

Allergen-specific IgE and IL-5 Are Essential for the Development of Airway Hyperresponsiveness

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The mechanisms underlying the development of airway hyperresponsiveness are not fully delineated. We addressed this question by studying the effects of passive sensitization with anti-OVA IgE on the development of altered airway responsiveness (AR) following local challenge with OVA in normal and athymic mice. Both normal and athymic BALB/c mice developed allergen-specific immediate cutaneous hypersensitivity after passive sensitization with anti-OVA IgE. In contrast, the combination of local challenge with allergen via the airways and passive sensitization triggered the development of airway hyperresponsiveness only in normal but not in athymic mice. Treatment of athymic mice with IL-5 significantly increased eosinophil accumulation in the lungs after local challenge with OVA; increased airway reactivity was only observed in athymic mice which received anti-OVA IgE, not an unrelated IgE, plus IL-5 treatment and airway challenge with OVA. These findings identify the requirement for allergen-specific IgE and IL-5 for the development of airway hyperresponsiveness following allergen challenge via the airways. **Hamelmann, E., A. Oshiba, J. Schwarze, K. Bradley, J. Loader, G. L. Larsen, and E. W. Gelfand. 1997. Allergen-specific IgE and IL-5 are essential for the development of airway hyperresponsiveness. *Am. J. Respir. Cell Mol. Biol.* 16:674–682.**

The development of airway hyperresponsiveness (AHR) is a central feature of bronchial asthma (1). The mechanisms responsible for the development of AHR are still not completely defined and are complex. In allergic bronchial asthma, the induction of allergen-specific IgE production is believed to be one of the elements contributing to development of increased airway reactivity (2). Elevated levels of IgE are found in atopic patients with bronchial asthma and IgE levels correlate with severity of the disease (3). IgE, bound to its receptors on mast cells and other cells, triggers the release of mediators such as histamine and leukotrienes, which can result in epithelial cell damage (4). In animal models, the requirement for mast cells in the development of AHR has been demonstrated (5).

The importance of an eosinophil inflammatory process in the lower airways has also been highlighted in the development of AHR (6, 7). Patients with bronchial asthma

show increased numbers of eosinophils in bronchoalveolar lavage fluid (BALF) and endobronchial biopsies (8, 9), and the number of eosinophils in the BALF (10) and the peripheral blood (11) correlates with the severity of the airway disease. Correspondingly, in animal models of AHR, local challenge of sensitized animals with allergen results in an eosinophil infiltration of airway tissues (12–14). For the induction of this eosinophil response, interleukin 5 (IL-5) appears to be the main promoting factor. IL-5 enhances the proliferation, migration, activation and survival of eosinophils in humans and animals (15–17). Elevated levels of IL-5 are found in the serum and BALF of patients with bronchial asthma (18), and messenger RNA for the IL-5 gene has been detected in BAL cells and lung biopsies (19). In some animal models, treatment with IL-5 enhances the induction of AHR (20, 21) whereas anti-IL-5 antibody treatment abolishes its development (22, 23).

To date, it has been difficult to dissociate the effects and interrelationships between IL-5 and allergen-specific IgE in triggering AHR. Sensitization to allergen normally evokes a T_H2 -like T-cell response (24), inducing both production of IL-4, which results in IgE production (25), and production of IL-5, which promotes eosinophil accumulation in the lungs (26). B-cell hybridomas producing anti-ovalbumin (OVA) specific antibodies that permitted passive sensitization of the mice were developed. We showed that passive sensitization with anti-OVA antibodies provokes the development of immediate cutaneous hypersen-

(Received in original form July 22, 1996 and in revised form November 18, 1996)

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Abbreviations: airway hyperresponsiveness, AHR; airway responsiveness, AR; bronchoalveolar lavage fluid, BALF; eosinophil peroxidase, EPO; fetal calf serum, FCS; immediate cutaneous hypersensitivity, ICH; immunoglobulin, Ig; interleukin, IL; intravenous, i.v.; mononuclear cells, MNC; ovalbumin, OVA.

sitivity (ICH) and, after at least two local challenges with allergen, AHR and antigen-specific T-cell responses (27). The aim of the present study was to isolate the requirements for T cells, T-cell products and allergen-specific IgE in the development of AHR. Using athymic (nude) mice lacking peripheral T cells and T-cell responses, we were able to analyze for the first time, the individual contributions of T cells, T-cell cytokines, and IgE in the development of AHR. Here, we show that passively sensitized nude mice fail to develop AHR following local challenge with allergen, despite their capacity for allergen-specific ICH. Additional treatment of passively sensitized nude mice with IL-5 prior to allergen challenge is necessary for the accumulation of eosinophils in the peribronchial regions and for the development of AHR, emphasizing the pivotal role of IL-5 in the presence of allergen-specific IgE for the induction of altered airway responsiveness (AR).

Materials and Methods

Animals

Female BALB/c and BALB/c nu/nu 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.

Generation of Anti-OVA Antibody-secreting Hybridoma Cell Lines

Hybridoma cell lines were prepared as previously described (27). Briefly, spleen cells from BALB/c mice immunized with OVA were fused with myeloma cells (myeloma cell line SP2/0; ATCC, Rockville, MD) and hybridoma cells secreting anti-OVA specific antibodies were recloned several times by limiting dilution. OVA-specific subclones from culture plates with cell growth in less than 30% of all wells were recultured for 7–10 days, supernatants were harvested and pooled. Hybridoma cell lines producing monoclonal anti-TNP IgE antibodies (ATCC) were used as a source of control antibodies.

Measurement of Anti-OVA Antibody and Total Ig Levels

Anti-OVA IgE and IgG antibody levels in culture supernatants of hybridomas and serum were measured by ELISA as previously described (28). The antibody titers of the samples were related to pooled standards that were generated in the laboratory. Total IgE and IgG levels were determined using the same method. Total Ig levels were calculated by comparison with known mouse IgE or IgG standards (PharMingen, San Diego, CA). The limit of detection was 100 pg/ml for IgE and 1 ng/ml for IgG.

IL-5 Purification and ELISA

IL-5 was purified from the supernatant of the T_H2-cell line D2 (kindly provided by Dr. R. Coffman, Palo Alto, CA) by affinity chromatography using an anti-IL-5 antibody (TRFK-5, provided by Dr. R. Coffman) on CNBr-activated Sepharose 4B.

IL-5 levels in the supernatant and in the serum were measured by ELISA as described (29). 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 the detecting antibody and the reaction was amplified with horseradish-peroxidase (Sigma, St. Louis, MO). 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.

Cell Preparation

Spleens and peripheral lymph nodes were harvested, mononuclear cells (MNC) 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 with PBS and resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY).

Proliferation Assay

MNC were cultured in 96-well round bottom plates at a concentration of 200,000 cells/well in the presence or absence of different concentrations of OVA and mitogen (concanavalin A 5 µg/ml) for 3 days. Thymidine incorporation was measured 6 h after addition of 1 µCi of [³H]thymidine (ICN, Irvine, CA).

Flow Cytometry

After preincubation with mouse serum, cells were incubated with fluorescein-conjugated anti-CD4, anti-CD8, anti-B220 antibodies (PharMingen) or isotype controls in staining buffer (PBS, 2% FCS, 0.1% sodium azide) for 30 min on ice. Following washing, cells were examined using an EPICS analyzer (Coulter Electronics, Hialeah, FL).

Experimental Design

Mice (3–4 mice/group/experiment) were passively sensitized by intravenous injection of anti-OVA IgE antibodies. Groups of normal or nude mice received intravenous (i.v.) injections on 3 consecutive days with hybridoma supernates containing 0.4 µg anti-OVA IgE in a volume of 200 µl. Age-matched control mice received similar volumes and protein amounts of culture supernates of the hybridoma-secreting anti-TNP IgE following the same protocol. Groups of nude mice were injected i.v. with IL-5 (20 ng per injection) in addition to anti-OVA or anti-TNP IgE on the same days. The passively sensitized mice were exposed to OVA (1% w/v diluted in PBS) by nebulization for 20 min daily, 3 and 4 days after the last injection of antibody. As a positive control group, normal mice were sensitized via the airways using the 10-day nebulization protocol with OVA as previously described (28).

Immediate Cutaneous Hypersensitivity

Development of ICH to OVA was assessed by intradermal skin testing one day after the last OVA challenge as described (30). 20 µl of test solution were injected intradermally on the shaved abdomen of the mice. Test solutions were OVA (500 µg/ml in PBS) and PBS as the negative control. Subsequent wheal reactions were measured by an investigator unaware of the sensitization status of the ani-

mals after 15 min and scored as positive if the wheal diameter exceeded 3 mm in any direction.

Determination of AR

Airway responsiveness was determined *in vitro* as described (31). 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 (EFS) 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. EFS elicits altered neural function of tracheal smooth muscle from sensitized mice due to increased release of acetylcholine as a result of muscarinic (M_2) autoreceptor dysfunction (32).

Lung Cell Isolation

Lung cells were isolated as previously described (33). 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 $\mu\text{g/ml}$ streptomycin via the right ventricle. Lungs were removed and cut into pieces of approximately 300 μm size. Four ml of HBSS containing 175 U/ml collagenase (type IA, Sigma), 10% FCS, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ 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 μm and 15 μm filters. Filters were washed with HBSS/2% FCS. Cells were resuspended in HBSS and counted with a hemocytometer, and cytospin slides were made. Slides were stained with Leukostat (Fisher) and the percentages of different cell types determined by counting at least 300 cells using light microscopy. Remaining cells were pelleted, resuspended in NP-40 (Sigma) 1% in distilled water at 2×10^5 cells/ml and samples were 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 (34). 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 and compared with blanks and negative controls. This method has been shown to be specific for the peroxidase activity of eosinophils and not of neutrophils (34).

Statistical Analysis

Values for all measurements are expressed as the mean $\pm$ standard deviation except values for ES_{50} which are presented as the mean $\pm$ standard error of the mean (SEM). The Tukey-Kramer HSD test was performed to compare multiple experimental groups. Student's two-tailed unpaired *t* test was used to determine the level of difference between two experimental groups.

Results

CD4⁺ and CD8⁺ T Cells Are Absent in the Peripheral Lymphoid Tissues of Athymic Mice

Nude mice fail to develop a functional thymus and thus lack the development and expansion of T cells. To ensure that the nude mice used in our experiments were T-cell deficient in the peripheral lymphoid tissues, we analyzed spleen and peripheral lymph node cells for the presence of CD4⁺ and CD8⁺ T cells. Figure 1 shows that spleen cells of normal BALB/c mice contained an average of $36 \pm 4\%$ B cells, $33 \pm 4\%$ CD4⁺ and $6.5 \pm 1.5\%$ CD8⁺ T cells. In contrast, in spleens of athymic BALB/c nu/nu mice, $68 \pm 8\%$ of the cells stained positive for the B-cell marker B220, whereas only $2.1 \pm 0.4\%$ and $2.3 \pm 1.5\%$ were CD4⁺ or CD8⁺, respectively. The positive staining for the two T-cell markers in athymic mice is likely explained by expression of these markers on cells other than lymphocytes. The results for peripheral lymph node cells of nude mice are similar in that the staining was negative for CD4 and CD8 (data not shown).

Proliferative Responses to Allergen or Mitogen Are Absent in Peripheral Lymphoid Cells of Athymic Mice

Allergen-stimulation leads to the expansion of T cells. To ensure the absence of T-cell responses in the athymic mice used in our experiments, we assessed the proliferative response of spleen cells after local allergen challenge with OVA. Sensitization of normal BALB/c mice via the airways following the 10-day sensitization protocol increased thymidine-incorporation of spleen cells in the presence of allergen by four-fold compared with cells cultured in medium alone (Figure 2). Passive sensitization of normal BALB/c mice with anti-OVA IgE followed by two allergen challenges via the airway failed to significantly increase allergen-specific proliferation above background levels, despite proliferative responses to mitogen (concanavalin A). In contrast, spleen cells of athymic mice pas-

Lymphocyte Staining of Spleen MNC of Normal and Athymic BALB/c Mice

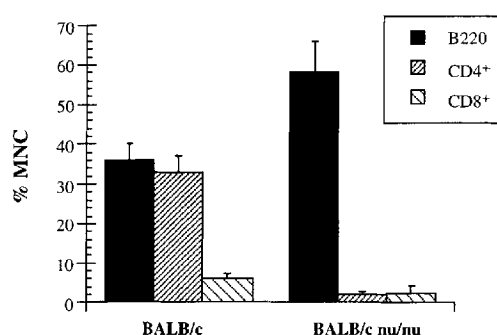


Figure 1. T cells are deficient in spleens of athymic (nude) BALB/c mice. Spleen MNC of normal ($n = 20$) and athymic ($n = 20$) mice were prepared by density gradient centrifugation and stained for the lymphocyte markers B220, CD4 and CD8. Expressed are the percentage $\pm$ SD of positively-stained cells from five independent experiments.

Spleen Cells of Athymic (Nude) Mice do not Proliferate After Antigen or Mitogen Stimulation

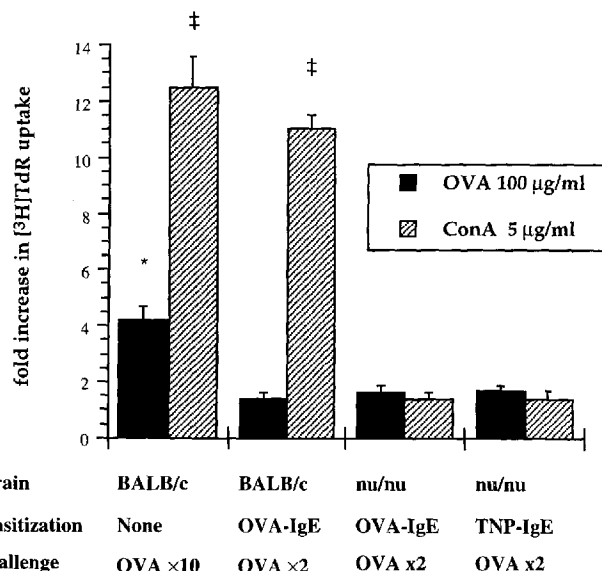


Figure 2. T-cell proliferative responses are absent in spleens of athymic (nude) BALB/c mice. Pooled spleen MNC (200,000 per well) of normal and athymic mice were prepared by density gradient centrifugation and cultured for three days in the presence of medium, OVA (100 µg/ml) or ConA (5 µg/ml). Cells were pulsed with thymidine (1 µCi/well) and harvested after 6 h. Expressed are the means $\pm$ SD of the fold increase in thymidine incorporation compared with non-stimulated conditions (medium alone) of cells from actively sensitized normal BALB/c mice (OVA 10 days; baseline 920 ± 70 cpm), passively sensitized normal BALB/c mice (baseline: 970 ± 52 cpm), and of passively sensitized nude mice receiving anti-OVA (baseline: 890 ± 60 cpm) or anti-TNP IgE (baseline: 850 ± 80 cpm) from three independent experiments ($n = 12$ for each group). * $P < 0.05$ versus the three other groups; † $P < 0.01$ versus the two nu/nu groups.

sively sensitized with anti-OVA IgE or with anti-TNP IgE failed to proliferate in response to allergen or mitogen (Figure 2).

Total and OVA-specific IgE Levels in Serum of Normal and Athymic Mice after Passive Sensitization

Serum levels of OVA-specific and total Ig levels were measured one day after the last nebulization. Passive sensitization with IgE resulted in measurable and similar serum levels of anti-OVA IgE in both normal and athymic BALB/c mice (Table 1). Allergen-specific antibody titers of other isotypes were not affected. Normal and nude mice passively sensitized with anti-OVA IgE showed significantly higher total IgE serum levels than non-sensitized control mice ($P < 0.05$) (Table 1). OVA challenge via the airways (on 2 days) without passive sensitization did not elicit detectable OVA-specific antibodies.

Passive Sensitization Induces Immediate Cutaneous Hypersensitivity in Normal and Athymic Mice

To determine if the injection of anti-OVA IgE antibodies leads to the development of ICH, we performed skin tests

TABLE 1
OVA-specific antibody and total Ig levels in the serum

Passive Sensitization	OVA-specific Antibody Levels (U/ml)			Total IgE Levels (ng/ml)
	IgE	IgG1	IgG	
None	< 10	< 10	< 10	24 $\pm$ 6.0
BALB/c: OVA-IgE	*679 $\pm$ 54	< 10	< 10	†121 $\pm$ 34
nu/nu: OVA-IgE	*434 $\pm$ 76	< 10	< 10	†139 $\pm$ 56

Serum titers for OVA-specific antibodies and total Ig were determined by ELISA in mice: control mice ($n = 8$), normal BALB/c mice passively sensitized with anti-OVA IgE (BALB/c: OVA-IgE, $n = 20$), nude mice passively sensitized with anti-OVA IgE (nude: OVA IgE, $n = 20$). Presented are the means $\pm$ SD.

*† Significant ($P < 0.05$) differences compared with non-sensitized group.

one day after completion of the sensitization or nebulization protocol. Normal and athymic BALB/c mice injected with anti-OVA IgE developed positive, allergen-specific responses to intradermal injection of OVA with or without additional OVA challenge via the airways; no wheal reactions were observed in non-sensitized mice (Table 2). Control animals receiving anti-TNP IgE and two consecutive airway challenges with OVA did not generate any OVA-specific skin test reactivity. Intradermal injection of PBS did not result in wheal formation in any of the mice. This indicates the normal capacity of the athymic mice to mediate mast cell degranulation in the presence of allergen-specific IgE antibodies and allergen.

Passive Sensitization Plus Airway Challenge Induces AHR in Normal but Not in Athymic Mice

To assess the effect of passive sensitization with anti-OVA IgE on the development of AHR after airway challenge with OVA, we measured *in vitro* reactivity of tracheal smooth muscle segments to electrical field stimulation. We monitored the electrical frequencies leading to 50% maximal contractions, the ES_{50} values in Hz. Non-sensitized control BALB/c mice had a mean ($\pm$ SEM) ES_{50} of 3.7 ± 0.2 Hz. Active sensitization of normal BALB/c mice via the airways on 10 consecutive days significantly increased AR and resulted in ES_{50} levels of 2.6 ± 0.3 Hz ($P < 0.01$)

TABLE 2
OVA-specific immediate cutaneous hypersensitivity

Strain	Passive Sensitization	Challenge	Mean wheal Diameter (mm) ($\pm$ SD)	Number of Positive Responders (%)
A. BALB/c	None	None	1.9 $\pm$ 0.3	0/8 (0)
	TNP-IgE	OVA $\times$ 2	2.2 $\pm$ 0.4	1/8 (12.5)
	OVA-IgE	OVA $\times$ 2	*8.4 $\pm$ 0.9	17/19 (89)
B. nu/nu	TNP-IgE	OVA $\times$ 2	2.5 $\pm$ 0.5	1/8 (12.5)
	OVA-IgE	None	*7.6 $\pm$ 0.5	8/8 (100)
	OVA-IgE	OVA $\times$ 2	*6.6 $\pm$ 0.4	15/19 (79)

Cutaneous reactivity to OVA was determined in groups of mice receiving passive sensitization with specific IgE antibodies. Skin tests were performed 1 day after completion of airway challenge. Following 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 ≥ 3 mm in diameter. Compared are the results from five independent experiments.

* $P < 0.01$ for wheal diameter compared with anti-TNP IgE sensitized animals.

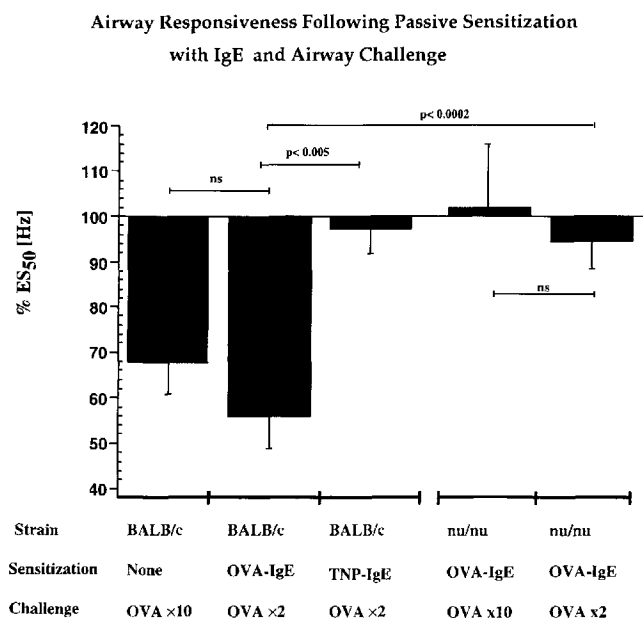


Figure 3. Passive sensitization plus allergen challenge triggers the development of AHR in normal but not in athymic mice. Positive controls were sensitized using the 10-day nebulization protocol (BALB/c: OVA $\times$ 10, $n = 14$). Normal BALB/c mice received anti-OVA IgE ($n = 19$) or anti-TNP IgE ($n = 8$) on days 1 to 3 of the protocol and were challenged with OVA via the airways on days 6 and 7 (BALB/c: OVA-IgE or TNP-IgE/OVA $\times$ 2). Nude mice were passively sensitized with anti-OVA IgE and challenged with OVA via the airways two ($n = 19$) or ten ($n = 9$) times (nu/nu: OVA-IgE/OVA $\times$ 2 or OVA $\times$ 10). One day after the last challenge, *in vitro* airway responsiveness was measured by electrical field stimulation of tracheal smooth muscle segments. The frequencies leading to 50% maximal contraction, $ES_{50} \pm SEM$ in Hz, were compared with non-sensitized, non-challenged mice where ES_{50} values were 3.7 ± 0.2 Hz and taken as 100%. Expressed are the data from five independent experiments. (P values as indicated on the graph.)

(Figure 3). Similarly, in normal BALB/c mice passively sensitized with anti-OVA IgE antibody, airway challenge with OVA on two consecutive days resulted in a significant decrease in the ES_{50} values (2.0 ± 0.2 Hz, $P < 0.01$, compared with non-sensitized controls). Injection of anti-OVA IgE in the absence of airway challenge did not alter AR (data not shown). Control mice receiving anti-TNP IgE injections and two challenges with OVA failed to develop AHR (ES_{50} 3.6 ± 0.2 Hz, $P < 0.01$ compared with anti-OVA IgE injected animals), indicating the allergen-specificity of the response.

In contrast to the findings in normal BALB/c mice, athymic BALB/c mice failed to develop AHR after passive sensitization with anti-OVA IgE. Passive sensitization of athymic BALB/c mice with anti-OVA IgE followed by two challenges with OVA resulted in ES_{50} levels of 3.5 ± 0.2 Hz ($P < 0.01$ compared with passively sensitized normal BALB/c mice) (Figure 3). Even an extension of the nebulization protocol to ten consecutive days after passive sensitization did not alter AR (ES_{50} 3.8 ± 0.5 Hz). These data indicate the inability of athymic mice to develop

AHR despite passive sensitization with allergen-specific IgE and airway challenge.

Injection of IL-5 Reconstitutes the Ability to Develop AHR in Athymic Mice

IL-5 (20 ng) was injected i.v. into nude mice on days 1 to 3 of the protocol in addition to anti-OVA IgE or anti-TNP IgE. Serum IL-5 levels were determined by ELISA at the end of the protocol to assess bioavailability of the injected IL-5. Mice receiving IL-5 in addition to anti-OVA IgE or anti-TNP IgE had serum IL-5 levels on day 8 of 45.6 ± 13.9 pg/ml and 73.25 ± 38.5 pg/ml, respectively (not significantly different between groups). The injections of IL-5 did not alter levels of anti-OVA IgE or ICH when compared to mice receiving anti-OVA IgE alone (data not shown).

Athymic mice passively sensitized with anti-OVA IgE and additionally treated with IL-5 prior to airway challenge developed AHR with significantly decreased ES_{50} values of 1.9 ± 0.3 ($P < 0.01$ compared with passively sensitized nude mice without IL-5 treatment) (Figure 4). To determine if this effect of IL-5 was nonspecific or related to the presence of anti-OVA IgE, we injected athymic mice with anti-TNP IgE and IL-5 prior to the two OVA challenges. These animals failed to develop AHR (non-significant differences compared with passively sensitized

**Airway Responsiveness Following Passive Sensitization
with IgE and IL-5 Treatment**

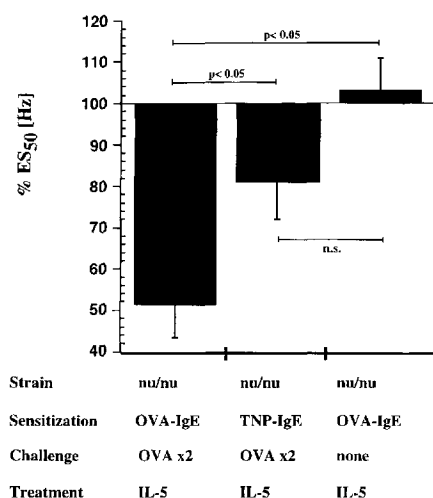


Figure 4. Treatment of athymic (nude) mice with IL-5 restores the ability to develop AHR after passive sensitization and local allergen challenge. Mice were treated and AHR was measured as described in Figure 3. Passively sensitized nude mice received 20 ng IL-5 i.v. on days 1 to 3 of the protocol in addition to anti-OVA IgE ($n = 12$) or anti-TNP IgE ($n = 12$) followed by two airway challenges, or IL-5 and anti-OVA IgE without airway challenge ($n = 8$). The frequencies leading to 50% maximal contraction, $ES_{50} \pm SEM$ in Hz, of three independent experiments were compared with non-sensitized, non-challenged mice where ES_{50} values were 3.7 ± 0.2 Hz and taken as 100%. (P values as indicated on the graph.)

nude mice without IL-5 treatment; $P < 0.05$ compared with nude mice passively sensitized with anti-OVA IgE plus IL-5 treatment). Nude mice passively sensitized with anti-OVA IgE and treated with IL-5 without allergen challenge also did not develop AHR (Figure 4). These data indicate that IL-5 results in the development of AHR in nude mice passively sensitized with allergen-specific IgE and locally challenged with allergen, but fails to significantly enhance the development of AHR in the absence of either allergen-specific IgE or local allergen challenge.

IL-5 Injection Increases Eosinophil Infiltration in Lungs of Nude Mice After Airway Challenge

Total leukocyte counts and differential counts for lung cells of individual mice were compared in nonsensitized, passively sensitized normal BALB/c mice, passively sensitized nude mice and passively sensitized nude mice receiving IL-5 treatment. All of the animals received two challenges with OVA via the airways. There were no significant differences in total leukocyte counts between passively sensitized and nonsensitized normal BALB/c mice (Figure 5A). The absolute numbers of macrophages/monocytes were lower and the lymphocyte numbers were higher in the passively sensitized group compared with nonsensitized mice. Few if any eosinophils were detected in nonsensitized normal and nude mice, but numbers were significantly increased in passively sensitized normal mice (Figure 5B).

Few eosinophils were detected in passively sensitized nude mice who received two airway challenges with OVA. Administration of IL-5 significantly increased the numbers of eosinophils in the lung tissue of nude mice after local allergen challenge by more than three-fold; administration of IL-5 in the absence of airway challenge had little effect on eosinophil accumulation (Figure 5B). The combination of passive sensitization with anti-OVA IgE, IL-5, and airway challenge further increased the number of eosinophils, although not significantly over IL-5 treatment and airway challenge alone or in combination with anti-TNP IgE (Figure 5B). Despite the accumulation of eosinophils in some of these conditions, altered AR was only observed with the combination of anti-OVA IgE, IL-5 treatment and airway challenge with OVA (Figure 4).

In parallel to numbers of eosinophils identified in lung cells, we measured eosinophil peroxidase (EPO) activity of the cell pellet and compared the optical densities (OD) at 450 nm wavelength for 250,000 lung cells. Concordant with eosinophil numbers, EPO activity of lung cells was significantly increased in lung cells of normal BALB/c mice passively sensitized with anti-OVA IgE and challenged with allergen ($OD\ 0.4 \pm 0.09$) compared to lung cells of non-sensitized animals ($OD\ 0.22 \pm 0.04$, $P < 0.05$). In contrast, nude mice passively sensitized with anti-OVA IgE and challenged with allergen twice failed to show a significant increase in the EPO activity of the lung cells ($OD\ 0.28 \pm 0.08$), unless treated with IL-5 in addition to passive sensitization ($OD\ 0.37 \pm 0.1$, $P < 0.05$ compared with nude mice not treated with IL-5).

Discussion

Allergic sensitization of mice triggers the development of AHR. We developed a murine model of adjuvant-free air-

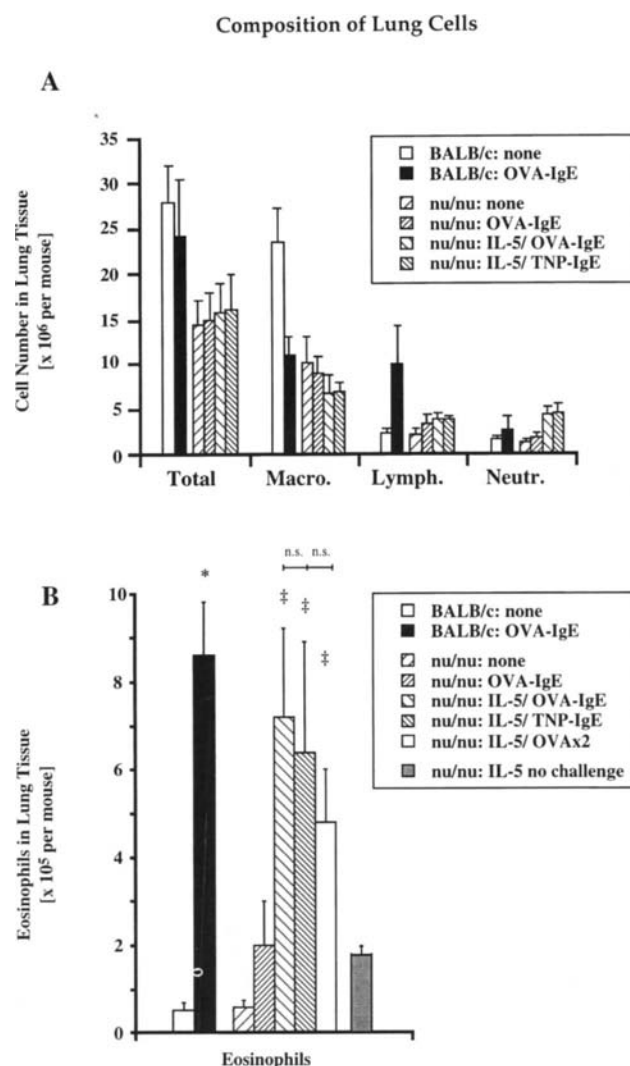


Figure 5. Distribution of lung cells. Relative numbers of different types of leukocytes isolated from lung tissue by digestion were determined in groups of mice following two airway challenges with OVA: nonsensitized normal mice (BALB/c: none, $n = 12$), BALB/c mice passively sensitized with anti-OVA IgE prior to OVA challenge (BALB/c: OVA-IgE, $n = 19$), nonsensitized normal nude mice (nu/nu: none, $n = 4$), nude mice passively sensitized with anti-OVA IgE prior to OVA challenge (nu/nu: OVA-IgE, $n = 19$), nude mice passively sensitized with anti-OVA IgE prior to OVA challenge with additional IL-5-treatment (nu/nu: OVA-IgE/IL-5, $n = 12$), nude mice passively sensitized with anti-TNP IgE prior to OVA challenge with additional IL-5 treatment (nu/nu: TNP-IgE/IL-5, $n = 12$), nude mice treated with IL-5 prior to OVA challenge (nu/nu: IL-5, $n = 12$) and IL-5 treated nude mice without OVA challenge (nu/nu: IL-5 no challenge, $n = 8$). Illustrated are numbers of total leukocytes, macrophages, neutrophils, and lymphocytes (A) and eosinophils (B). Shown are the means $\pm$ SD of the percentage of the different cell types from three separate experiments. * $P < 0.05$ compared with non-sensitized control mice. † $P < 0.05$ compared with passively sensitized nude mice without IL-5 treatment. There were no significant differences between mice receiving IL-5 and airway challenge with or without passive sensitization with anti-OVA or anti-TNP IgE.

way sensitization to study the pathogenesis of allergen-induced AHR (28). Sensitization in this way leads to the expansion of specific T-cell subsets (35), a T_H2 -type response, including increases in IL-4 (36), elevated levels of allergen-specific IgE (28, 30, 31, 35) and increased IL-5 production (37), leading to eosinophilic airway inflammation. In this model of active airway sensitization, both the production of allergen-specific IgE and the IL-5 dependent eosinophilic inflammation are critical for the development of AHR: the use of a low IgE-responder mouse strain (SJL) (31), depletion of IL-5-producing CD8⁺ T cells (38) or treatment with anti-IL-5 antibody (37), which results in reduced eosinophil infiltration, all were associated with a failure to develop AHR. Recent reports using different strains of mice sensitized in a different fashion have examined the role of either IgE or IL-5 in the development of AHR, but the conclusions are somewhat contradictory (39–42). In each example, because of the ability of the animals to respond immunologically to the sensitization, it is difficult to define the interplay between the factors involved in AHR. Importantly, the interaction between allergen-specific IgE and IL-5 triggered eosinophil accumulation and activation can not be addressed in this way. The present study was designed to delineate the inter-relationships between allergen-specific IgE, T-cell activation and eosinophil infiltration and to isolate their involvement in the development of AHR. To independently investigate the requirements for allergen-specific IgE and T cells or T-cell products in the development of AHR, we passively sensitized athymic (nude) mice lacking peripheral T cells and T-cell responses with allergen-specific IgE. This allowed us to analyze the influences of IgE and the requirements for T-cell responses in the development of increased AR.

Passive sensitization with IgE induced a similar degree of allergen-specific immediate cutaneous responsiveness in normal and athymic mice. This confirmed the presence of an intact IgE receptor and functional mast cells in the dermis of athymic mice and, importantly, demonstrated the similarity of IgE-triggered cutaneous responses in normal and athymic mice. After two airway challenges, passively sensitized normal BALB/c mice showed eosinophil infiltration around the small and middle-sized airways and increased EPO levels in lung cells, and developed increased AR. In contrast, passively sensitized nude mice failed to develop airway inflammation or altered AR after airway challenge, despite elevated serum levels of allergen-specific IgE and immediate cutaneous reactivity. Treatment of passively sensitized nude mice with IL-5 reconstituted their ability to respond to allergen airway challenge with eosinophil airway infiltration around the small and middle-sized airways and with development of AHR. This demonstrates the requirements for increased AR in this model: IL-5, passive sensitization with allergen-specific IgE, and local airway challenge with the relevant allergen. Deletion of one of these three components resulted in the inability to develop AHR. The provision of IL-5 together with antigen challenge resulted in eosinophilic infiltration of the lungs; antigen specific IgE appeared to provide little additional stimulus for eosinophil accumulation but appeared to be essential for AHR.

The pathogenic role of IgE in the development of AHR

is not well defined. A major target cell for IgE, the mast cell, not only releases histamine and other proteins capable of damaging the airway epithelium (4), but also releases cytokines such as IL-4 and IL-5 that may be involved in eosinophil infiltration of the lung (43). The requirement for mast cells in the development of AHR is nonetheless controversial. There are few studies in mast cell deficient mice that have monitored airway function (5). It is also unclear how important mast cells are as a source of IL-4 or IL-5 (44). In nude mice passively sensitized with IgE and capable of normal mast cell degranulation, airway challenge with allergen did not provoke an eosinophil response, implying that IL-5, at least in sufficient quantities, was not released by activated mast cells.

The combination of antigen-specific IgE and antigen has been demonstrated to augment allergen-specific T-cell responsiveness and T-cell recruitment to the site of allergen challenge (27). In the absence of T cells, nude mice were incapable of developing AHR, even after passive sensitization with allergen-specific IgE and antigen challenge. The pivotal role of T cells in the development of allergic airway inflammation and AHR has been emphasized (9, 13, 24, 31, 45). T cells can enhance the development of AHR through at least two possible mechanisms. Allergic sensitization of the airways induces a T_H2 -type T cell response (24). This results in IL-4 production, prerequisite for the production of allergen-specific IgE (25). Moreover, IL-4 upregulates the expression of the adhesion molecules involved in eosinophil infiltration of the lungs (46).

Secondly, T_H2 -type T cells are a major source of IL-5 (19), a primary, if not essential factor in the recruitment and activation of eosinophils (15–17). Our data support the importance of IL-5 in the induction of eosinophil accumulation and AHR. Administration of IL-5 (together with IgE) appeared to circumvent the need for activated T cells. Treatment of nude mice with IL-5 increased eosinophil accumulation by more than three-fold after airway challenge and, if given in conjunction with OVA-specific IgE antibodies, triggered AHR. In IL-5 treated animals without airway challenge, the increase in eosinophil numbers in lung tissue was marginal, underscoring the contributions of local allergen challenge in stimulating the migration of eosinophils to the site of the challenge. The role of local allergen challenge in the recruitment of eosinophils is not delineated but may trigger an increase in the release of chemokines (RANTES, MIP-1 α , eotaxin), known chemoattractants for eosinophils (47).

Importantly, IL-5 treatment of challenged nude mice alone or in combination with an unrelated rather than allergen-specific IgE did not induce AHR, despite a similar increase in eosinophil numbers in the lung. There are two possible explanations for this observation. First, allergen-specific IgE and activated eosinophils could both be required, as parallel but independent factors causing epithelial damage leading to altered airway function. The degree of eosinophil infiltration reached after IL-5 treatment of the nude mice in our experiments might not in itself have been sufficient to result in AHR. Additional tissue damage brought about by IgE-mediated mast cell activation may have been required. Alternatively, it is the combination of allergen-specific IgE, allergen and (activated) eosi-

nophils which results in AHR. The exact mechanism underlying eosinophil activation and degranulation remains unclear and controversial. Several reports imply activation of eosinophils via different IgE receptors (48–50) or enhancement of *in vitro* degranulation of eosinophils by allergen-specific IgG1 and IgG3 (51). Taken together, these data provide evidence for an enhancing effect of allergen-specific antibodies on degranulation and cytotoxic activity of eosinophils. Additional studies are needed to correlate AHR with eosinophil accumulation and eosinophil activation. This has been somewhat difficult in the mouse since there is no murine equivalent of the anti-eosinophil cationic protein (ECP) antibody, EG₂, identifying activated human eosinophils (52). Although measurements of EPO levels correlate with eosinophil numbers, it has been difficult to use this parameter to assess eosinophil activation (53). Assessing degranulation of eosinophils by electron microscopy may correlate with activation, but does not correlate with development of AHR (54).

In summary, our data provide evidence for the requirement of allergen-specific IgE, IL-5 and allergen challenge via the airways in the development of AHR. In T-cell deficient mice, passive sensitization with IgE prior to airway challenge is insufficient to provoke AHR. However, treatment of passively sensitized nude mice with IL-5 prior to airway allergen challenge effectively reconstitutes the ability to recruit eosinophils to the airways and to develop increased airway responsiveness. Knowledge of these specific components which are essential to the development of AHR provides opportunities for direct, targeted interventions.

Acknowledgments: This work was supported by grants AI-29704 (E.W.G.) and HL-36577 (E.W.G. and G.L.) from the National Institutes of Health. Eckard Hamelmann is a fellow of the Deutsche Forschungsgemeinschaft (Ha 2162/1-1) and recipient of the 1996 Janssen Pharmaceutica Research Award of the American Academy of Allergy, Asthma and Immunology. The writers thank Dr. R. Coffman, DNAX Institute, Palo Alto, CA, for his kind gift of the IL-5 secreting T cells and anti-IL-5 antibody, and Dr. Christoph Persin for his assistance in the purification of IL-5. The assistance of Ms. Diana Nabighian in the preparation of this manuscript is gratefully acknowledged.

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