

Anti-interleukin (IL)-4 and -IL-5 antibodies downregulate IgE and eosinophilia in mice exposed to *Aspergillus* antigens

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The effect of multiple divided doses compared with single-dose injections of antibodies to murine interleukin (IL)-4 and IL-5 in their respective downregulation of IgE and eosinophilia developing in a model of allergic aspergillosis is investigated. BALB/c mice were exposed to *Aspergillus fumigatus* antigens (Af) before and along with anticytokine antibodies. The kinetics of blood eosinophils, eosinophil peroxidase (EPO) in bone-marrow cells, serum levels of IgE and Af-specific antibodies, Af-induced cytokine production and mRNA, and lung histology were studied. The results indicate that only multiple anti-IL-5 antibodies were effective in maintaining baseline levels of blood eosinophils. Multiple anti-IL-4 antibodies also downregulated eosinophils in the bone marrow, lung, and peripheral blood, although to a lesser extent than in anti-IL-5 antibody-injected mice. Significant correlation between the EPO activity and the eosinophil numbers in anticytokine antibody-treated mice was observed. The different anti-IL-4 antibody treatments downregulated IgE to the same extent. We conclude that multiple divided doses of anti-IL-5 antibodies are required to sustain normal eosinophil levels in murine allergic aspergillosis. This information may be significant in the therapy of pulmonary allergic diseases.

V. P. Kurup¹, P. S. Murali¹, J. Guo¹, H. Choi², B. Banerjee¹, J. N. Fink¹, R. L. Coffman³

¹Allergy and Immunology Division, Departments of Medicine and ²Pathology, Medical College of Wisconsin, and ³Research and ²Laboratory Services, VA Medical Center, Milwaukee, Wisconsin; ³DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California, USA

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Viswanath P. Kurup, PhD
VA Medical Center
Research Service 151-I
5000 West National Avenue
Milwaukee, WI 53295
USA

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In previous studies, we have demonstrated that the *Aspergillus fumigatus* (Af) antigen invokes characteristic features of allergic aspergillosis in mice. These include elevated serum IgE level, Af-specific serum antibodies, and peripheral blood and lung eosinophilia (1–4). These manifestations are a result of activation of CD4⁺ T-helper 2 (Th2) cells by *Aspergillus* antigens. Interleukin-4 (IL-4), a major cytokine produced by Th2 cells, is responsible for both IgE antibody isotype switching and enhanced IgG1 production in primed mice (5, 6). IL-5, another Th2 cytokine, expressed in response to Af antigen, is involved in eosinophil differentiation, activation, and survival (7). However, cytokines expressed by CD4⁺ T-helper 1 cells (Th1), particularly interferon (IFN)- γ , downregulate both IgE and eosinophil responses induced by Af antigens (4). Our previous results have shown that mice exposed to Af antigens modulated specific immune responses when anticytokine antibodies (Ab) were administered.

Anti-IL-5 Ab abrogated the peripheral blood and lung eosinophilia, and anti-IL-4 Ab markedly limited the increase in total serum IgE, but failed to decrease the serum levels to baseline in Af-exposed mice. On the other hand, anti-IFN- γ Ab enhanced both IgE and eosinophils in animals challenged with *Aspergillus* antigens (4). These studies were carried out in animals with ongoing eosinophilia and strong total and specific IgE responses to Af antigens.

In the present study, we investigated the effect of administration of anticytokine Ab before exposure of mice to Af and compared a single high dose of anticytokine Ab with multiple low Ab doses. A pronounced suppression of IgE and eosinophils after multiple low doses of anti-IL-4 Ab and anti-IL-5 Ab administration was detected. This indicates that any attempt to control allergy caused by Af with anticytokine Ab should focus on prolonged maintenance of the neutralizing effect of anticytokine Ab with multiple divided doses.

Material and methods

BALB/c mice

Specific pathogen-free BALB/c mice, 10–12 weeks old (purchased from Sasco), were selected for these studies. The mice were housed in the animal facility at the Milwaukee VA Medical Center. Food and water were provided *ad libitum*. The Institutional Committee on Animal Studies has approved this project.

A. fumigatus antigen (Af) exposure

The Af antigen used was a mixture of culture filtrate and mycelial extracts from *A. fumigatus*. The antigen was extracted from cultures of *A. fumigatus*, as previously reported (8, 9). Two groups of mice were exposed to the *A. fumigatus* antigen, as shown in Fig. 1. Mice were anesthetized with Metofane (Methoxyflurane, Pitman-Moore, Mundelein, IL, USA) before exposure to antigen intranasally (1). Groups of five mice each were treated similarly and studied. The anticytokine Ab (2 mg per mouse) was given intraperitoneally (i.p.), either as a single dose (group I, Fig. 1) given 1 day before the antigen exposure or as multiple doses of 1 mg Ab (group II, Fig. 1). The mice were killed by CO₂ inhalation, and blood was collected from the heart. The spleen and a femur were aseptically removed from each mouse and placed in Hanks' balanced salt solution (HBSS) to study the cellular responses and eosinophil peroxidase content, respectively. The lungs were preserved in 10% buffered formalin for histologic studies.

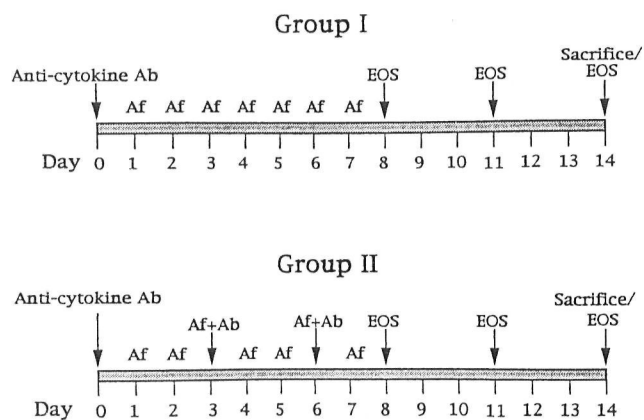


Fig. 1. Experimental details on exposure of mice to *A. fumigatus* antigen (Af) and treatment of anticytokine antibodies (Ab). Eosinophils in peripheral blood were studied on days marked with EOS.

Anticytokine antibodies

Rat mAb to murine IL-4 (11B11), IL-5 (TRFK5), and IFN- γ (XMG1.2) and an isotype control antibody (GL-113; IgG2a) were injected i.p., as shown in Fig. 1. The optimum concentrations of anticytokine Ab for abrogation of the appropriate cytokine have been previously studied (4). We used both single injections of high doses and multiple low doses of anticytokine Ab to maintain sustained effect for a prolonged period of time. A control group of mice that received PBS was also studied to determine the baseline cytokine and Ab responses.

Spleen-cell isolation and culture

Spleens were minced and single cells were isolated, as described previously (2), by gentle mashing of the tissue over a wire mesh with the plunger of a 10-ml sterile syringe. The cells were washed and passed through a nylon mesh to remove the larger clumps. The single cells were washed in HBSS and resuspended in the same fluid. The cells were counted with a hemacytometer, and 2×10^6 cells/ml were cultured in complete RPMI 1640 medium with 10% fetal bovine serum for 7 days with Af (5 μ g/ml) or without stimulants. The supernatants were collected and frozen at -20° until total IgE and Af-specific Ab were studied.

Bone-marrow cells

The femur was cleaned and the ends cut off, and the marrow was forced out with HBSS, a syringe and a 20-gauge needle being used. The single cells were washed three times with HBSS and cultured in complete RPMI 1640 medium, and eosinophil peroxidase was estimated as described below.

Kinetics of peripheral blood eosinophilia

Blood eosinophils were counted after the last exposure to Af antigen. Peripheral blood was collected from the tail vein in tubes containing heparin (25 U/ml) and stained with Discombe's fluid (5 mg eosin Y dissolved in 1 ml acetone and further diluted to 20 ml with distilled water), as previously described (1, 2). The stained eosinophils were then counted with a hemacytometer.

EPO assay

Eosinophil peroxidase was studied as a measure of eosinophil activity and was estimated in bone-marrow cells, as previously described (2, 3, 9). Briefly, 100 μ l of bone-marrow cells (5×10^5 /ml)

from mice was cultured in complete RPMI 1640 medium in 96-well, sterile, round-bottom plates. After 48 h of incubation, the plates were centrifuged at 1000 rpm for 10 min, and the medium was aspirated and replaced with 100 μ l of 1 mM *o*-phenylenediamine in 0.05 Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and 1 mM hydrogen peroxide. After incubation for 30 min at room temperature, the color reaction was stopped by the addition of 50 μ l of 4 M sulfuric acid, and the absorbance at 490 nm was determined with an MR 700 Microplate reader (Dynatech Lab, Chantilly, VA, USA). Results were expressed in optical density (OD) units in comparison with blanks.

Serum IgE

Total serum IgE was measured with a rat anti-mouse IgE mAb, as previously reported (1). The levels of IgE were expressed in ng/ml.

Af-specific antibodies

Af-specific IgG1 and IgG2a antibodies were determined by ELISA (1, 4). Briefly, the method is as follows. Af antigen was coated on 96-well microtiter plates (Immulon-2) at a concentration of 5 μ g/ml. Serum (diluted 1:100, 1:250, or 1:1000) and culture supernatants (undiluted) were incubated for 3 h, followed by washing with PBS containing Tween 20 (PBST). The plates were then incubated with biotin-conjugated rabbit anti-mouse IgG isotype subclass (1:1000 dilution). After incubation for 1 h and washing with PBST, horseradish peroxidase coupled to streptavidin was added and incubated for 1 h. The color was then developed with *o*-phenylenediamine and H₂O₂, and read at 490 nm with an ELISA plate reader. Data are presented as OD values at 490 nm after subtraction of the blank values.

mRNA transcription in spleen cells

Total RNA was extracted from 10 $\times$ 10⁶ spleen cells by a guanidinium isothiocyanate phenol chloroform single-step method, as described earlier (10). The cDNA was prepared from the mRNA with Moloney's murine leukemia virus (MMLV) reverse transcriptase (RT). Specific primers of β -actin, IL-4, IL-5, IFN- γ , and IL-10 were used to amplify the gene by polymerase chain reaction (PCR), as previously described (10). PCR products were run on a 2% agarose gel and visualized after staining with ethidium bromide. The size of the amplified fragment was determined by comparison with a 1-kb DNA ladder. The band intensity was compared with a scanning densitometer, and the

mRNA levels were semiquantitatively measured in comparison with β -actin bands. The values were normalized and transformed into an OD index by the following formula:

$$\text{OD index} = (\text{OD of cytokine band} / \text{OD of } \beta\text{-actin band from respective group}) \times 100$$

Lung histology

Lungs were fixed in 10% buffered formalin, and the tissue was processed and embedded in paraffin. Sections were cut and stained with hematoxylin-eosin for microscopic examination. The pathologic reactions were read blindly, and the results were compared. The eosinophils were counted in a number of high-power magnification fields (hpf), and the average was calculated. The eosinophilic infiltrates were graded as follows: 0: no eosinophils, 1–3: occasional, 4–10: few, 11–30: many, and over 30: numerous.

Statistical analysis

Student's *t*-test (two-tailed, independent samples) was used to compare mean values from test mice with controls. Correlation analysis was performed with the Spearman rank order correlation test. A *P* value less than 0.05 was considered statistically significant.

Results

Peripheral blood eosinophils

The peripheral blood eosinophils were monitored in all groups of mice after the last exposure to antigen (Fig. 2). Both groups of animals showed a similar pattern of change in the eosinophil numbers on treatment with a different anticytokine Ab, except for TRFK5. There was a more prolonged suppression of eosinophilia in mice receiving three divided lower doses of TRFK5 (group II) than in those receiving a single high dose (group I) (Fig. 2). The

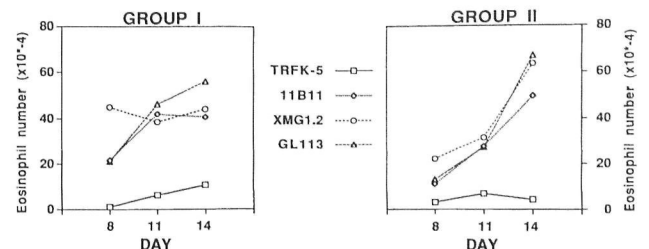


Fig. 2. Kinetics of peripheral blood eosinophilia in Af antigen-exposed mice receiving either single high dose (group I) or multiple low doses (group II) of anticytokine antibodies (see Material and methods for details). Each point on graph represents mean value obtained from five mice receiving similar treatment.

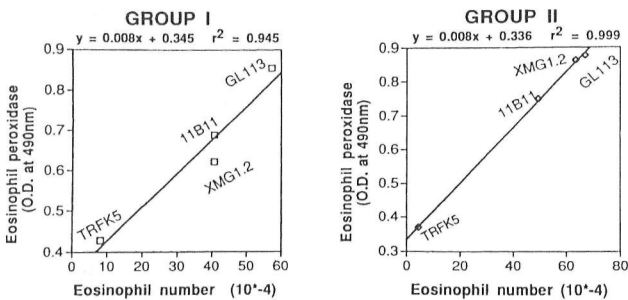


Fig. 3. Correlation between bone-marrow eosinophil peroxidase and peripheral blood eosinophil numbers in mice (see Fig. 2 and Material and methods for details). Each point on graph represents mean value obtained from five mice receiving similar treatment.

eosinophil numbers in these animals were similar to that of uninoculated normal control mice.

EPO

Eosinophil peroxidase was estimated colorimetrically in bone-marrow cells and compared with the peripheral-blood eosinophil counts obtained on day 14. In both test groups, EPO levels were elevated in anti-IL-4 Ab-, anti-IFN-γ Ab-, and control Ab-treated mice. The TRFK-5-treated mice in both groups showed much lower EPO levels than other Ab-treated mice (Fig. 3). Furthermore, the levels of EPO and eosinophil numbers showed good correlation in all groups of animals (Fig. 3). These data suggest that sustained levels of anti-IL-5 Ab inhibit eosinophil differentiation in this mouse model.

Total serum IgE

Af antigen-exposed mice from both groups I and II showed significantly elevated total serum IgE levels compared to controls, with the exception of anti-IL-4 Ab-treated mice (Table 1). Anti-IL-4 Ab-injected mice showed a decrease in the

total serum IgE in both groups (group I, 519 ± 169 ng/ml; group II, 601 ± 87 ng/ml). Normal mice treated with PBS, but not antigens, showed IgE levels similar to those treated with anti-IL-4 Ab.

Aspergillus-specific antibody in the sera of mice

No major differences in *Aspergillus*-specific IgG1 levels could be detected in the different groups where the Ab levels were higher in the sera of animals exposed to a single dose of anticytokine Ab than in those exposed to multiple doses. IgG2a was elevated in animals treated with anti-IL-4 Ab irrespective of single or multiple doses. Anti-IL-5 Ab-treated animals belonging to both groups showed only low levels of IgG2a and IgG1 (Table 1).

mRNA in spleen cells

Animals receiving a single injection of anticytokine Ab failed to show any difference in the respective cytokine mRNA levels compared to controls. The mRNA transcription in spleen cells of different animal groups receiving multiple treatment of anticytokines is shown in Fig. 4. A strong IL-5 band was seen only in anti-IFN-γ Ab-treated animals. However, multiple injections of anticytokine Ab reduced the levels of mRNA for the corresponding cytokine by more than 60% when the band intensity of the PCR product was quantitated (Table 2). Normal, untreated animals showed no mRNA of IL-4 and IL-5, while occasionally showing very weak levels of IFN-γ mRNA.

Lung histology

Single or multiple doses of anti-IL-5 Ab showed only occasional eosinophils, indicating significantly lower eosinophil levels in lung tissue (Fig. 5). Eosinophilic infiltrates were not observed in lung from normal control mice (Fig. 6). Mice receiving an isotype control Ab (GL-113) showed numerous

Table 1. Total serum IgE and *Aspergillus*-specific IgG1 and IgG2a in sera of mice

Treatment	IgE (ng/ml)		IgG1 (OD)		IgG2a (OD)	
	Group I	Group II	Group I	Group II	Group I	Group II
Af + TRFK5	2733 ± 622¶	2977 ± 47*	0.532 ± 0.08	0.545 ± 0.16§	0.212 ± 0.02	0.252 ± 0.02¶
Af + 11B11	519 ± 169	601 ± 87	1.301 ± 0.45	1.203 ± 0.12	0.564 ± 0.28	0.473 ± 0.05
Af + XMG1.2	1764 ± 270	3746 ± 1340	1.063 ± 0.28	0.421 ± 0.13¶	0.281 ± 0.02	0.222 ± 0.01¶
Af + GL113	2597 ± 363	2221 ± 651	1.183 ± 0.19	0.535 ± 0.09¶	0.330 ± 0.02	0.26 ± 0.01¶
PBS (normal)	348 ± 11	482 ± 11	0.195 ± 0.02*	0.176 ± 0.02	0.241 ± 0.01	0.202 ± 0.01

P values as compared to Af + 11B11 group; * < 0.001, ¶ < 0.01, § < 0.02.

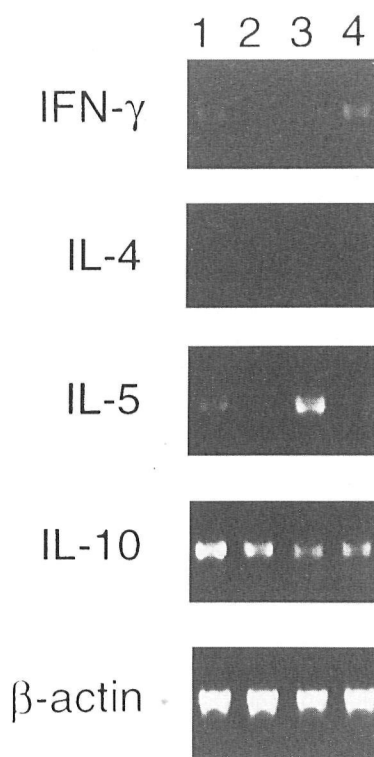


Fig. 4. Cytokine mRNA by RT-PCR in spleen cells from mice. Repeated administration (group II) of anticytokine antibodies to IL-4 (1), IL-5 (2), IFN- γ (3), or isotype control antibody (4) treated mice exposed to *A. fumigatus* antigen.

eosinophils in the lung (Fig. 7). Animals given single doses of anti-IL-4 Ab demonstrated marked lung eosinophilia with numerous eosinophils (over 30 hpf) (Fig. 8), while mice given multiple doses showed fewer eosinophils. An increase in the number and severity of granulomas in lungs was observed in mice receiving multiple injections of anti-IFN- γ Ab. Mice treated with the control Ab also exhibited granuloma formation, albeit to a lesser extent. Multiple anti-IL-4 Ab or anti-IL-5 Ab exposure resulted in fewer or no granulomas in the lungs. Mice given a single dose of Ab showed a moderate to severe interstitial infiltrate of chronic inflammatory cells, with lymphocytes, plasma cells, and his-

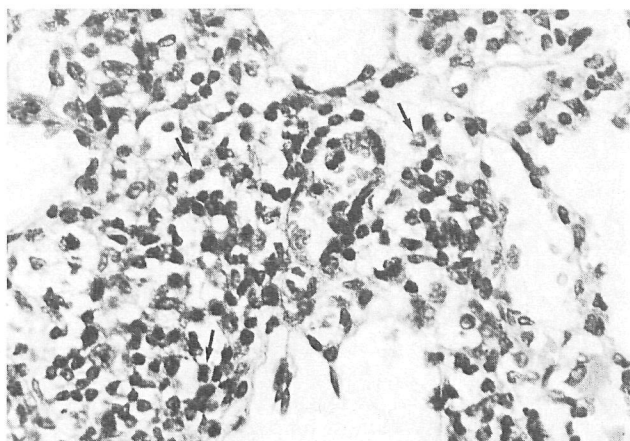


Fig. 5. Lungs from group II mice treated with anti-IL-5 antibody showing interstitial chronic inflammatory infiltrate with lymphocytes, plasma cells, and histiocytes. Only very few eosinophils (arrows) are demonstrable (HE $\times 400$).

tiocytes, with peribronchiolar and perivascular accentuation, including large numbers of eosinophils. These inflammatory infiltrates were less severe in the mice treated with multiple doses of the different anticytokine Ab.

Discussion

The purpose of the present study was to clarify the role of anti-IL-4 Ab and anti-IL-5 Ab in regulating two major features of allergic aspergillosis, namely, elevation of IgE and eosinophilia. Administration of multiple low doses of anticytokine Ab was more effective in abrogating peripheral blood eosinophilia than a single high dose. Anti-IL-5 Ab significantly lowered pulmonary eosinophilia in mice exposed to Af antigen. Eosinophils also showed a slight reduction in mice receiving multiple doses of anti-IL-4 Ab. Our results concur with others (11, 12) showing that IL-5 is the most important cytokine regulating eosinophils and that a prolonged or continuous presence of neutralizing anti-IL-5 Ab has therapeutic potential in controlling Af-induced allergy.

Table 2. Cytokine mRNA transcripts in spleen cells from group II mice exposed to *Aspergillus* antigens

	β -Actin Units	IL-4		IL-5		IFN- γ		IL-10	
		Units	I*	Units	I*	Units	I*	Units	I*
11B11	3309	22	0.66	734	22.18	676	20.43	2451	74.07
TRFK5	3211	180	5.61	0	0.00	345	10.74	2149	66.93
XMG1.2	3137	236	7.52	2000	63.76	287	9.15	1369	43.64
GL113	3031	216	7.13	315	10.39	1052	34.71	1410	46.52
Control	3076	376	10.25	326	10.25	726	22.87	1454	45.5

I*: OD index = $100 \times (\text{units from cytokine bands} / \text{units from } \beta\text{-actin band from respective group})$.

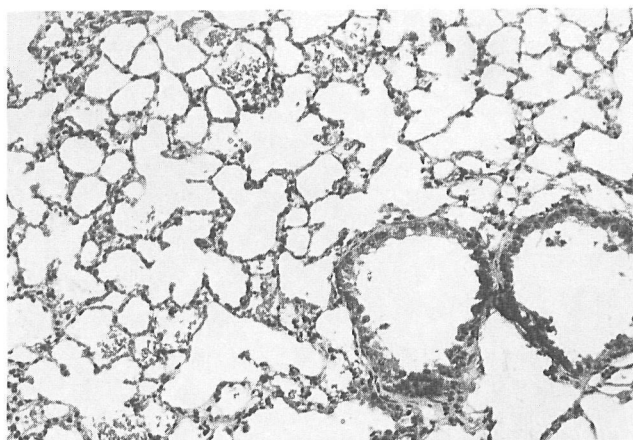


Fig. 6. Lung section of normal control mouse showing normal histology (HE $\times 160$).

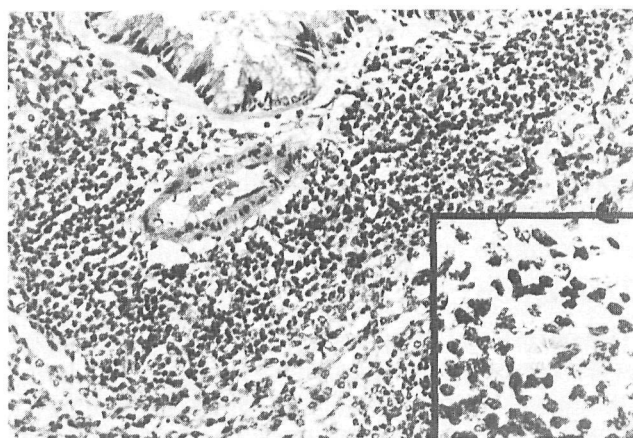


Fig. 7. Lung section of mouse treated with control antibody (GL113) showing severe peribronchiolar and perivascular infiltrate with numerous eosinophils (HE $\times 160$; inset $\times 400$).

Good correlation was obtained between eosinophils in peripheral blood and EPO in bone marrow in response to different anticytokine Ab treatments. Both single and multiple treatments gave essentially the same results, although the single dose showed a reduced effect over 14 days. Hence, we speculate that anti-IL-5 Ab may act at the bone-marrow level in the downregulation of eosinophilia. Eosinophils are formed in the bone marrow, and their life cycle is continued through blood and finally in tissues (13). The kinetics of changes in eosinophils in different lymphoid compartments was the focus of a recent study (14). In the present study, we have shown that peripheral blood eosinophilia is concurrent with enhanced EPO in bone marrow, and both responses are identically regulated subsequent to treatment with anti-IL-5 Ab.

Mice exposed to Af antigens had significantly elevated serum IgE compared to unexposed mice.

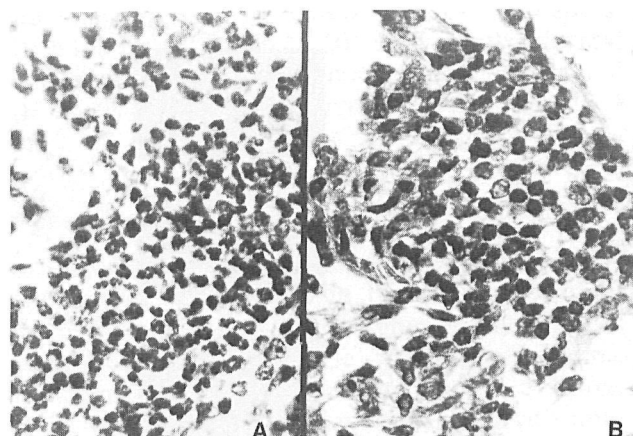


Fig. 8. A) Lungs from group I mouse treated with anti-IL-4 antibody showing many eosinophils along with moderate interstitial chronic inflammatory infiltrate (HE $\times 400$). B) Lungs from group II mouse treated with anti-IL-4 antibody demonstrating fewer eosinophils than group I mice (HE $\times 400$).

Neutralization of IL-4 by antibody in this model leads to normal levels of IgE in serum. In the present model, the number of doses of anti-IL-4 Ab, unlike those of anti-IL-5 Ab, had no effect on the final serum level of IgE. This suggests that a single dose of anti-IL-4 Ab given before exposure to Af antigen can control any increase of serum IgE levels during the entire period of the experiment. In a previous study, we found that anti-IL-4 Ab administered after priming with Af antigens failed to lower the high IgE levels in the serum (4). Thus, from these results, it can be concluded that anti-IL-4 Ab failed to lower or halt the expression of IgE Ab by already committed B cells, although switching of uncommitted cells to IgE-secreting cells was completely retarded.

In the present model, Af antigen induced elevated serum levels of Af-specific IgG1. This Ab response was significantly enhanced in anticytokine Ab-treated mice of group II. However, mice treated with anti-IL-4 Ab also showed elevated Af-specific IgG1 and IgG2a levels. IL-4-deficient mice infected with parasites demonstrated significant increases in both total and parasite-specific IgG2a (15–17). It has been reported that anti-IL-4 Ab (11B11) strongly inhibits IgE production in *Nippostrongylus brasiliensis*-infected mice. However, similar treatment did not affect IgG1 production (18). Moreover, other studies have shown that IgE production is uniquely dependent upon IL-4, whereas IgG1 secretion can be induced in the absence of IL-4 (5, 16). IL-4 may have a regulatory role in eosinophil production, as it acts as a growth factor for CD4+ cells and particularly for Th2 cells. It has been shown that, compared to the wild type, IL-4 knockout mice exhibit lower levels of both IL-5 and eosinophils (17).

Depletion of IFN- γ , a Th1 cytokine, via multiple doses of anti-IFN- γ Ab, leads to enhanced granuloma formation in the lung, while mice receiving anti-IL-4 Ab or anti-IL-5 (Th2 cytokines) Ab have very few granulomas. This supports the previous studies (19, 20) on the protective role of a Th1-type response in murine, as in human, diseases.

In a murine model of allergic aspergillosis, we have demonstrated that administration of multiple doses of anti-IL-5 Ab and anti-IL-4 Ab can produce a cytokine-deficiency state effectively abrogating eosinophils in peripheral blood, bone marrow, and lungs and IgE in blood.

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