

Antiinterleukin-5 Antibody Prevents Airway Hyperresponsiveness in a Murine Model of Airway Sensitization

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Eosinophils play a central role in the inflammatory response associated with bronchial asthma. We studied the involvement of eosinophils in the development of airway hyperresponsiveness (AHR) in a mouse model of allergic airway sensitization. Sensitization of BALB/c mice to OVA via the airways induced allergen-specific T-cell responses, IgE production, immediate cutaneous hypersensitivity (ICH), and increased airway reactivity. Airway sensitization was associated with eosinophil infiltration of the airways and increased production of interleukin-5 (IL-5) in cultures of peribronchial lymph node cells. Treatment of OVA-challenged animals with anti-IL-5 antibody during the sensitization protocol completely abolished the infiltration of eosinophils into the lung tissue and prevented the development of AHR without affecting levels of allergen-specific IgE, cutaneous hypersensitivity and allergen-specific T cell responses. These findings demonstrate that infiltration of lung tissue by eosinophils, triggered by increased IL-5 production, is a major factor in the development of AHR in this mouse model of airway sensitization. **Hamelmann E, Oshiba A, Loader J, Larsen GL, Gleich G, Lee J, and Gelfand EW. Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization.**

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Airway hyperresponsiveness (AHR) is a cardinal feature of bronchial asthma and allergic airway sensitization (1). The pathology of AHR is complex, and many factors contribute to its development. There is increasing evidence for the pivotal role of eosinophil infiltration of lung tissues in the development of increased airway reactivity (AR) (2). The infiltration of eosinophils has been linked to the production of interleukin 5 (IL-5), the major maturation factor for eosinophils in humans and animals (3-5). To directly investigate the role of eosinophils and IL-5 in the development of AHR, animal models are necessary. In guinea pig and monkey models of AHR, treatment with monoclonal antibody against IL-5 blocked the infiltration of eosinophils into the lungs and prevented the development of AHR after allergen challenge (6-8). In contrast to these findings, the role of IL-5 in mouse models of AHR remains controversial. To date, two studies evaluating the effects of anti-IL-5 antibody treatment on the development of AHR in normal mice (BALB/c) have been reported (9, 10). Surprisingly, both studies show that antibody treatment inhibited the allergen-induced increase of eosinophils

in the lung but had no effect on the development of AHR (9, 10), suggesting that AHR may develop independently of eosinophil infiltration of lung tissue in allergen sensitized BALB/c mice. In contrast, genetically IL-5 deficient C57BL/6 (B6) mice were unable to develop bronchial eosinophilia or AHR after systemic sensitization and airway challenge (11), indicating a pivotal role for IL-5-induced eosinophilia in these mice. This controversy may reflect the different immunologic backgrounds of the BALB/c and B6 mice, the former depending on IgE-mediated mast cell activation and the latter on IL-5-triggered eosinophil infiltration (12).

The purpose of the present study was to clarify the role of eosinophils in the development of AHR in BALB/c mice and to define the effects of anti-IL-5 antibody treatment on immunologic reactivity after allergic sensitization (immunoglobulin production, immediate cutaneous hypersensitivity, T-cell responsiveness, cytokine production). In the present model, BALB/c mice are sensitized exclusively via the airways after 10 d of airway exposure to ovalbumin (OVA), mimicking a more "natural" mode of allergic sensitization to aeroallergens (13). Airway sensitization in this way induces allergen-specific IgE (13) and T-cell activation (14), associated with immediate cutaneous hypersensitivity (15) and increased airway reactivity (16). Here we show for the first time that sensitization of BALB/c mice via the airways leads to significant eosinophil infiltration of lung tissue and increased local production of IL-5 during the development of AHR. Treatment with anti-IL-5 antibody during sensitization completely blocked eosinophil mobilization from the bone marrow and accumulation in lung tissue and prevented the development of AHR without altering T- and B-cell responses.

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METHODS

Animals

Female BALB/c mice from 8 to 12 wk of age were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Center for Immunology and Respiratory Medicine.

Anti-IL-5 Antibody

Anti-IL-5 antibody was purified from supernates of hybridoma cells (TRFK-5) (17) by protein G separation. Antibody was resuspended in PBS at a concentration of 1 mg/ml and stored at -70°C .

Airway Sensitization and Antibody Treatment

Mice (three to four mice/group/experiment) were sensitized via the airways by nebulization of OVA (Sigma; 1% in PBS) on 10 consecutive days for 20 min daily as described (13). Control animals were exposed to nebulized PBS following the same protocol. Mice in one treatment group received 100 μg of anti-IL-5 antibody (TRFK-5) in 200 μl PBS intravenously on Days 4, 6, and 8 of the sensitization protocol. This treatment protocol was shown to be most effective in preventing pulmonary eosinophilia in preliminary experiments. Allergen-specific IgE production and T-cell responses began to develop on Day 4 of the sensitization protocol. Age-matched control mice received similar amounts of a control rat IgG (Sigma) following the same protocol. There were no differences between OVA-sensitized mice with or without rat IgG treatment in any parameter tested. AR was assessed on Day 12, 2 d after the last nebulization, and the mice were killed to obtain tissues and cells for further assays.

Measurement of Anti-OVA Antibody and Total Ig Levels

Anti-OVA Ig serum levels were measured by ELISA as previously described (13). The antibody titers of the samples were related to pooled standards that were generated in the laboratory. Total IgE levels were determined using the same method as previously described (13). Total Ig levels were calculated by comparison with known mouse IgE standards (PharMingen, San Diego, CA). The limit of detection was 100 pg/ml for IgE.

Immediate Cutaneous Hypersensitivity

Development of immediate cutaneous hypersensitivity to OVA was assessed by intradermal skin testing 1 d after the last OVA challenge (15). Test solutions (20 μl) were injected intradermally on the shaved abdomen of the mice. Test solutions were OVA (500 $\mu\text{g}/\text{ml}$ in PBS) and PBS as the negative control. Subsequent wheal reactions were measured by an investigator unaware of the sensitization status of the animals after 15 min and scored as positive if the wheal diameter extended 3 mm in any direction.

Cell Preparation and Culture

Peribronchial lymph nodes (PBLN) were harvested, mononuclear cells were purified by passing the tissue through stainless steel mesh, followed by density gradient centrifugation (Organon Teknika, Durham, NC). The cells were washed three times in PBS and resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY). Cells were plated in 96-well round-bottom plates at 200,000 cells/well and cultured in the presence or absence of OVA and nitrogen at 37°C for 48 h. Cell-free supernates were harvested and stored at -20°C .

Proliferation Assay

PBLN MNC were cultured in 96-well round-bottom plates at 200,000 cells/well in the presence or absence of OVA and mitogen: 10 nM phorbol 12,13-dibutyrate plus 0.5 μM ionomycin (P/I) (Sigma Chemical, St. Louis, MO) for 5 d. Incorporation was measured 6 h after addition of 1 μCi of [^3H]thymidine (ICN, Irvine, CA).

IL-5 ELISA

IL-5 levels in the culture supernates of PBLN cells were measured by ELISA as described (17). Briefly, ELISA plates (Immulon, Chantilly,

VA) were coated with anti-IL-5 antibody (TRFK-5 in PBS/3% BSA) and blocked with PBS/1% BSA overnight. Biotinylated anti-IL-5 (TRFK-4; PharMingen) was used as detecting antibody, and the reaction was amplified with horseradish-peroxidase (Sigma). IL-5 levels were calculated by comparison with known IL-5 standards (PharMingen). The limit of detection in the assay system was 4 pg/ml.

Determination of AR

Airway responsiveness was determined *in vitro* as described (16). Briefly, tracheal smooth muscle segments of about 0.5 cm in length were placed in Krebs-Henseleit baths suspended by triangular supports transducing the force of contractions. Electrical field stimulation was delivered with increasing frequencies until peak contractile responses were reached. ES_{50} , the frequency leading to 50% of maximal contractions, was calculated from linear plots and compared for the different treatment groups.

Isolation of Lung Cells

Lung cells were isolated as previously described (18). Lungs were perfused with warmed (37°C) calcium- and magnesium-free HBSS containing 10% FCS, 0.6 mM EDTA, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin via the right ventricle at a rate of 4 ml/min for 4 min. Lungs were removed and cut into 300- μm pieces. Four ml of HBSS containing 175 U/ml collagenase (type IA; Sigma), 10% FCS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin were added to the minced lungs and incubated for 60 min in an orbital shaker at 37°C . The digested lungs were sheared with a sterile 20-gauge needle and filtered through 45- and 15- μm filters. Filters were washed with HBSS/2% FCS (45 μm : $1 \times 10\text{ ml}$; 15 μm : $2 \times 10\text{ ml}$). Cells were resuspended in HBSS and counted with a hemocytometer, and cytospin slides were prepared. Slides were stained with Leukostat (Fisher Diagnostics, Fair Lawn, NJ), and cell differentiation percentages were determined by counting at least 300 cells using light microscopy. Remaining cells were pelleted, resuspended in distilled water containing 1% NP-40 (Sigma) at 2×10^6 cells/ml, and samples stored at -20°C for measurement of EPO activity.

Determination of Eosinophil Peroxidase (EPO) Activity

Levels of EPO in pellets from lung cells were determined as previously described (19). Briefly, 100 μl of the substrate solution (0.1 mM *o*-phenylene-diamine-dihydrochloride, 0.1% Triton X-100, 1 mM hydrogen peroxide in 0.05 M TRIS-HCl; all reagents from Sigma) were added to 100 μl of sample in microtiter plates and incubated for 30 min at 37°C . The reaction was stopped by adding 50 μl of 4 M sulfuric acid, and the optical densities were read in a microtiter autoreader at 492 nm. This method has been shown to be specific for the peroxidase activity of eosinophils and not of neutrophils (19).

Immunohistochemistry

After perfusion via the right ventricle, lungs were inflated through the tracheas with 2 ml and fixed in 10% formalin. Major basic protein (MBP) in lung sections was localized as described (20). Blocks of the left lung tissue were cut around the main bronchus and embedded in paraffin blocks. Tissue sections 5 μm thick were affixed to microscope slides, deparaffinized, and incubated in normal rabbit serum for 2 h at 37°C . The slides were then stained with either rabbit-antimouse MBP or with normal rabbit control serum and incubated overnight at 4°C . After washing and incubation in 1% chromotrope 2R (Harlesco, Gibbstown, NJ) for 30 min, the slides were placed in fluorescein-labeled goat antirabbit IgG for 30 min at 37°C . The slides were examined in a blinded fashion with a Zeiss microscope equipped with a fluorescein filter system. Numbers of eosinophils in the submucosal tissue around central airways were evaluated using the IPlab2 software (Signal Analytics, Vienna, VA) for the Macintosh counting four different sections per animal.

Determination of Eosinophil Numbers in the Bone Marrow

Bone marrow cells were collected from the right femur of each animal. Cytospins were prepared, and slides were stained with Leukostat (Fisher Diagnostics). Cell differentiation percentages were determined by counting at least 300 cells using light microscopy by a person unaware of the experiment. Eosinophils were defined as all cells containing eosinophilic granules.

TABLE 1
OVA-SPECIFIC ANTIBODY LEVELS IN THE SERUM*

Sensitization	Treatment	OVA-specific Antibody Levels (EU/ml)	
		IgE	IgG1
PBS	None	< 10	< 10
OVA ×10	Rat IgG	45 ± 33 [†]	1,260 ± 430 [†]
OVA ×10	TRFK-5	60 ± 45 [†]	1,090 ± 350 [†]

* Serum titers for OVA-specific antibodies were determined by ELISA in mice sham-sensitized with PBS (PBS, n = 8) or sensitized with OVA and treated with either rat IgG (n = 12) or with TRFK-5 (n = 12) antibody. Presented are the means ± SD (in ELISA units/ml) from three independent experiments.

[†] Significant (p < 0.05) differences compared with PBS group.

Statistical Analysis

Analysis of variance was used to determine the levels of difference between all groups. Pairs of groups were compared by Student's *t* test. Comparisons for all pairs were performed by the Tukey-Kramer HSD test for airway responsiveness and histology data; *p* values for significance were set to 0.05. Values for all measurements are expressed as the mean ± standard deviation (SD), except values for ES₅₀, which are presented as the mean ± standard error of the mean (SEM).

RESULTS

Total and OVA-specific IgE and IgG Serum Levels after Sensitization

Serum levels of OVA-specific and total immunoglobulins were measured 1 d after completion of the nebulization protocol. Sensitization with OVA via the airways resulted in significantly increased serum levels of anti-OVA IgE and IgG1 in BALB/c mice (Table 1). Sensitization did not significantly alter total IgE and IgG serum levels (data not shown). Treatment of mice with anti-IL-5 antibody on Days 4, 6, and 8 of the protocol did not affect the increases in allergen-specific Ig levels after sensitization.

Immediate Cutaneous Hypersensitivity

To confirm that sensitization to OVA leads to immediate cutaneous hypersensitivity, we performed skin tests 1 d after completion of the sensitization protocol. In parallel to the production of anti-OVA IgE, BALB/c mice developed positive, allergen-specific skin responses to intradermal injections of OVA after sensitization to OVA; no OVA responses were observed in non-sensitized mice exposed to PBS (Table 2). Treatment of mice with anti-IL-5 did not reduce or enhance the development of cutaneous hypersensitivity compared with sensitized animals receiving control rat IgG.

Proliferative Responses to OVA

To assess T-cell proliferative responses to OVA after sensitiza-

TABLE 2
OVA-SPECIFIC IMMEDIATE CUTANEOUS HYPERSENSITIVITY*

Sensitization	Treatment	Mean Wheal Diameter	Positive Responders	
		(mm ± SD)	(n)	(%)
PBS	None	1.9 ± 0.2	0/8	0
OVA ×10	Rat IgG	8.3 ± 0.3 [†]	10/12	83
OVA ×10	TRFK-5	8.0 ± 0.3 [†]	9/12	75

* Cutaneous reactivity to OVA was determined in groups of mice sensitized with PBS or OVA and treated with rat IgG or TRFK-5 antibodies three times during sensitization. Skin tests were performed 1 d after completion of airway challenge. After intradermal injection of OVA, the size of the wheal response was assessed 15 min later. A positive test response was defined by active development of a wheal of ≥ 3 mm in diameter. Compared are the results from three independent experiments.

[†] p < 0.01 for wheal diameter compared with PBS sham-sensitized animals.

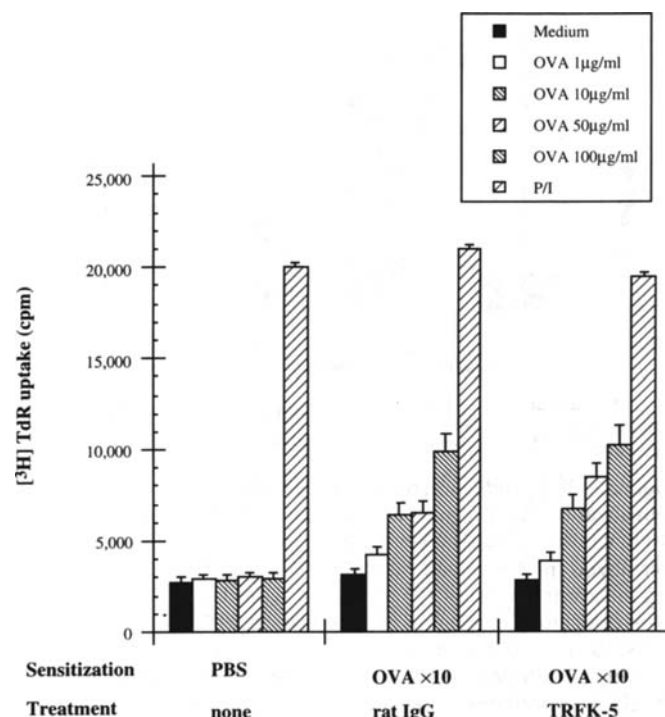


Figure 1. Proliferative responses of peribronchial lymph node cells. BALB/c mice were sensitized to OVA via the airways using the 10-d sensitization protocol. Control mice were nebulized with PBS (n = 8). OVA-sensitized mice received rat IgG (n = 12) or TRFK-5 antibody (100 µg intravenously) (n = 12) on Days 4, 6, and 8 of the 10-d protocol. Two days after completion of sensitization, PBLN MNC (2 × 10⁵ per well) were cultured in triplicate in the absence or presence of OVA or mitogen (phorbol 12,13-dibutyrate/ionomycin) for 5 d. Thymidine uptake was measured after pulsing the cells with 1 µCi [³H]TdR for 6 h. Expressed are the mean cpm ± SD of one of three representative experiments.

tion, MNC were cultured for 5 d in the absence or presence of OVA or mitogen. Sensitization to OVA via the airways resulted in dose-dependent OVA-specific responses in mononuclear cells prepared from peribronchial lymph nodes (PBLN) (Figure 1) as well as spleen (data not shown). Responses to mitogen were similar for sensitized animals and (PBS-sensitized) control animals. Treatment of mice with anti-IL-5 antibody during the period of OVA-sensitization did not affect OVA-specific responsiveness or proliferation to mitogens.

IL-5 Production by Local Lymph Node Cells

IL-5 production by PBLN cells, cultured for 48 h in the absence or presence of OVA or mitogen (phorbol/ionomycin), was measured in the supernates by ELISA. Sensitization via the airways significantly enhanced *in vitro* IL-5 production by PBLN compared with nonsensitized animals (Figure 2). Increases in IL-5 production were observed after stimulation with both mitogen and OVA. Treatment of mice with anti-IL-5 antibody did not significantly alter the capacity of PBLN cells to produce IL-5.

Airway Reactivity

To assess the effect of airway sensitization to OVA on the development of AHR, we measured the reactivity of tracheal smooth muscle segments to electrical field stimulation. We compared the electrical frequencies that resulted in 50% maximal contraction, the ES₅₀ value in Hz. Non-(PBS-) sensitized control animals had

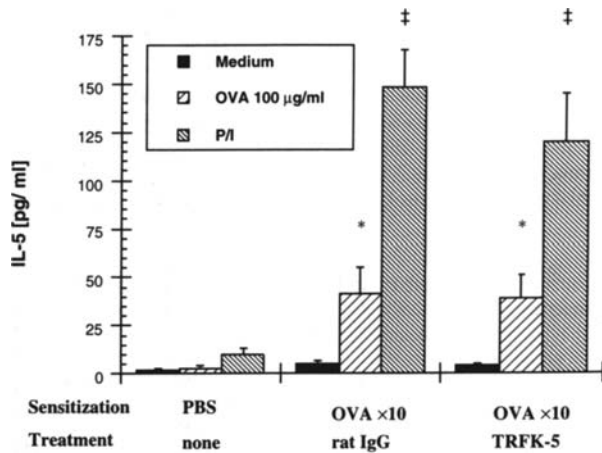


Figure 2. IL-5 production by peribronchial lymph node cells after OVA sensitization. BALB/c mice were sensitized to OVA via the airways for 10 d. Control animals were exposed to nebulized PBS ($n = 8$). OVA-sensitized mice received rat IgG ($n = 12$) or TRFK-5 antibody (100 µg intravenously) ($n = 12$) on Days 4, 6, and 8 of the 10-d protocol. Two days after completion of sensitization, PBLN cells were cultured (2×10^5 cells per well) in the absence or presence of OVA (100 µg/ml) or mitogen (PDB 10 µM, ionomycin 0.5 µM) for 24 h. IL-5 levels in supernates were determined by ELISA. Expressed are the mean $\pm$ SD of IL-5 (pg/ml) from three independent experiments: $p < 0.05$ compared with PBS controls after OVA- (*) or mitogen (†) stimulation (Tukey-Kramer HSD).

mean ($\pm$ SEM) ES_{50} levels of 3.7 ± 0.3 Hz. Sensitization of BALB/c mice via the airways on 10 consecutive days significantly increased airway reactivity, resulting in ES_{50} levels of 2.1 ± 0.4 Hz ($p < 0.04$ compared with PBS control animals). Treatment with rat IgG did not affect AHR as untreated mice sensitized

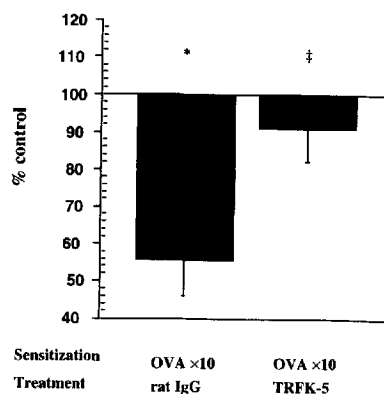


Figure 3. Airway sensitization to OVA induces and treatment with anti-IL-5 antibody prevents the development of airway hyperresponsiveness. BALB/c mice were sensitized to OVA via the airways for 10 d. Control animals were exposed to nebulized PBS ($n = 8$). OVA-sensitized mice received rat IgG ($n = 12$) or TRFK-5 antibody (100 µg intravenously) ($n = 12$) on Days 4, 6, and 8 of the 10-d protocol. Two days after the last challenge, *in vitro* airway reactivity was measured by electrical field stimulation of tracheal smooth muscle segments. Compared are the frequencies leading to 50% maximal contraction, the $ES_{50} \pm$ SEM in Hz, from three independent experiments, as a percent of control ES_{50} value (100%) in non-(PBS-)sensitized animals (3.8 ± 0.2 Hz). * $p < 0.04$ versus PBS; † $p < 0.02$ versus 10-d OVA/rat IgG. OVA-sensitized animals without rat IgG treatment had ES_{50} values of 2.2 ± 0.2 Hz, similar to rat IgG-treated animals; (2.2 ± 0.4 Hz).

to OVA had similar ES_{50} levels of 2.2 ± 0.2 Hz (Figure 3). Treatment of OVA sensitized mice with anti-IL-5 antibody on Days 4, 6, and 8 of the 10-d protocol prevented the development of AHR (ES_{50} , 3.5 ± 0.3 Hz; $p < 0.02$ compared with OVA-sensitized animals receiving rat control IgG).

Lung Cell Composition after Airway Sensitization

Total leukocyte and differential counts in isolated lung cells of individual mice were compared for the following groups: non-(PBS-)sensitized mice, OVA-sensitized mice receiving control rat IgG, and OVA-sensitized mice receiving anti-IL-5 antibodies on Days 4, 6, and 8 of the 10-d sensitization protocol. The absolute numbers of leukocytes, macrophages/monocytes, lymphocytes and neutrophils were essentially the same in OVA-sensitized animals and nonsensitized mice (Figure 4). Numbers of eosinophils were significantly increased in OVA-sensitized mice by more than 5-fold. Treatment with anti-IL-5 antibody during OVA-sensitization did not significantly reduce the total number of leukocytes or numbers of macrophages, lymphocytes, and neutrophils, but significantly reduced eosinophil numbers after allergen-challenge to background levels (Figure 4). Numbers of eosinophils in the lungs after OVA sensitization correlated well with AHR as measured with EFS (Figure 5).

EPO Activity in Lung Cells

Activity of EPO in lysed pellets of lung cells of individual lungs were measured by ELISA for the different groups. In parallel to the increase in eosinophil numbers, sensitization to OVA via the airways significantly increased EPO activity in lung cells compared with non-(PBS-)sensitized animals (Figure 6). Concordant with the reduction in eosinophil numbers, treatment of mice with anti-IL-5 antibody inhibited the increase in EPO activity after airway sensitization with allergen.

Anti-IL-5 Antibody Inhibits Eosinophil Accumulation in the Lung

Formalin-fixed, paraffin-embedded lung sections were stained with rabbit-anti-MBP antibody to localize and quantitate numbers of eosinophils. Sensitization to OVA via the airways signifi-

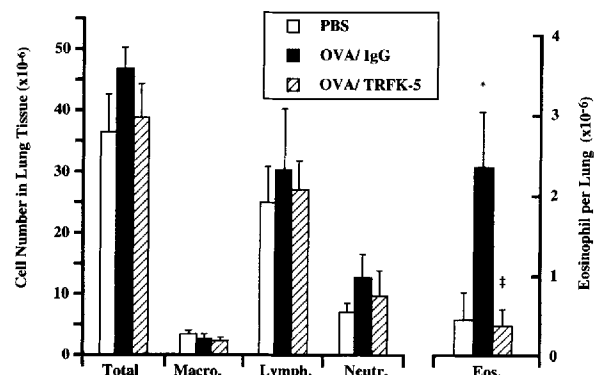


Figure 4. Airway sensitization to OVA increases and treatment with anti-IL-5 antibody prevents eosinophil infiltration of young cells. BALB/c mice were sensitized to OVA via the airways for 10 d. Control animals were exposed to nebulized PBS ($n = 8$). OVA-sensitized mice received rat IgG ($n = 12$) or TRFK-5 antibody (100 µg intravenously) ($n = 12$) on Days 4, 6, and 8 of the 10-d protocol. Relative numbers of different types of leukocytes isolated by lung digestion were determined by light microscopy. Expressed are the mean $\pm$ SD of the percentage of the different cell types. * $p < 0.001$ versus PBS; † $p < 0.007$ versus 10-d OVA/rat IgG.

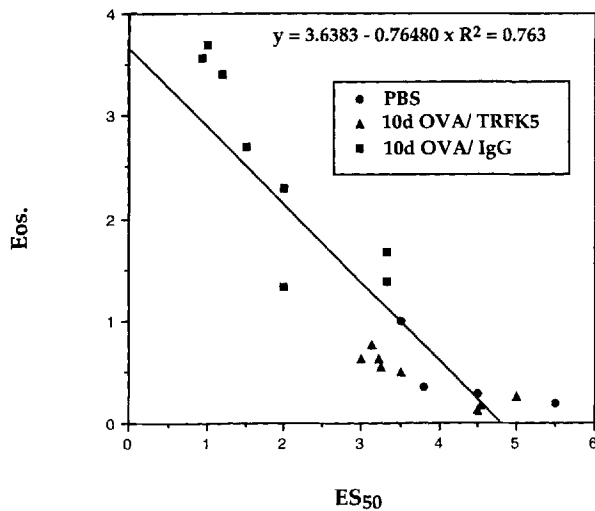


Figure 5. Correlation between eosinophil numbers in the lungs and AHR. Numbers of eosinophils ($\times 10^3$) per lung were correlated with ES_{50} values for individual mice from two independent experiments. Compared are PBS-nebulized ($n = 4$), OVA-sensitized mice receiving rat IgG ($n = 8$), and OVA-sensitized mice receiving TRFK-5 antibody ($n = 8$) on Days 4, 6, and 8 of the 10-d protocol.

cantly increased the numbers of eosinophils per area in the peribronchial tissue (Figure 6); nonsensitized animals showed very few eosinophils in these sites. Treatment of mice with anti-IL-5 antibody on Days 4, 6, and 8 of the 10-d sensitization protocol completely abrogated the infiltration of eosinophils into the peribronchial tissue after airway sensitization with allergen (Figure 7). Eosinophils were reduced by five-fold compared with mice receiving control rat IgG and were virtually absent in the tissue after anti-IL-5 treatment.

Numbers of eosinophils in the bone marrow were compared after completion of sensitization in the different groups. Airway sensitization increased the number of eosinophils in the bone marrow: numbers of eosinophils per femur increased by more

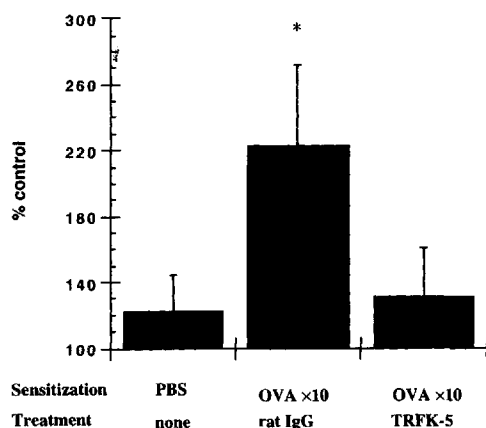


Figure 6. Levels of eosinophil peroxidase in lung cells after sensitization to OVA. BALB/c mice were sensitized to OVA via the airways for 10 d. Control animals were exposed to nebulized PBS ($n = 8$). OVA-sensitized mice received rat IgG ($n = 12$) or TRFK-5 antibody ($100 \mu\text{g}$ intravenously) ($n = 12$) on Days 4, 6, and 8 of the 10-d protocol. EPO levels of lysed lung cells were determined as described in METHODS. Expressed are the mean $\pm$ SD of the optical densities from three independent experiments as a percent of control baseline readings from wells containing $100 \mu\text{l}$ HBSS and $100 \mu\text{l}$ substrate buffer (0.18 ± 0.02). * $p < 0.05$ versus PBS or 10-d OVA/TRFK-5.

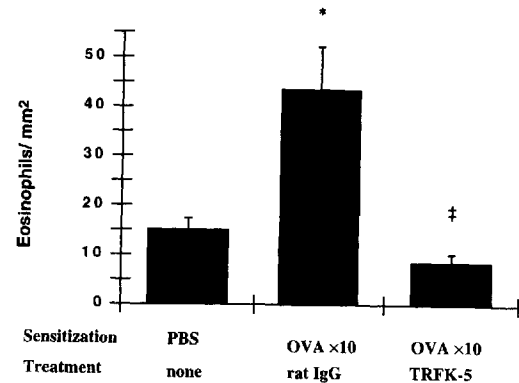


Figure 7. Localization of eosinophils in peribronchial tissue after airway sensitization with OVA. BALB/c mice were sensitized to OVA for 10 d. Control animals were exposed to nebulized PBS ($n = 8$). OVA-sensitized mice received rat IgG ($n = 12$) or TRFK-5 antibody ($100 \mu\text{g}$ intravenously) ($n = 12$) on Days 4, 6, and 8 of the 10-d protocol. Sections of peribronchial tissue were stained with rabbit antimouse MBP antibodies. Four sections around similar-sized central airways were chosen randomly, and eosinophils in the connecting tissue were counted in a blinded fashion. Expressed are the mean $\pm$ SD numbers of eosinophils per mm^2 lung tissue from three independent experiments. * $p < 0.01$ versus PBS; † $p < 0.005$ versus 10-d OVA/rat IgG.

than two-fold, from $0.47 \pm 0.05 \times 10^6$ to $0.99 \pm 0.04 \times 10^6$ ($p < 0.01$), with no significant changes in total cell count ($10.4 \pm 0.5 \times 10^6$ in sensitized versus $9.3 \pm 0.8 \times 10^6$ bone marrow cells/femur in nonsensitized mice). Treatment of mice with anti-IL-5 antibody during allergic airway sensitization inhibited this increase ($0.34 \pm 0.07 \times 10^6$ eosinophils per femur; $p < 0.01$ compared with untreated and sensitized mice) without significantly affecting total cell counts ($9.4 \pm 0.7 \times 10^6$ cells/femur). These data indicate that after airway sensitization, eosinophil numbers in the bone marrow increase as well as in the lung tissue, and that these increases are inhibited by anti-IL-5 antibody treatment during sensitization.

DISCUSSION

After airway sensitization, increased airway responsiveness (AR) is accompanied by eosinophil infiltration of lung tissue (21, 22). For the induction of this inflammatory response, the production of T_H2 -type cytokines by lymphocytes, mast cells, eosinophils, and other cells appears critical (2, 23). Among these, IL-5 has been defined as the main promoting factor for eosinophil accumulation (24). In the present study, we determined the importance of local IL-5 production and eosinophil infiltration for the development of AHR in a mouse model of airway sensitization and examined the effects of anti-IL-5 antibody treatment on immunologic reactivity after sensitization. BALB/c mice were sensitized exclusively via the airways and responded with allergen-specific IgE production, T-cell responsiveness, development of cutaneous hypersensitivity, and increased AR. The development of AHR after airway sensitization (in the absence of adjuvant) was accompanied by increased IL-5 production by peribronchial lymph node cells, the local draining lymph nodes of the airways, and significant increases in numbers of eosinophils in the bone marrow and peribronchial regions of the lungs. Treatment of sensitized mice with anti-IL-5 antibody during the course of sensitization blocked eosinophil mobilization from the bone marrow and eosinophil lung infiltration and inhibited the development of AHR without altering B-cell (antibody production), T-cell (cytokine production, proliferative responses), or mast cell function (ICH).

The mechanism underlying the anti-IL-5 activity seems to be the blocking of the maturation and mobilization of eosinophils from the bone marrow since all other immunologic reactions after sensitization were not affected by antibody treatment. The increase in eosinophils observed in the bone marrow differs from the findings published by Kung and colleagues (25), who showed that airway challenge of sensitized mice reduced eosinophil numbers in the bone marrow through an initial "wash-out" effect. This difference may be explained by the repeated airway sensitization in our protocol, leading to a continuous stimulus for maturation, proliferation, and differentiation of eosinophil bone marrow progenitor cells. We previously demonstrated that AHR as measured by electrical field stimulation is caused by increased acetylcholine release (26). Activated eosinophils release cytotoxic cationic proteins such as MBP and EPO (22), which are allosteric antagonists of the M_2 muscarinic receptor (27). In guinea pigs, allergen-challenge of sensitized animals results in a dysfunction of the inhibitory M_2 receptors, thus causing increased acetylcholine release and AHR, and pretreatment with anti-IL-5 antibody prior to allergen challenge prevented the loss of M_2 receptor function (28). The inhibition of AHR by anti-IL-5 antibody as shown in the present study may reflect the same underlying mechanisms.

The importance of IL-5 for eosinophil accumulation in the lungs and AHR has been suggested in other animal models of AHR (6–8). Mouse models of allergic sensitization and inflammation have the unparalleled advantages of well-characterized murine immunology and the availability of genetically altered mouse strains and immunologic tools. In contrast to studies in guinea pigs and monkeys (6–8), the findings in mouse models of AHR are controversial. In the recent report by Foster and colleagues (11), genetically IL-5-deficient B6 mice sensitized by allergen plus adjuvant did not develop increased bronchial eosinophilia and AHR after airway challenge. Two other studies investigated the effect of anti-IL-5 antibody treatment on the development of AHR in allergen-sensitized normal (BALB/c) mice (9, 10). They demonstrated that antibody treatment prior to airway challenge (9) or during a 4-wk sensitization period (10) significantly reduced eosinophil airway infiltration but had no effect on AHR. In a third study, only hyper-IgE mice, but surprisingly not normal BALB/c mice, developed AHR after standard systemic sensitization plus airway challenge, and alterations in AR in the mutant mice were inhibited by anti-IL-5 antibody treatment (29). These seemingly controversial results may reflect differences in the underlying mechanisms leading to the development of AHR in different mouse strains (12). AHR development in BALB/c mice has been proposed to be caused by IgE-mediated mast cell activation; in B6 mice, which are deficient in the tryptase-like mast cell protease 7 (30) and in the secretory phospholipase A_2 (31), eosinophil infiltration might be the precipitating event. Our data from the present study demonstrate for the first time that in BALB/c mice, IL-5-mediated eosinophil infiltration of lung tissue is a major requirement for the development of AHR.

In the present study, three injections of anti-IL-5 antibody (150 μ g) were administered during airway sensitization, and this regimen resulted in a reduction of the pulmonary eosinophilia to background levels. The degree of pulmonary eosinophilia was assessed using three independent measurements: analysis of dispersed lung cells, eosinophil peroxidase (EPO)-activity of lung cell pellets, and *in situ* staining of eosinophils with an antibody specific for mouse MBP. This enabled us not only to quantify the number of eosinophils but also to localize the eosinophils to the peribronchial regions. In the report by Corry and coworkers (10), mice were treated with a very low amount of anti-IL-5 antibody (8 μ g total), and only during the 4 wk of sensitization, but not before or during the subsequent airway allergen chal-

lenges. Nagai and coworkers (9) treated systemically sensitized mice with anti-IL-5 antibody shortly before three airway challenges, but eosinophil accumulation was only partially inhibited. That the amount of anti-IL-5 antibody is crucial in dictating the outcome has been shown by Mauser and colleagues (7), who demonstrated that higher amounts of anti-IL-5 antibody prevented AHR, whereas low-dose antibody treatment only inhibited the pulmonary eosinophilia.

On the other hand, pulmonary eosinophilia and the development of AHR might not always correlate but represent two distinct phenomena resulting from allergic sensitization that are not interrelated. There is indeed evidence for a dissociation of airway eosinophilia and AHR (7), and reports of the successful prevention of AHR without affecting eosinophil numbers (32, 33). Intraperitoneal treatment of guinea pigs with IL-5-producing cells resulted in AHR as early as on Day 3 after injection of the cells, whereas measurable increases in eosinophil numbers in BAL fluid or peripheral blood only occurred a week after treatment (6). This suggests that an increase in eosinophil numbers may not be the sole critical factor in the development of AHR. Indeed, the activation of eosinophils by IL-5 in the target tissue may be more critical in the development of increased AR, and data on the activation status of eosinophils are necessary. Measurements of EPO activity only correlated with eosinophil numbers and not necessarily with activation status (34). Future work must delineate if IL-5 is responsible in the pathogenesis of AHR for triggering not only pulmonary eosinophilia but for eosinophil activation in the peribronchial tissue and airways after airway challenge.

We have demonstrated that development of AHR in mice after sensitization exclusively via the airways is accompanied by increased local IL-5 production and eosinophil infiltration of the submucosal tissue around the small and middle-sized airways. We demonstrate that treatment of BALB/c mice with anti-IL-5 antibody during the course of airway sensitization not only inhibited this eosinophil infiltration but moreover prevented the development of AHR. In contrast, anti-IL-5 antibody treatment did not alter T- or B-cell responses to allergen challenge. These findings directly show the important role for IL-5 in the migration of eosinophils to the site of allergen challenge and support approaches interfering with anti-IL-5 antibody in the treatment of allergen-induced AHR.

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