Correlation between eosinophilia induced by CD4+ T cells and bronchial hyper-responsiveness

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Abstract

The relationship between CD4+ T cell-mediated airway eosinophilic inflammation and bronchial hyper-responsiveness (BHR) was investigated. Ovalbumin-reactive Th0 clones were adoptively transferred to unprimed BALB/c mice and then the mice were challenged by inhalation of the relevant antigen. Upon antigen provocation, infused Th clones infiltrated into the airways, followed by the accumulation and degranulation of eosinophils, goblet cell hyperplasia, edema and increase in bronchial responsiveness to acetylcholine. Transfer of several clones that differed in the levels of IL-5 production revealed that the magnitude of in vivo eosinophilia strongly correlated with the IL-5-producing capacity of the infused Th clones. Administration of anti-IL-5 mAb almost completely suppressed antigen-induced eosinophilic inflammation and BHR. Administration of anti-IL-4 mAb or anti-IFN-γ mAb enhanced the eosinophilia and BHR, whereas anti-IL-2 mAb did not affect them. The number of accumulated eosinophils significantly correlated with the intensity of BHR. Our present results clearly demonstrated that CD4+ T cells induced BHR as a result of eosinophilic inflammation. IL-5 totally regulated both responses.

Introduction

Bronchial hyper-responsiveness (BHR) to non-specific stimuli is a hallmark of bronchial asthma. Accumulating evidence suggests that the development of BHR is closely related to eosinophilic inflammation of the bronchial mucosa, a prominent pathological feature of asthma (1–8). Eosinophils release toxic granule proteins such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein and eosinophil-derived neurotoxin (4), all of which are detected at the damaged respiratory epithelium of asthmatic patients (9). Toxic concentrations of MBP were also demonstrated in the sputum obtained from the asthmatic patients (10). Direct instillation of MBP into the bronchus of monkeys caused a significant increase in the bronchial responsiveness to methacholine (11). Epithelial damage has been clinically related to BHR (12). These findings strongly suggest that BHR stems from the eosinophilic inflammation of the bronchial mucosa. However, few reports have directly demonstrated that eosinophils infiltrating into the airways really induce BHR, while quite a few animal studies reported a dissociation of airway eosinophilia from BHR (13–16).

Activated Th2 cells and Th2 cytokines, including particularly IL-5, are crucial for the local infiltration and activation of eosinophils (2,17–19). IL-5 promotes the terminal differentiation of committed eosinophil precursors (20) and prolongs the survival of eosinophils (21). IL-5 concentration in the bronchoalveolar lavage fluid (BALF) and serum of asthmatic patients is increased compared to normal subjects (22,23). CD4+ T cells isolated from the BALF of allergic asthmatics express elevated levels of mRNA for IL-4 and IL-5 (24), a pattern of cytokine production consistent with the Th2 phenotype. IL-5 production by peripheral CD4+ T cells is also enhanced in atopic and non-atopic asthmatics compared
with normal control subjects (25). Administration of anti-IL-5 mAb totally abrogated the induction of late-phase airway eosinophilic inflammation in antigen-sensitized mice, clearly indicating that eosinophilic inflammation is dependent on IL-5, a T cell cytokine (19,26–28). As to BHR, quite a few animal models of asthma have indicated that IL-5 is a prerequisite for the development of BHR as well (29–31). Administration of anti-IL-5 mAb completely inhibited the development of BHR without affecting the level of antigen-specific IgE or T cell responses (26). IL-5 deficiency abolished antigen-induced BHR and the reconstitution of IL-5 production completely restored it (32).

The involvement of several other cytokines including IL-4, on the other hand, has been implicated in eosinophilic inflammation and BHR in murine models (13,33). Administration of anti-IL-4 mAb reduced the airway eosinophilic infiltration in response to parasite antigen in CBA/J mice (33). The essential requirement of IL-4 for the ovalbumin (OVA)-induced inflammation and BHR, which was modulated independently by IL-4 and IL-5 (34). Hessel et al. showed that IFN-γ directly induced BHR (16). The discrepancies observed among these studies in the association versus dissociation between eosinophil infiltration and BHR, and in the relative dependency on the contribution of cytokines might reflect a complex situation in which multiple cell types, cytokines and chemical mediators are involved to mount allergic eosinophilic inflammation and BHR.

Recently, we have developed a new animal model suitable for the analysis of the interaction among CD4+ T cells, cytokines, eosinophilic inflammation and BHR. Allergic eosinophilia, which was accompanied by BHR, was reconstituted in the airways of unprimed mice by the adoptive transfer of CD4+ T cells, cytokines, eosinophilic inflammation and BHR. Allergic eosinophilia, which was accompanied by BHR, was reconstituted in the airways of unprimed mice by the adoptive transfer of CD4+ T cells, despite the absence of humoral immune factors such as IgG, IgA and IgE antibodies (35). CD4+ T cell-mediated eosinophil infiltration was completely dependent on IL-5 produced by the infused Th clones, as the responses were abolished by the administration of anti-IL-5 neutralizing antibody (35). To further clarify the mechanism of BHR in allergic asthma, the present study focused on the relationship between airway eosinophilia and BHR, and also on the role of T cell-derived cytokines in the development of BHR using our CD4+ T cell-mediated asthma model.

**Methods**

**Animals**

Specific pathogen-free male BALB/c mice, 10–14 weeks of age, were obtained from Japan SLC (Hamamatsu, Japan). These animals were housed in an environmentally controlled room (temperature, 23 ± 2°C; humidity, 55 ± 5%; illumination time, from 7:00 to 19:00) with food and water available ad libitum for 1 week prior to the experiment.

**Reagents**

Anti-IL-2 (S4B6-1), -IL-4 (11B11) and -IFN-γ (R4-6A2) mAb were purified by affinity chromatography from abdominal dropsy of mice injected with corresponding hybridomas. Purity of the mAb was confirmed by SDS-PAGE. Anti-IL-5 mAb (TRFK-5) was prepared from the supernatant of hybridoma cells (TRFK-5) (36). Other reagents were as follows: Ficoll-Paque (Pharmacia, Uppsala, Sweden), AIM-V medium (Gibco/BRL, Gaithersburg, MD), pentobarbital sodium (Tokyo Kasei, Tokyo, Japan), acetylcholine chloride (Nacalai Tesque, Kyoto, Japan), pancuronium bromide (Sankyo, Tokyo, Japan), OVA (Sigma, St Louis, MO) and 5- (and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR).

**Cytokine production by Th clones**

OVA-reactive Th clones (FP3 or another clones) were established from the regional lymph node cells of immunized BALB/c mice as previously described (35). To determine the profiles of cytokine production, cells were harvested 10–14 days after the last antigenic stimulation, purified by centrifugation over Ficoll-Paque (37), suspended in AIM-V medium (5×10^5/ml) and cultured in 96-well culture plates with X-ray-irradiated spleen cells (5×10^5/ml) with or without OVA (100 µg/ml) for 24 h. The resulting supernatants were assayed for IL-2, IL-4, IL-5 and IFN-γ by specific ELISAs as described previously (38).

**Cell transfer and challenge procedure**

Th clones (2×10^6, 5×10^6 or 2×10^7 cells) suspended in 0.5 ml HBSS were injected into the tail vein of normal recipient mice. In some experiments, injected T cells were labeled with fluorescein-based dye, CFSE, as described by Lyones et al. (39) and Banks et al. (40). Briefly, cells were suspended in medium (2.5×10^7/ml) containing 5 µM CFSE, incubated at 37°C for 10 min, washed 3 times and then injected. Twenty-four hours after cell transfer, mice were individually placed in 50 ml plastic tubes and made to inhale aerosolized OVA (100 mg/ml) dissolved in 0.9% saline delivered by a DeVilbiss 646 nebulizer (DeVilbiss, Somerset, PA) driven by compressed air at 18 l/min for 60 min. As a control, 0.9% saline alone was administered by the nebulizer. In some animals, antibodies against IL-2, IL-4, IL-5 or IFN-γ (2 mg/body) were i.v. administered 4 times, 30 min before, and 24, 48 and 120 h after the challenge.

**Measurement of BHR**

BHR was assessed as the bronchoconstriction following infusion of acetylcholine by determining changes in respiratory overflow volume, essentially following the method described by Konzett and Rössler (35). Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and their trachea and femoral vein were cannulated. The tracheal cannula was connected to a rodent ventilator (model 680-683; Harvard, South Natick, MA) and then the mice were mechanically ventilated at 60 strokes/min with a stroke volume of 1 ml after neuromuscular blockade with i.v. administered 1 mg/kg pancuronium bromide. The changes in respiratory overflow volume were measured with a differential pressure transducer (TP-602; Nihon Kohden, Tokyo, Japan) connected to a T-tube on the tracheal cannula. The increase in respiratory overflow volume provoked by acetylcholine (30–3000 µg/kg) is represented as a percentage of the maximal overflow volume (100%) obtained by totally occluding the tracheal cannula.
The acetylcholine dose that increased respiratory overflow volume to 50% of the maximal value (PD_{50}) was calculated from the log dose–peak response curves and expressed as log value of geometric mean ± SEM.

Measurement of eosinophil number and EPO activity in BALF

After the measurement of BHR, BAL was performed in the same animals. The trachea was re-cannulated with a polyethylene tube through which the lungs were gently lavaged with 0.5 ml HBSS 4 times (2 ml total). On average, 1.9 ml BALF was recovered and then centrifuged at 1500 r.p.m. for 5 min. Then, 1 ml supernatant was stored to measure EPO activity. The pellet obtained was immediately suspended in 250 µl HBSS and total cell numbers in BALF were counted by an automatic cell counter (Celltac MEK-5158; Nihon Kohden, Japan). Differential cell counts were made by microscopic examination of centrifuged preparations stained with May–Giemsa, counting 200 cells in each animal.

The level of free EPO in the supernatant from BALF was determined as an index of eosinophil activation according to the method described by Strath et al. (41) with some modifications. Briefly, 50 µl samples were incubated with 200 µl assay buffer and 100 µl o-phenylenediamine hydrochloride (1 mM)/H_2O_2 (0.8 mM) for 5 min at 37°C, and the reaction was stopped by adding 200 µl H_2SO_4 (4 M). The optical density was then measured at 492 nm. Samples containing contaminating hemoglobin in the supernatant were discarded. Standard curves for horseradish peroxidase (0–20 mU/ml) and human myeloperoxidase (0–100 mU/ml) were plotted to check the specificity of the assay.

Histologic examination

Saline- or OVA-challenged mice were killed humanely by excess i.p. administration of sodium pentobarbital at designated times. Lungs fixed with formaldehyde were embedded in paraffin, sectioned at 4 µm thickness and stained with hematoxylin & eosin. Lung sections prepared from the mice with transfer of CFSE-labeled FP3 were observed with a confocal laser scanning microscope (Leica; TCS-NT).

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was performed using Student’s t-test for comparison between two groups and one-way analysis of variance with Bonferroni’s method for three groups or more. Values of P < 0.05 were considered to be statistically significant.

Results

Antigen-induced IL-2, IL-4, IL-5 and IFN-γ production by OVA-specific murine T_h clone in vitro

Profiles of IL-2, IL-4, IL-5 and IFN-γ production of a T_h clone, FP3, are shown in Table 1. Production of all cytokines that we investigated was significantly increased upon antigen stimulation. FP3 was classified as T_h0 phenotype based on its production of IL-2, IL-4, IL-5 and IFN-γ.

Table 1. Antigen-induced cytokine production by a murine T_h clone, FP3

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cytokine production (pg/ml)</th>
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<tr>
<td></td>
<td>No stimulation</td>
</tr>
<tr>
<td>IL-2</td>
<td>&lt;10</td>
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<td>IL-4</td>
<td>&lt;20</td>
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<td>IL-5</td>
<td>430</td>
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<td>IFN-γ</td>
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FP3 cells (5×10^5/ml) were cultured with X-ray-irradiated spleen cells (5×10^5/ml) in the presence or absence of OVA for 24 h. The concentrations of IL-2, IL-4, IL-5 and IFN-γ in the culture supernatants were measured by specific ELISAs. Data are shown as the means of quadruplicate cultures (SEM < 10%). The minimum detectable concentrations were 10, 20, 10 and 20 pg/ml for IL-2, IL-4, IL-5 and IFN-γ respectively.

Antigen-induced accumulation of T_h clones in the lung

Accumulation of the infused T_h clones in the lungs upon antigen challenge was first confirmed. CFSE-labeled FP3 cells were only slightly detectable around the vessels and bronchi upon saline challenge (Fig. 1A). Inhalation of the relevant antigen significantly increased the number of CFSE-labeled clones in the airway submucosa 24 h after antigen challenge (Fig. 1B). The infiltration of the infused T_h clones into the bronchi peaked at 48 h and persisted even after 96 h (data not shown).

Antigen-induced airway eosinophilia and the increase of EPO activity in the BALF of T_h clone-transferred mice

Late-phase eosinophil recruitment into the lung was induced upon antigen inhalation in the T_h clone-transferred mice, as we previously reported (35). To determine whether the accumulated eosinophils were activated and degranulated, the number of eosinophils and EPO activity in the BALF of FP3-transferred mice was measured. As shown in Fig. 2, the number of BALF eosinophils significantly increased 24 h after antigen challenge, reached a maximum at 192 h and then declined. EPO activity also increased following antigen challenge and reached a maximum 96 h after challenge, indicating that the accumulating eosinophils were activated and degranulated, despite the absence of antigen-specific Ig. Neither eosinophil number nor EPO activity was significantly changed in saline-challenged mice (data not shown).

Induction of BHR by antigen inhalation following airway eosinophilia

The kinetics of the bronchial responsiveness to acetylcholine was next examined to identify the chronological relationship between BHR and eosinophilic inflammation induced by antigen provocation in this model. As shown in Fig. 3, bronchial responsiveness to acetylcholine was significantly elevated 48 h after antigen provocation, reached a maximum 192 h after challenge and then gradually declined. It recovered to the pre-challenge level 4 weeks after challenge. The intensity of BHR was quite comparable compared with that induced in the ordinary antigen-sensitized murine asthma models (28).
Fig. 1. Antigen-induced accumulation of Th clones in the lungs. CFSE-labeled FP3 cells (2×10^7 cells/head) were transferred to unprimed mice by i.v. injection. After 24 h, these animals were challenged with inhaled OVA (100 mg/ml). Lung specimens were taken 24 h after saline (A) or antigen (B) provocation from mice with transfer of FP3 and examined under a confocal laser scanning microscope. The results shown are representative of four separate experiments. Bar = 50 µm.

No significant change in bronchial responsiveness occurred upon saline challenge (data not shown).

**Relationship between cytokine productions and BALF eosinophilia induced by various Th clones**

To verify the role of IL-5 produced by Th clones in the induction of airway eosinophilia, we tested several clones that produced various amounts of IL-5 upon activation. Th clones (5×10^5/ml) were cultured with irradiated spleen cells and OVA (100 µg/ml) to evaluate in vitro production of IL-2, IL-4, IL-5 and IFN-γ. These clones (5×10^6 cells) were transferred into unprimed mice and then the mice were challenged by antigen inhalation. The number of BALF eosinophils was enumerated 96 h after the challenge. There was a significant correlation between the amounts of IL-5 produced by the clones in vitro and the BALF eosinophils of the Th clone-transferred mice upon antigen inhalation (r² = 0.92, Fig. 4A). In contrast, the amounts of IL-2 (r² = 0.12, Fig. 4B), IL-4 (r² = 0.23, Fig. 4C) and IFN-γ (r² = 0.0070, Fig. 4D) produced by the Th clones did not significantly correlate with the airway eosinophilia. Bronchial responsiveness to acetylcholine was
IL-5-mediates bronchial hyper-responsiveness

Fig. 4. Relationship between IL-5 (A), IL-2 (B), IL-4 (C) and IFN-γ (D) production by various clones and eosinophil number in BALF of Th clone-transferred mice. To measure the cytokine productions, Th clones (5×10^5/ml) were cultured with X-ray-irradiated spleen cells (5×10^5/ml) in the presence or absence of OVA for 24 h. The concentration of IL-5 in the culture supernatants was measured by ELISA. To estimate OVA-induced airway eosinophilia of Th clone-transferred mice, various clones (2×10^7 cells/head) were transferred to unprimed mice by i.v. injection. After 24 h, these animals were challenged with inhaled OVA (100 mg/ml) for 60 min. Eosinophil numbers in BALF were measured 192 h after antigen provocation. Data are presented as the mean (n = 3–4). Each clone represents the respective symbol.

significantly up-regulated upon antigen challenge in the mice transferred with the clone that produced a large amount of IL-5 (closed circle in Fig. 4), whereas the clone that produced only a small amount of IL-5 (open triangle in Fig. 4) failed to induce BHR (data not shown).

Effects of neutralizing antibodies against IL-2, IL-4, IL-5 and IFN-γ on antigen-induced airway eosinophilia and BHR

To determine which cytokine might be responsible for the eosinophilic inflammation and BHR induced solely by the adoptive transfer of Th cells, neutralizing antibodies against IL-2, IL-4, IL-5 and IFN-γ were administered in vivo. Bronchial responsiveness to acetylcholine as well as BALF eosinophils was induced in FP3-transferred mice upon antigen challenge (Fig. 5). Administration of anti-IL-5 mAb almost completely inhibited the development of both airway eosinophilia and BHR. In contrast, mAb against IL-4 and IFN-γ augmented these responses, whereas anti-IL-2 mAb did not affect them. None of the mAb affected BALF eosinophils or bronchial responsiveness to acetylcholine of saline-challenged mice (data not shown). Of note, the number of BALF eosinophils correlated well with the magnitude of BHR (r^2 = 0.97, Fig. 5), indicating that BHR induced upon Th cell activation is related to eosinophilia.

Histologic examination

Histopathological examinations were performed to confirm the above findings (Fig. 6). Lung sections were prepared
Our present study clearly indicated that the adoptive transfer of T_δ cells into unprimed recipients successfully induced airway eosinophilic inflammation and BHR upon inhalation of the relevant antigen in an IL-5-dependent manner. CSFE-labeled T_δ cells accumulated into the bronchial mucosa and submucosa after antigen challenge. The magnitude of eosinophilic infiltration was well correlated with the extent of BHR, in good accordance with the close relationship between the two parameters reported in human asthma. Administration of anti-IL-4 mAb and anti-IFN-γ mAb aggravated both eosinophilia and BHR, whereas that of anti-IL-2 neutralizing antibody did not significantly affect them, indicating that IL-5 alone is essential for the development of eosinophilic inflammation, although IL-5 and IL-4 are often coordinately expressed by T_δ cells. A unique feature of our model system is that airway eosinophilia and BHR developed in the absence of humoral immune factors such as IgG, IgA and IgE antibodies. On the other hand, ordinary experimental asthma models employing actively sensitized animals cannot discriminate the roles played by T cells and antigen-specific Ig (13,16,26,32).

Moreover, airway eosinophilia and BHR was induced after repetitive antigen provocation in several asthma models using actively sensitized mice (13,16,26,32) and T_δ clone-transferred mice (42). Repetitive antigen challenges might well induce active sensitization of the animals and accordingly confounded the definite interpretation as to the mechanism of BHR. Our model is quite different from experimental asthma reported by others in that airway eosinophilia and BHR was induced solely by the infused T_δ clones upon single antigen challenge, and thus clearly demonstrated the casual relationship among T cell cytokines, airway eosinophilia and BHR.

Our findings are consistent with the previous report of Hogan et al. showing that CD4+ T_δ cells reconstituted the eosinophil infiltration and BHR in IL-5-deficient mice (43). The findings obtained in the present study, i.e. (i) anti-IL-5 mAb abrogated not only the inflammatory responses including eosinophil infiltration but also the induction of BHR (Figs 5 and 6), (ii) the number of BALF eosinophils highly correlated with the magnitude of BHR (Fig. 5) and (iii) BHR emerged after the increase of EPO activity which paralleled with eosinophil infiltration into the airways (Figs 2 and 3), support the view that the activated eosinophils that accumulated into the lung mainly regulate the development of BHR by causing airway epithelial damage through the production of toxic granule proteins. Moreover, the demonstration of the increase in EPO activity clearly indicates that the accumulating eosinophils were activated and degranulated in vivo. The finding that eosinophils could degranulate in the absence of antigen-specific Ig is especially intriguing, considering that secretary IgA and IgG have been postulated as potent inducers of degranulation for human eosinophils (44). The precise mechanism of eosinophil activation in our model warrants further investigation, as several other molecules such as platelet activating factor, CSa, cytokines, etc., also induced degranulation at least in vivo (45). The possibility that the release of EPO into the surrounding tissue may result from eosinophil cytolysis seems less likely, since the kinetics of the EPO activity preceded that of the eosinophil accumulation. In addition, shrivelled-shaped or damaged eosinophils were hardly observed by the histologic examinations (Fig. 6), suggesting that BALF EPO activity mainly stemmed from the active degranulation.

Discussion

Our present study clearly indicated that the adoptive transfer of T_δ cells into unprimed recipients successfully induced airway eosinophilic inflammation and BHR upon inhalation of the relevant antigen in an IL-5-dependent manner. CSFE-labeled T_δ cells accumulated into the bronchial mucosa and submucosa after antigen challenge. The magnitude of eosinophilic infiltration was well correlated with the extent of BHR, in good accordance with the close relationship between the two parameters reported in human asthma. Administration of anti-IL-4 mAb and anti-IFN-γ mAb aggravated both eosinophilia and BHR, whereas that of anti-IL-2 neutralizing antibody did not significantly affect them, indicating that IL-5 alone is essential for the development of eosinophilic inflammation, although IL-5 and IL-4 are often coordinately expressed by T_δ cells. A unique feature of our model system is that airway eosinophilia and BHR developed in the absence of humoral immune factors such as IgG, IgA and IgE antibodies. On the other hand, ordinary experimental asthma models employing actively sensitized animals cannot discriminate the roles played by T cells and antigen-specific Ig (13,16,26,32).

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Our present findings pose a discrepancy in several important aspects with previous reports made by others that showed dissociation between eosinophilic inflammation and BHR (13–16,46). Martin et al. reported that the administration of anti-IgE antibody resulted in BHR in normal littermate mice but not in the mast cell-deficient mice (46). In their studies, activation of mast cells resulted in BHR in the absence of detectable eosinophilic infiltration into the lung (15,46). It was also reported that the administration of anti-IL-4 mAb
Fig. 6. Anti-IL-5 mAb prevented induction of eosinophilic inflammation in the lungs of T₈ clone-transferred mice. FP3 cells (2×10⁵) were transferred to normal mice by i.v. injection. After 24 h, these animals were challenged with inhaled antigen. Lung specimens were taken from saline-challenged mice (A), OVA-challenged mice (B and C), and OVA-challenged mice with anti-IL-5 mAb (D), anti-IL-2 mAb (E), anti-IL-4 (F) and anti-IFN-γ (G) treatment 192 h after challenge, and stained with hematoxylin & eosin. Arrow heads show infiltrated eosinophils. The results shown are representative of three separate experiments. Bar = 50 µm.
abrogated BHR without any effects on airway eosinophilia (13). Administration of IFN-γ completely inhibited the induction of BHR without affecting airway eosinophilia (16). These investigations implicated that BHR observed in their models was independent of eosinophilic inflammation, which is rather a hallmark of human asthma.

The reason for the discrepancy is not fully clear at this moment, but bronchial responsiveness was examined only at a very early stage (~24 h) in most studies which reported a dissociation between eosinophilia and BHR. BHR in our study became evident relatively late in its kinetics (Fig. 3). BHR, which develops independently of eosinophil accumulation, might be essentially different in nature from that accompanied by eosinophilic inflammation. We recently demonstrated that mast cells contributed to the infiltration of eosinophils into the lung only at the early stage and, besides, CD4+ T cells mostly regulated it (47). The protracted BHR observed in our model was dependent on CD4+ T cells and IL-5. Considering that the severity of asthma symptoms and BHR is reported to be well correlated with the magnitude of eosinophilic infiltration (4,11) and activation of CD4+ T cells (18,22), our model seems to better reflect the pathophysiology of human asthma compared to the preceding investigations.

It has been suggested, on the other hand, that CD4+ T cells have the potential to directly affect bronchial responsiveness independent of eosinophilic inflammation (48,49). De Sanctis et al. reported that transfer of CD4+ T cells of a BHR strain of mice to a hypo-responsive strain conferred BHR (49). In contrast, BHR was induced only upon antigen challenge in our model, whereas the transfer of Th2 clones by itself did not confer BHR at all. In addition, the transfer of CD4+ T cells that produced a small amount of IL-5 failed to induce airway eosinophilia (Fig. 4) and BHR (35). Our present result supports a notion that BHR is a consequence of activation of ‘competent’ T cells. In humans, severity of asthma was well correlated with the activation markers of T cells such as expression of CD25 and various cytokines (18,24).

The mode of action of IL-5 on the development of BHR could be multifold. Anti-IL-5 mAb did not affect the infiltration of lymphocytes into the lung (Fig. 6D), suggesting that the suppression of BHR by anti-IL-5 mAb was not caused by the inhibition of CD4+ T cell activation. There is another possibility that IL-5 directly induces BHR independently of the eosinophil infiltration into the airways, although, IL-5 concentration in BALF reached a maximum 48 h after antigen provocation and then immediately declined (35). The time course of BHR induction, which peaked relatively late (192 h) in the present study, rather excluded the direct contribution of IL-5 to BHR.

Mediators other than IL-5 have also been implicated in the induction of airway eosinophilia and BHR. For example, IL-4 might play pivotal roles in orchestrating the inflammatory response in animal models (13,33), as IL-4 is essential for IgE production and Th2 development in vivo (50,51). It also enhances endothelial cell expression of VCAM-1, which may be involved in the selective recruitment of eosinophils (24,52–55). Antigen-induced BHR was abolished in IL-4-deficient mice (50) and mice treated with anti-IL-4 mAb (13). On the contrary, Th2 clones derived from IL-4-deficient mice did induce BHR without causing eosinophilia when transferred into unprimed mice, whereas Th1 clones derived from the control mice that produced IL-4 induced both airway eosinophilia and BHR (42). The role of IL-4 and eosinophilia in the development of BHR seems complex. So, it is intriguing to find that the administration of anti-IL-4 neutralizing mAb rather aggravated both eosinophil infiltration and BHR in our model (Fig. 5). Previous reports showing the contribution of IL-4 on eosinophil infiltration only investigated the very early phase of eosinophilia and BHR (13) or employed IL-4-deficient mice (50,51), complicating a direct comparison with our results. We have reported that mast cells do transiently play a minor role only in the early phase of pulmonary eosinophilia (47). Preliminary histologic analysis suggests that anti-IL-4 mAb inhibited early-phase (48 h) eosinophil accumulation (data not shown). The contribution of IL-4 to the eosinophilic inflammation might be different, depending on the kinetics and the relative cellular composition of the response following the antigen challenge. Therefore, further examinations, such as a time-course study using anti-IL-4 mAb, will be needed to fully explain the discrepancy between our present findings and previous reports, and it is currently underway.

Recently, Hawker et al. demonstrated that IL-4 inhibits the proliferation of airway smooth muscle cell in vitro (56). Hypertrophy of airway smooth muscle might contribute to the development of BHR by narrowing the airway caliber (57). There is a possibility that the administration of anti-IL-4 mAb might restore the proliferation of airway smooth muscle in our model, thus aggravating BHR; or else, IL-4 might be involved in BHR by promoting IgE production and accordingly activating mast cells, since histamine and tryptase produced by mast cells enhance the proliferation of airway smooth muscle (58,59), resulting in BHR independently of eosinophilia. The potentially ambiguous contribution of IL-4 to the development of BHR might be simplified due to the lack of the contribution of IgE/mast cell system.

Anti-IFN-γ mAb enhanced both airway eosinophilia and BHR in our model (Fig. 5), consistent with the findings made by others that the administration of IFN-γ suppressed both eosinophil recruitment (60,61) and BHR (62,63), although there is one report that indicated that IFN-γ directly induced BHR (16). Iwamoto et al. reported that IFN-γ regulates eosinophil recruitment into the airways by inhibiting the infiltration of CD4+ T cells using OVA-sensitized mice (60). In addition, IFN-γ-producing Th1 clones significantly suppressed airway eosinophilia induced upon antigen challenge when co-transferred with Th2 clones into recipients (64). Our present finding confirmed their results in a totally different situation. IFN-γ produced by Th1 clones might inhibit further infiltration of themselves in an autocrine manner, thus regulating eosinophil accumulation and BHR.

The capacity of Th1 clones to produce IL-4 and IFN-γ in vitro did not correlate with the severity of airway eosinophilia and BHR (Fig. 4), whereas the administration of mAb against IL-4 and IFN-γ exacerbated both airway eosinophilia and BHR (Fig. 5). The discrepancy cannot be fully explained at this moment, but there is a possibility that relatively small amounts of IL-4 and IFN-γ may be sufficient to display their effects on the airway infiltration of eosinophils and BHR; or the involvement of multiple cytokines and cells on the complex responses might
complicate the in vivo analysis, leaving the relatively large contribution of IL-5 alone to be significant.

Goblet cell hyperplasia and mucus hypersecretion are the typical pathological features of bronchial asthma in humans. Shimura et al. suggested that enhanced mucus secretion in the asthmatic airways might clinically contribute to BHR (65). Several reports demonstrated the dissociation between mucus secretion and airway eosinophilia/BHR in mice. Administration of leukotriene antagonists inhibited airway mucus secretion and eosinophilia, without affecting BHR in murine asthma (14). Intravenous administration of anti-CD49d neutralizing mAb prevented eosinophil recruitment into the lung, but did not inhibit BHR or mucus hypersecretion (66). Selective expression of IL-4 in the bronchial epithelium elicited an inflammatory response characterized by the epithelial cell hypertrophy and the accumulation of macrophages, lymphocytes, eosinophils and neutrophils in the absence of apparent BHR (67). The lack of the induction of airway eosinophilia and BHR in STAT6-deficient mice was overcome by the administration of IL-5, although the mucus production at the airway epithelium was not restored (68). The present finding that the administration of anti-IL-5 mAb did not inhibit goblet cell hyperplasia or mucus hypersecretion (Fig. 6D) is intriguing, considering the complex nature of airway remodeling.

Our present findings demonstrating the critical role of IL-5 in the development of airway eosinophilic inflammation associated with BHR seem to be inconsistent with the clinical finding recently reported by Leckie et al. (69) that administration of anti-IL-5 mAb to asthmatic patients failed to affect the development of early asthmatic response and late asthmatic response after allergen exposure. The neutralization experiments can reach definite conclusions only when the neutralization achieved by either inhibitors or antibodies is complete. The extent of neutralization reported by clinical trial of anti-IL-5 mAb is apparently not complete, as significant amounts of eosinophils still remained locally in the sputum, although some decrease was reported. In our current study, animals were administered 100 mg/kg mAb, whereas their ‘high-dose’ group was administered 10 mg/kg mAb. The lack of the clinical effect of the antibody could be explained by the lack of neutralization of IL-5 produced locally in the airways or by the lack of contribution of IL-5 to clinical asthma, as they mentioned. The clinical observation may provide some implication but warrants cautious interpretation. The possibility that anti-IL-5 therapy might have any effect on the infiltration of inflammatory cells including eosinophils and T cells into the airway mucosa and submucosa of asthma patients should be explored by immunohistochemical examinations to reach a firm conclusion.

The experiments using several T\textsubscript{H} clones with various IL-5-producing capacities and neutralizing antibodies against cytokines collectively indicated that BHR induced by the infused T\textsubscript{H} clones upon antigen stimulation is related to the eosinophil infiltration which is also IL-5 dependent. In conclusion, our present findings support the notion that CD4\textsuperscript{+} T cells are responsible for both airway eosinophilic inflammation and BHR through the production of IL-5. BHR seemed to be essentially related to the eosinophils. Agents that down-regulate IL-5 production seem to control airway eosinophilic inflammation in allergic disorders.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BHR</td>
<td>bronchial hyper-reactiveness</td>
</tr>
<tr>
<td>CFSE</td>
<td>5- (and-6)-carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
</tr>
<tr>
<td>MBP</td>
<td>major basic protein</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
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<tr>
<td>PD\textsubscript{50}</td>
<td>50% of the maximal value</td>
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References
IL-5 mediates bronchial hyper-responsiveness


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