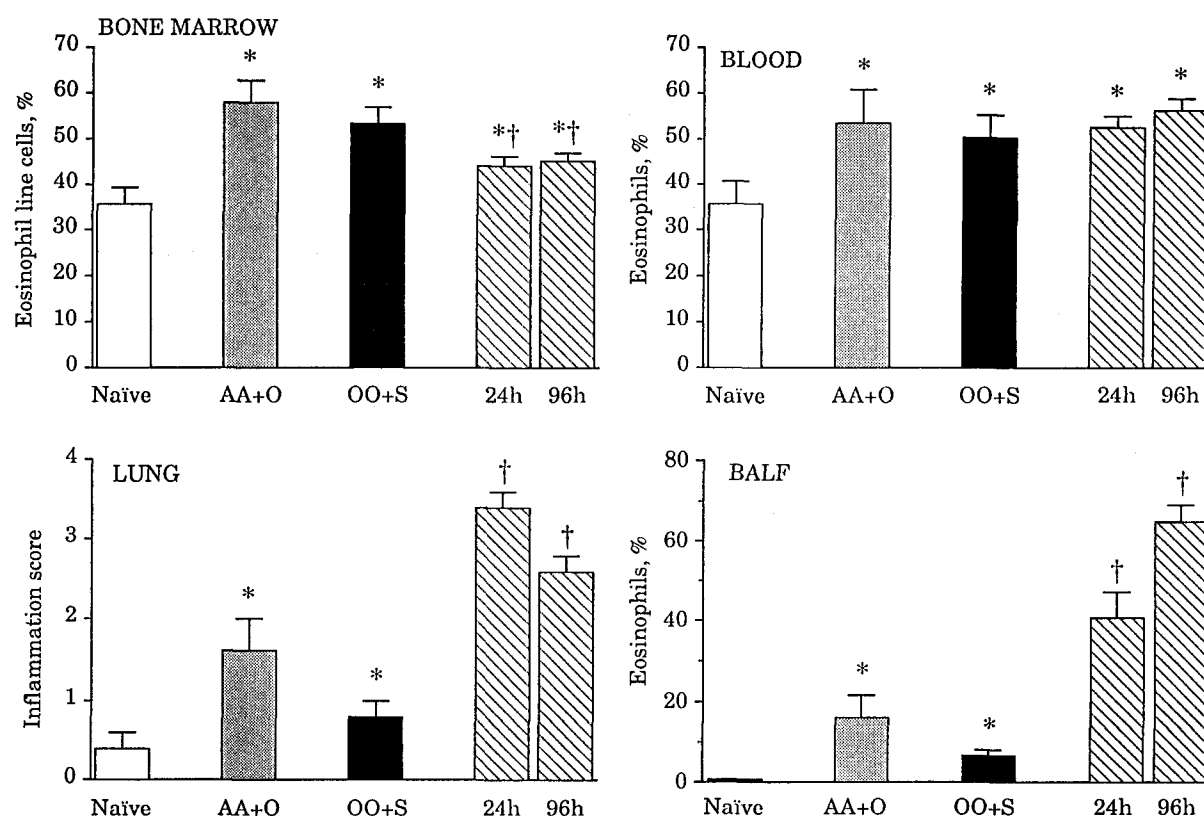


FIG. 5. Eosinophils in bone marrow, blood, and BALF as compared with intensity of lung inflammation in IL-5 transgenic mice. AA+O, Sham-immunized and ovalbumin-challenged mice; OO+S, ovalbumin-immunized and saline-challenged mice. Hatched columns correspond to values obtained at indicated time points after challenge of immunized mice with ovalbumin. * $p \leq 0.05$ as compared with values for naive mice. † $p \leq 0.05$ as compared with immunized and saline-challenged mice.

epithelium) noted. Rather, swollen epithelial cells were noted in half of the animals 4 and 7 days after challenge.

The booster injection of ovalbumin on day 7 after immunization was not required to support the antigen-induced lung inflammation, because nonboosted mice had a score of 2.2 ± 0.4 versus 2.5 ± 0.4 for mice immunized with two injections of ovalbumin. This differs from the results obtained in the BALF, in which the percent of eosinophils doubled in the mice immunized with two injections of ovalbumin.

Lungs and airways from naive CBA mice contained occasional inflammatory cells, particularly mononuclear cells (Fig. 7, A), whereas those from naive IL-5 transgenic mice contained eosinophils (Fig. 7, C). In both instances moderate inflammation was seen in lungs from saline-immunized and ovalbumin-challenged or ovalbumin-immunized and saline-challenged animals (Figs. 4 and 5, lower left panels). A marked inflammation was triggered

at 24 and 96 hours after provocation with allergen, as shown in Fig. 7.

Interference of dexamethasone with eosinophilia in Swiss mice

Eosinophils accounted for $10.5\% \pm 2.7\%$ of total cells in the BALF 24 hours after the antigenic provocation and for $8.4\% \pm 2.4\%$, $4.7\% \pm 2.7\%$, and $2.3\% \pm 1.4\%$ of total cells in mice pretreated with 1.25, 2.5, and 5 mg/kg dexamethasone, respectively. Similarly, after 96 hours, eosinophils accounted for $23\% \pm 8\%$ of total cell numbers in control mice and for $13.8\% \pm 6.0\%$, $3.0\% \pm 1.5\%$, and $3.7\% \pm 1.3\%$ of total cell numbers in mice pretreated with 1.25, 2.5, and 5 mg/kg dexamethasone, respectively. Dexamethasone also decreased blood eosinophilia in naive and immunized saline-challenged mice and 6 hours after challenge (Fig. 8). The percent of eosinophil line cells in the bone marrow of ovalbumin-immunized saline-challenged mice was also reduced by dexamethasone.

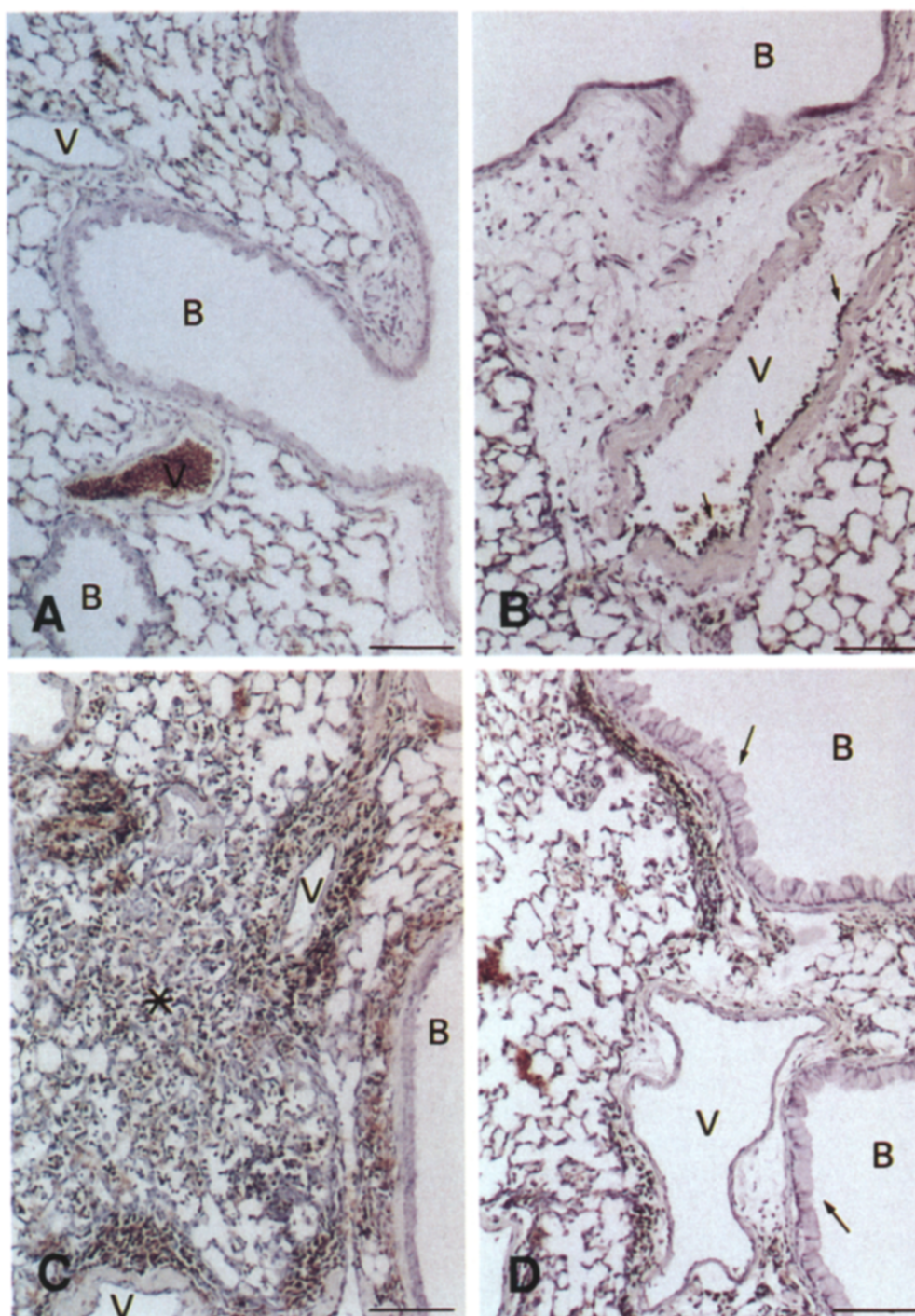


FIG. 6. Photomicrographs of lung sections from immunized and challenged Swiss mice. **A**, Normal aspect of the lung from an immunized animal, 2 hours after challenge. **B**, Six hours after challenge, eosinophils appear marginated along the endothelium (*arrows*). **C**, Twenty-four hours after challenge, a marked eosinophil infiltration is present around the bronchi and blood vessels and in the alveoli (*asterisk*). **D**, Four days after challenge, eosinophils are still present in the lungs. The epithelium is swollen (*arrows*). Paraffin sections. The staining for cyanide-resistant eosinoperoxidase shows eosinophils as nucleated brown cells. Bar = 100 μ m. B, Bronchi; V, vessels.

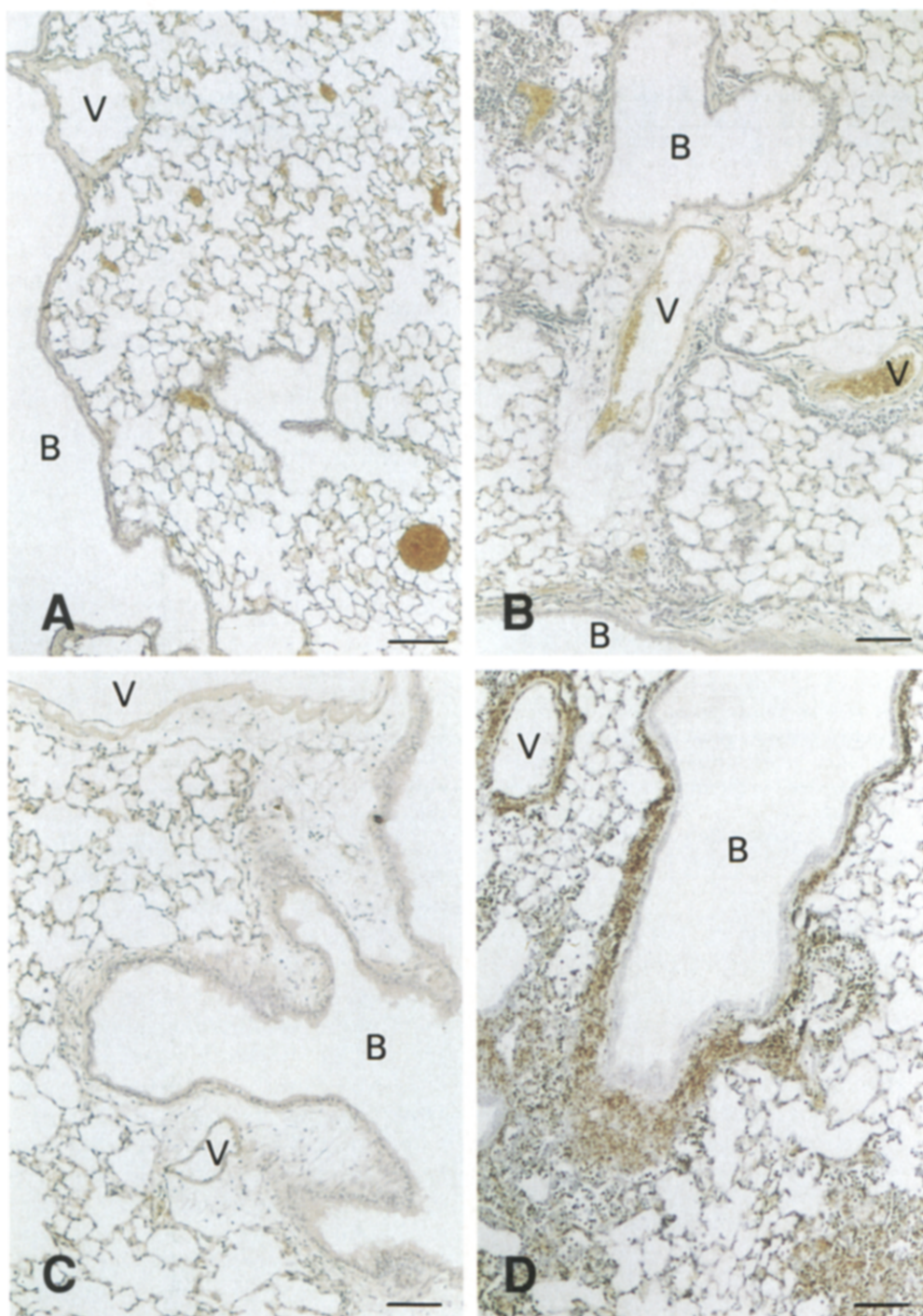


FIG. 7. Photomicrographs of lung sections from immunized and challenged CBA and IL-5 transgenic mice. **A**, Normal aspect of the lung from a naive CBA mouse. **B**, Twenty-four hours after challenge of an immunized CBA mouse. **C**, Normal aspect of the lung from a naive IL-5 transgenic mouse. Note that inflammatory cells, particularly eosinophils, are present around bronchi and blood vessels. **D**, Twenty-four hours after challenge of an immunized IL-5 transgenic mouse. Note the intense recruitment of eosinophils (*in brown*). Paraffin sections. The staining for cyanide-resistant eosinoperoxidase shows eosinophils as nucleated brown cells. Bar = 100 μ m. B, Bronchi; V, vessels.

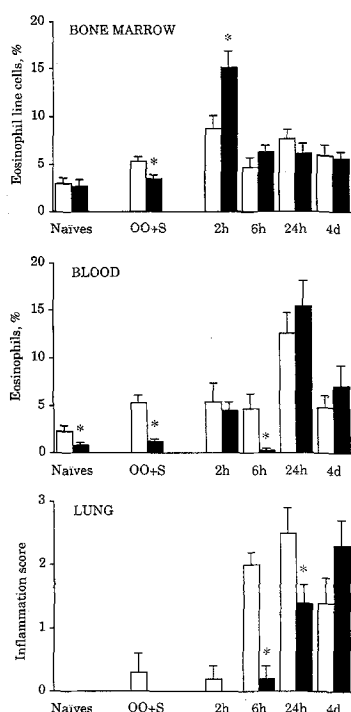


FIG. 8. Interference of dexamethasone with antigen-induced effects on eosinophils in bone marrow and in blood as compared with lung inflammation in Swiss mice. Dexamethasone was administered at 2.5 mg/kg by the subcutaneous route just before challenge, and counts and histologic examination were performed at the indicated time points. OO+S, Ovalbumin-immunized and saline-challenged mice. * $p \leq 0.05$ as compared with the untreated group. Open columns, Control mice; filled columns, dexamethasone-treated mice.

In contrast, the proportion of cells of the eosinophil line doubled in the bone marrow of those mice 2 hours after challenge. Lung inflammation, evaluated histologically 6 hours after challenge, was suppressed by dexamethasone and was significantly decreased after 24 hours, with alveolitis being abolished, but was as intense as in untreated challenged animals after 4 days.

DISCUSSION

Eosinophils and bronchopulmonary hyperresponsiveness

Despite its likelihood, there is no proof of a causal relationship between eosinophils and allergic bronchopulmonary hyperresponsiveness.^{11, 12} Allergic challenge is followed by eosinophil and lymphocyte accumulation around vessels and alveolar septa in human beings,¹ and, as shown here, also in Swiss, CBA, and IL-5-transgenic mice after intranasal challenge. In fact, eosinophil recruitment does not always follow the challenge, and

mice immunized with inhaled ovalbumin (in the absence of adjuvant), which display augmented T lymphocyte numbers in lymph nodes,¹³⁻¹⁵ show no increase in eosinophil counts, but are reported to become hyperresponsive.¹⁴ In contrast, Saloga et al.¹⁶ showed that the elevation of IgE and IgG₁ titers after transcutaneous immunization of BALB/c mice with ovalbumin was only followed by in vitro hyperresponsiveness if the animals were challenged through the airways. Garssen et al.¹⁷ demonstrated tracheal hyperresponsiveness and in vivo augmentation of the basal pulmonary resistance 1 week after the intranasal provocation with allergen in BALB/c mice immunized subcutaneously. Our experiments demonstrate that the presence of eosinophils in the airways of allergen-challenged Swiss, CBA, or IL-5 transgenic mice is not sufficient to induce bronchial hyperresponsiveness. In fact, secretion of cytotoxic materials from eosinophils is claimed to be essential for epithelial damage in human beings¹¹ and guinea pigs.¹² Eosinophils were not identified in the bronchial epithelium, which appeared intact at all time points studied, even near sites of strong eosinophil infiltration, as described for human beings.¹⁹ The only epithelial change noted was a late swollen appearance, also described by Garssen et al.,²⁰ who claimed that it might be related to bronchoconstriction, but no hyperresponsiveness was seen at this time point. It is thus likely that in our experiments the eosinophils recruited into the airways were not activated, and indeed, we failed to identify eosinophil peroxidase in BALF (not shown). Eosinophil recruitment by itself does not induce bronchopulmonary hyperresponsiveness in guinea pigs, but the subsequent eosinophil activation in the airways, which is not found in nonhyperresponsive but otherwise hypereosinophilic BALF, does so.⁷ Thus even though our experiments show that the recruitment of eosinophils to mouse airways does not induce hyperresponsiveness, the results do not rule out the possibility that hyperresponsiveness could be present after eosinophil secretion in situ. It should also be recognized that other forms of airway hyperresponsiveness exist, which may be independent from eosinophils.^{13, 14, 21}

Effects of immunization and antigenic provocation on blood and bone marrow eosinophil content

Immunization alone increased the eosinophil numbers in mouse blood and bone marrow (Figs. 3 and 4), as reported for patients with allergy¹⁹ and

immunized guinea pigs (unpublished data). In contrast, the eosinophil counts in the blood and bone marrow of IL-5 transgenic mice was increased by sham immunization to a level not further augmented by immunization or challenge with ovalbumin, suggesting that those animals are primed for those events.

The proportion of eosinophils in the blood of Swiss mice doubled 24 hours after challenge but was already augmented in the bone marrow within 2 hours and in the lungs within 6 hours (Fig. 3). Because Swiss mice showed increased basal eosinophil counts in bone marrow and blood because of prior immunization, the further rapid increment in those numbers in the bone marrow probably resulted from late proliferation of already differentiated precursors. In fact, the bone marrow increase in eosinophil numbers varied according to the protocol of immunization, because no such changes were noted when mice were immunized with a single injection of ovalbumin, in contrast to boosted mice. Thus the increased eosinophil proliferation requires at least two administrations of antigen. One may speculate that the first antigen administration involves predominantly (IL-3- and granulocyte-macrophage colony-stimulating factor-dependent?) proliferation and differentiation of early precursors, whereas the challenge operates as a stimulus for the selective proliferation of eosinophils, possibly induced by IL-5. It is noteworthy that IL-3 and granulocyte-macrophage colony-stimulating factor are generated in the airways of subjects with asthma.^{22, 23} Immunization might prime hematopoietic precursors for proliferation, whereas the challenge might switch eosinophil production, expressed by the increased counts in blood, bone marrow, and lungs. Such biphasic processes were suggested in parasitic infections in which nonspecific T-cell reactions appear to be followed by antigen-specific T-cell reactions.²⁴

In contrast to Swiss and CBA mice, eosinophil counts in the blood and bone marrow of IL-5 transgenic mice were not augmented 24 or 96 hours after challenge. It is likely that the very high numbers of eosinophils of IL-5 transgenic mice limit the possibility of further expansion, suggesting the presence of inhibitors, such as soluble forms of IL-5 receptors.²⁵ IL-5 transgenic mice infected with a parasite retain inducibility for the production of high levels of IL-5, but the eosinophil counts are not further increased, suggesting a control mechanism.²⁶

Effects of immunization and antigenic provocation on lung inflammation: Mechanisms of lung recruitment of eosinophils

Lung inflammation after provocation peaked at 24 hours (i.e., before the full augmentation of the BALF content of eosinophils). Unexpectedly, mild inflammation occurred in the lungs of ovalbumin-immunized and saline-challenged CBA mice, which had increased eosinophil counts in the bone marrow and blood, and in sham-immunized and ovalbumin-challenged mice, in which those values did not differ from those of naive mice (Fig. 4). Because lung inflammation can occur in the absence of noticeable central eosinophil proliferation, it is possible that lung eosinophils multiply *in situ* after the interaction of the allergen with immune cells, such as CD4+ T lymphocytes, a recognized source of IL-5, which have been identified in the bronchial wall of human beings with allergy,²³ immunized mice,⁶ and guinea pigs.²⁷ IL-5 activity is present in the airways of allergen-challenged guinea pigs (Pretolani et al. Manuscript in preparation), and Amorim et al.²⁸ have shown that the increased eosinophil numbers found in the peritoneal cavity of BALB/c mice after antigenic provocation is suppressed selectively by the depletion of CD4+, but not CD8+, T lymphocytes.⁶

The mechanisms of eosinophil recruitment by antigen into lungs are multiphased. Thus a single immunization with ovalbumin supported the appearance of half the number of eosinophils in the bone marrow and BALF of mice after intranasal provocation, as compared with immunized and boosted mice. In contrast, blood and lung eosinophil contents were the same in both procedures of immunization. It is likely that the first immunization without the booster induced the recruitment of eosinophils to the blood and lungs, but that a second stimulation was required to make the eosinophils cross the epithelium into the BALF. The secondary upregulation of adhesion proteins on repeated provocations may account for this dissociation.

The infiltration of eosinophils into the lungs of asthmatic subjects may be caused by or linked to the release of IL-5 in the airways. It is thus likely that an early signal, which stimulates eosinophil differentiation in bone marrow and possibly in the lungs, is generated by a lung target that recognizes the antigen, whereas cell diversion into the circulation and pulmonary recruitment occur later, possibly by distinct mechanisms. To this extent, exper-

imental (and human) asthma may well be primarily a local pulmonary disease, which secondarily operates distally to ensure the continuous availability of immune and effector cells. In support of this hypothesis, Corris and Dark²⁹ recently demonstrated that in nonasthmatic recipients of asthmatic lungs, asthma developed after transplantation; whereas in asthmatic recipients of normal lungs, asthma had not developed for up to 3 years after transplantation. Samter et al.,³⁰ in 1953, demonstrated the presence of a factor in anaphylactically shocked guinea pig lungs, which induces blood eosinophilia in normal animals, indicating the presence of a pulmonary source capable of turning on eosinophil production. Very recently, Takamoto and Sugane³¹ demonstrated that an "eosinophil-differentiating activity," most likely IL-5, is produced by lung cells, possibly other than T lymphocytes, from normal and congenitally athymic nude mice infected with *Toxocara canis*. It is thus likely that a lung source, such as mast cells, generates IL-5,³² which may either operate locally on precursors or at distance on the bone marrow. In both cases eosinophils would be recruited to the lungs.

Interference of glucocorticosteroids with eosinophil stimulation

Glucocorticosteroids are the most widely used agents in the treatment of asthma.³³ Dexamethasone dose-dependently suppressed the increased eosinophil content of BALF for at least 96 hours, confirming the results of Wang et al.³⁴ for eosinophilia caused by *Aspergillus* antigen. At 2.5 mg/kg, it reduced blood eosinophil counts in naive and immunized saline-challenged Swiss mice and inhibited blood eosinophilia 6 hours after challenge, but the 2-hour point was unaffected. Lung inflammation was reduced at 6 hours and 24 hours and was unaffected at 96 hours. Thus the transepithelial migration of eosinophils, as reflected by the increased counts in BALF, was inhibited for a longer time interval than its recruitment to blood and to the bronchial wall. It is unclear whether failure of dexamethasone to modify the increased blood counts results from a short-lasting effect or whether the antigen-induced increment in blood eosinophilia is in part steroid-resistant. Furthermore, even though dexamethasone slightly decreased the eosinophil counts in the bone marrow of immunized saline-challenged mice, the antigen-induced augmentation of the bone marrow eosinophil content was not reduced by dexamethasone, suggesting that it does not operate in the bone

marrow through the stimulation of apoptosis, as previously described.³⁵

Two hours after challenge, when the stimulating effects of antigen on bone marrow (but not on blood and lungs) were already apparent, eosinophil counts were in fact augmented, as reported by Hudson.³⁶ Dexamethasone increases lymphocyte accumulation in the bone marrow³⁷ and decreases migration to blood by reducing chemotaxis.³⁸ The increased bone marrow counts may result from the combination of these effects.

In summary, our results show that eosinophilia in mouse lungs, induced by antigen and/or by transgenicity to IL-5, is not accompanied by bronchopulmonary hyperresponsiveness. The mechanisms of allergen-induced eosinophil recruitment to lungs may involve, according to the protocol of immunization (and to the pattern of immunization in the case of asthmatic patients), different balances between local (pulmonary) and bone marrow eosinophil proliferation and differentiation. Those sites, as well as the mechanisms of hyper-eosinophilia in blood and its final diversion into BALF, offer new targets for pharmacologic intervention.

We thank Mrs. Rose Jacobessen and Mr. Dominique Godfrin for their participation in the early part of these studies.

REFERENCES

1. Beasley R, Roche WR, Roberts JA, Holgate S. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 1989;139:806-17.
2. Azzawi M, Bradley B, Jeffery PK, et al. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthmatics. *Am Rev Respir Dis* 1990;142:1407-13.
3. Djukanovic R, Roche WR, Wilson JW, et al. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990;142:434-57.
4. Motojima S, Frigas E, Loegering DA, Gleich GJ. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. *Am Rev Respir Dis* 1989;139:801-5.
5. Lellouch-Tubiana A, Lefort J, Simon MT, Pfister A, Vargaftig BB. Eosinophil recruitment into guinea-pig lungs after PAF-acether and allergen administration. Modulation by prostacyclin, platelet depletion and selective antagonists. *Am Rev Respir Dis* 1988;137:948-54.
6. Nakajima H, Iwamoto I, Tomoe S, et al. CD4+ T-lymphocytes and Interleukin-5 mediate antigen-induced blood eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* 1992;146:374-7.
7. Pretolani M, Ruffe C, Joseph D, et al. Role of eosinophil activation in the bronchial reactivity of allergic guinea-pigs. *Am J Respir Crit Care Med* 1994;149:1167-74.
8. Dent LA, Strath M, Mellot AL, Sanderson CJ. Eosinophils in transgenic mice expressing interleukin 5. *J Exp Med* 1990;172:1425-31.

9. Zucker-Franklin D, Grusky G. The identification of eosinophil colonies in soft-agar cultures by differential staining for peroxidase. *J Histochem Cytochem* 1976;24:1270-2.
10. Ten RM, Pease LR, McKean DJ, Bell MP, Gleich GL. Molecular cloning of the eosinophil peroxidase. Evidence for the existence of a peroxidase multigene family. *J Exp Med* 1989;169:1757-69.
11. Gleich GJ, Frigas F, Loegering DA, Wasson DL, Steinmuller D. Cytotoxic properties of the eosinophil major basic protein. *J Immunol* 1979;123:2925-7.
12. Frigas F, Loegering DA, Gleich GJ. Cytotoxic effects of guinea-pig eosinophil major basic protein on tracheal epithelium. *Lab Invest* 1980;42:35-43.
13. Larsen GL, Renz H, Loader JE, Bradley KL, Gelfand EW. Airway response to electrical field stimulation in sensitized inbred mice. *J Clin Invest* 1992;89:747-52.
14. Renz H, Smith HR, Henson JR, Ray BS, Irvin CG, Gelfand EW. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J ALLERGY CLIN IMMUNOL* 1992;89:1127-38.
15. Renz H, Bradley K, Saloga J, Loader J, Larsen GL, Gelfand W. T cells expressing specific V β elements regulate immunoglobulin E production and airways responsiveness in vitro. *J Exp Med* 1993;177:1175-80.
16. Saloga J, Renz H, Larsen G, Gelfand EW. Increased airways responsiveness in mice depends on local challenge with antigen. *Am J Respir Crit Care Med* 1994;149:65-70.
17. Garssen J, van Loveren H, van der Vliet H, Bot H, Nijkamp FP. T-cell-mediated induction of airway hyperreactivity and altered lung functions in mice are independent of increased permeability and mononuclear cell infiltration in mice. *Am Rev Respir Dis* 1993;147:307-13.
18. Levitt RC, Mitzner W. Expression of airway hyperreactivity to acetylcholine as a simple autosomal recessive trait in mice. *FASEB J* 1988;2:2605-8.
19. Laitinen LA, Laitinen A, Heino M, Haahtela T. Eosinophilic airway inflammation during exacerbation of asthma and its treatment with inhaled corticosteroid. *Am Rev Respir Dis* 1991; 143:423-7.
20. Garssen J, Nijkamp FP, Van Der Vliet H, Van Loveren H. T-cell-mediated induction of airway hyperreactivity in mice. *Am Rev Respir Dis* 1991;144:931-8.
21. Martin TR, Tkeishi T, Katz HR, Austen KF, Drazen JM, Galli SJ. Mast cell activation enhances airway responsiveness to methacholine in the mice. *J Clin Invest* 1993;91: 1176-82.
22. Broide DH, Paine MM, Firestein GS. Eosinophils express interleukin-5 and granulocyte-macrophage-colony-stimulating factor messenger RNA at sites of allergic inflammation in asthmatics. *J Clin Invest* 1992;90:1414-24.
23. Walker C, Virchow JC, Bruijnzeel PL, Blaser K. T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. *J Immunol* 1991;146:1829-35.
24. Higa A, Maruyama H, Abe T, Ohashi M, Nawa Y. Effects of *Toxocara canis* infection on hemopoietic stem cells and hemopoietic factors in mice. *Int Arch Allergy Appl Immunol* 1990;91:239-43.
25. Kiruchi Y, Migita M, Takaki S, Tominaga A, Takatsu K. Biochemical and functional characterization of soluble form of IL-5 receptor α (sIL-5R α). *J Immunol Methods* 1994;167:289-98.
26. Strath M, Dent L, Sanderson C. Infection of IL5 transgenic mice with *Mesocricetus* corti induces very high levels of IL5 but depressed production of eosinophils. *Exp Hematol* 1992;20:229-34.
27. Lapa e Silva JR, Bachelet C-M, Pretolani M, Vargaftig BB. Immunopathological alterations in the bronchi of immunized guinea-pigs. *Am J Respir Cell Mol Biol* 1993;9:44-53.
28. Amorim C, Leduc D, Vargaftig BB, Pretolani M. Modulation by rm interferon γ and CD4+ T-lymphocytes of allergic eosinophil accumulation in the mice peritoneal cavity. *Ann NY Acad Sci* 1994;725:34-43.
29. Corris PA, Dark JH. Aetiology of asthma: lessons from lung transplantation. *Lancet* 1993;341:1369-77.
30. Samter M, Kofoed MA, Pieper W. A factor in lungs of anaphylactically shocked guinea-pigs which can induce eosinophilia in normal animals. *Blood* 1953;8:1078-90.
31. Takamoto M, Sugane K. Mechanisms of eosinophilia in *Toxocara canis* infected mice: in vitro production of interleukin 5 by lung cells of both normal and congenitally athymic nude mice. *Immunology* 1993;15:493-500.
32. Plaut M, Pierce JH, Watson CJ, Hanley-Hyde J, Nordan RP, Paul WE. Mast cell lines produce lymphokines in response to cross-linkage of Fc ϵ RI or to calcium ionophores. *Nature* 1989;339:64-7.
33. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. *Trends Pharmacol Sci* 1993;14:436-40.
34. Wang JM, Denis M, Fournier M, Laviolette M. Cyclosporin A increases the pulmonary eosinophilia induced by inhaled *Aspergillus* antigen. *Clin Exp Immunol* 1993;93:323-30.
35. Wallen N, Kita H, Weiler D, Gleich GJ. Glucocorticoids inhibit cytokine-mediated eosinophil survival. *J Immunol* 1991;147:3490-5.
36. Hudson G. Eosinophil granulocyte reactions. In: Yoffey JM, ed. *Bone marrow reactions*. London: Edward Arnold, 1966:86-104.
37. Issekutz TB. Effects of anti-inflammatory agents on lymphocyte migration stimulated by the interferons, tumor necrosis factor and cutaneous inflammation. *Int J Immunopharmacol* 1989;11:725-32.
38. Altman LC, Hill JS, Hairfield WM, Mullarkey MF. Effects of corticosteroids on eosinophil chemotaxis and adherence. *J Clin Invest* 1981;67:28-36.