Original Article

Effect of repeated antigen inhalation on airway inflammation and bronchial responsiveness to acetylcholine in interleukin-5 transgenic mice

Hiroyuki Tanaka,¹ Naoki Kawada,¹ Kenji Kawada,² Kiyoshi Takatsu³ and Hiroichi Nagai¹

¹Department of Pharmacology, Gifu Pharmaceutical University, ²Gifu College of Medical Technology, Gifu and ³Department of Immunology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

ABSTRACT

Bronchial hyperresponsiveness (BHR) is a characteristic feature of bronchial asthma, yet its precise mechanism remains obscure. Human studies have demonstrated that T helper 2 cytokines, including interleukin (IL)-4 and IL-5, are involved in the development of airway inflammation and BHR. In the present study, we examined the role of IL-5 in the onset and aggravation of bronchial responsiveness to acetylcholine in two strains of IL-5 transgenic (Tg) mice, derived from the C3H/HeN and BALB/c strains. Three inhalations of antigen (ovalbumin) caused an increase in the number of eosinophils in bronchoalveolar lavage fluid (BALF) and a significant elevation in serum IgE in wild-type mice. In contrast with wild-type animals, systemic overproduction of IL-5 resulted in massive airway eosinophilia, especially around the peribronchi and perivascular regions of the tissues, after repeated antigen provocation. In C3H/HeN background IL-5 Tg mice, repeated antigen provocation did not induce BHR similar to that of wild-type mice. In contrast, antigen-induced BHR was observed in BALB/c-background mice, but there were no significant differences between the magnitude of BHR in wild-type and IL-5 Tg mice. Furthermore, antigen-induced IL-5 production in BALF was detected in both C3H/HeN and BALB/c mice and was elevated to a similar degree in both wild-type and IL-5 Tg mice. These findings suggest that systemic overproduction of IL-5 or airway eosinophilia is not, by itself, important in the development or aggravation of antigen-induced BHR in mice.

Key words: bronchial hyperresponsiveness, eosinophils, interleukin-5, mice.

INTRODUCTION

Bronchial hyperresponsiveness (BHR) is a characteristic feature of most asthmatics and it correlates with the severity of the disease.¹ Recent investigations have demonstrated that eosinophilic inflammation of the airway is another important feature of asthma² and airway inflammation is thought to be a causative factor in the development of BHR. Among the candidate factors, which include cytokines and inflammatory mediators, particular interest has been paid to interleukin (IL)-5 because IL-5 is a crucial factor in the control of eosinophil functions, such as differentiation,³ prolongation of survival,⁴ activation and degranulation.⁵ Moreover, eosinophil-derived mediators appear to play an important role in the development of BHR.⁶⁻⁸

Recent studies have investigated the role of IL-5 in the development of BHR using monoclonal antibodies (mAbs),⁹⁻¹⁴ transgenic (Tg) mice¹⁵⁻¹⁷ and gene-disrupted mice;¹⁸ however, the correlation between airway eosinophilia and airway function remains controversial.¹²,¹⁹,²⁰ Furthermore, evidence for a role of IL-5 in the development of BHR is conflicting.⁹,¹⁰,¹⁴,¹⁵,¹⁷,¹⁸
We have recently demonstrated that repeated antigen inhalation leads to an increase in the level of T helper (Th) 2 cytokines, including IL-4 and IL-5, in the number of inflammatory cells, mainly eosinophils, in bronchoalveolar lavage fluid (BALF) and in BHR to acetylcholine (ACH) with a significant elevation in serum antigen-specific IgE in sensitized BALB/c mice. Furthermore, the development of antigen-induced BHR is T lymphocyte dependent and mast cell dependent, suggesting that a Th2 pattern of cytokine production and IgE-mediated responses may be associated with the development of these allergic responses. We have also investigated the role of IL-5 in the onset of airway eosinophilia and BHR using a mouse model. However, anti-IL-5 mAb and IL-5 soluble receptor α chain administered during antigen inhalation, failed to inhibit BHR in sensitized mice. In contrast, we examined the role of IL-5 receptor α chain using mice that are genetically deficient in the receptor α chain. As a result, the IL-5 receptor α chain and IL-5 showed a crucial role in the onset of airway eosinophilia and BHR, suggesting that IL-5 is involved in the development of allergen-induced BHR, although it appears to be difficult to regulate its function during the effector phase. Strategies of gene manipulation, such as ‘knockout’ or ‘transgenic’, are important methods to understand the roles of certain molecules, especially in mice. However, recent studies have reported that the genetic background may directly influence the interpretation of the effect of gene disruption in some cases. Regarding bronchial responsiveness to stimuli, several studies have reported on different strains of inbred mice with differences in airway responsiveness. Of nine common inbred mice, A/J and AKR/J mice showed the greatest responsiveness to ACh injected exogenously and BALB/c showed a moderate responsiveness, whereas C57BL/6, SJL/J and C3H/HeJ mice exhibited the least responsiveness. In addition to airway responsiveness, it has been reported, especially in mice, that the production of IgE also depends on the genetic background.

To date, however, no studies have investigated the role of IL-5 using different strains of IL-5 Tg mice in the same experimental protocol. Thus, the purpose of present study was to clarify the role of IL-5 in the development of aeroallergen-induced airway inflammation and BHR using two different strain backgrounds of IL-5 Tg mice, namely C3H/HeN and BALB/c.

METHODS

Animals

Seven-week-old male IL-5 Tg mice (derived from the C3H/HeN and BALB/c strains) weighing 25–30 g were prepared and maintained as described previously. Age-matched male animals were used as wild-type controls. All animals were housed in plastic cages in an air-conditioned room at 22 ± 1°C with a relative humidity of 60 ± 1%, were fed a standard laboratory diet and were given water ad libitum. Experiments were performed following the 1987 guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animals Science.

Agents

Ovalbumin (OA; Seikagaku Kogyo, Tokyo, Japan), acetylcholine chloride (ACh; Nacalai Tesque, Kyoto, Japan), bovine serum albumin (BSA; Seikagaku Kogyo), Turk solution (Wako Pure Chemical Industries, Osaka, Japan), pancronium bromide (Sigma Chemical Co., St Louis, MO, USA), pentobarbital sodium (Abbott Laboratories, Chicago, IL, USA), disodium ethylenediaminetetraacetic acid (EDTA-2Na; Nacalai Tesque), Diff-Quick solution (International Reagent Corp., Kobe, Japan), monoclonal antimouse IgE antibody (LO-ME-3; Serotek, Oxford, UK), monoclonal antidinitrophenol IgE antibody (anti-DNP IgE, Clone SPE-7; Sigma Chemical Co.), peroxidase-conjugated streptavidin (Dakopatts a/s, Glostrup, Denmark) and peroxidase-labeled antimouse IgE (Nordic Immunology, Tilburg, Netherlands) were purchased commercially.

Sensitization and antigen challenge

Mice were actively sensitized by intraperitoneal injections of 50 µg OA with 1 mg alum on days 0 and 12. Starting on day 22, mice were exposed to OA (1% w/v diluted in sterile saline) for 30 min three times every 4th day according to a previously reported method. Non-sensitized animals received 1 mg alum and were exposed to saline in a similar manner. In a previous study, we compared the effect of sensitization on airway function and airway inflammation in saline-exposed animals; there were no significant differences in these parameters. Thus, we chose two groups for the present study. The aerosol (particle size 2.0–6.0 µm) was generated by a nebulizer (ultrasonic nebulizer TUR-3200; Nihon Koden, Tokyo,
Japan) driven by filling a perspex cylinder chamber (5.5 cm diameter, 12 cm height) with a nebulized solution.

**Measurement of immunoglobulins**

Immediately before the last antigen challenge, blood was collected and the serum was obtained by centrifugation and was stored at −80°C. Antigen-specific IgE and total IgE in mouse serum were measured using ELISA, as described previously.23 Briefly, serum OA-specific IgE was measured by coating monoclonal rat antimouse IgE antibody (LO-ME-3) at a concentration of 5 µg/mL. After blocking with 1% BSA, serum dilutions were incubated for 1 h followed by biotinylated-OA and peroxidase-conjugated streptavidin. Sequentially diluted monoclonal anti-OA IgE was used as a standard. Serum total IgE was measured by coating monoclonal rat antimouse IgE antibody (LO-ME-3) at a concentration of 5 µg/mL. After blocking with 1% BSA, serum dilutions were incubated for 1 h followed by peroxidase-labeled antimouse IgE. Sequentially diluted monoclonal anti-DNP IgE antibody (SPE-7) was used as a standard. Optical densities of the enzymatic reactions were measured using an automatic ELISA plate reader (Titertek Multiscan MCC/340; Lab Systems Oy, Helsinki, Finland) at 450 nm (reference wavelength 690 nm). The detection limit was 10 ng/mL.

**Bronchoalveolar lavage study**

To evaluate airway inflammation, we studied the accumulation of inflammatory cells in bronchoalveolar lavage fluid (BALF). Experiments were performed according to previously described methods.9,21 Briefly, 24 h after the last inhalation of antigen (30 days after the first immunization), test animals were killed by intraperitoneal injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and the air lumen was washed four times with 1 mL calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.1% BSA and 0.05 mmol/L EDTA-2Na. The BALF obtained from each animal was pooled in a plastic tube, cooled in ice and centrifuged (150 g) at 4°C for 10 min. Cell pellets were resuspended in the same buffer (1 mL) and the procedure was repeated three times (total volume 3 mL; recovery > 85%). The number of nucleated cells in BALF stained with Turk solution was counted in a Bürker chamber and a differential count under a microscope (× 500) was made on a smear of at least 300 cells prepared with a cytocentrifuge (Cytospin II, Shandon, England) and stained with Diff-Quick solution (based on standard morphologic criteria). The amount of IL-5 in the sera or BALF was measured using ELISA (Endogen Inc., Cambridge, MA, USA).

**Measurement of airway function**

Measurement of airway responsiveness to ACh was performed according to methods described previously with some modifications.23 Briefly, in order to measure airway responsiveness to ACh, mice were anesthetized with sodium pentobarbitone (80 mg/kg, i.p.) and the jugular vein was cannulated for intravenous injection of ACh. Mice were injected with pancronium bromide (0.1 mg/kg, i.v.) to suppress spontaneous respiration and animals were ventilated with a rodent ventilator (New England Medical Instruments, Medway, MA, USA) with air supplemented with oxygen at 60 strokes/min, with a stroke volume of 0.6 mL/animal. Bronchoconstriction was measured according to the overflow method described by Konzett and Rössler,34 using a bronchospasm transducer (Ugo Basile 7020, Milano, Italy) connected to the tracheal cannula. To measure the bronchial responsiveness to ACh, changes in the respiratory overflow volume were measured using increasing doses of ACh. The increase in respiratory overflow volume provoked by ACh is represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. Area under the curve (AUC) was calculated from the data of the dose–response curve for ACh (range 125–2000 µg/kg), as described previously.23

**Histologic study**

Mice were killed 24 h after the third antigen inhalation and whole lungs were distented by instillation of 10% buffered formalin via the trachea, removed and immersed in the same fixative with the trachea clamped for 24 h. Tissues were sliced and embedded in paraffin and 6 µm sections were stained with hematoxylin and eosin for light microscopic examination.

**Statistical analyses**

Values are presented as the mean±SEM. Statistical significance between saline- and OA-inhaled animals or between wild-type and Tg animals was estimated using the two-tailed Student’s t-test or the Aspin–Welch test. *P* < 0.05 was considered statistically significant.
RESULTS

Effect of IL-5 transgene on BAL leukocyte influx and local IL-5 production in the airways

To test whether systemic IL-5 overproduction influences BAL and airway leukocyte accumulation and also local IL-5 production in the airways, IL-5 Tg mice were immunized with OA and exposed to OA aerosol three times and compared with age- and sex-matched wild-type mice. On day 31, 24 h after the last antigen inhalation, BAL was performed.

In IL-5 Tg mice, repeated antigen provocation caused striking BAL eosinophilia and also marked increases in the number of total leukocytes and lymphocytes compared with saline-injected control groups (Fig. 1). In contrast with each IL-5 Tg mouse, analysis of BAL cells of OA-exposed wild-type mice revealed a selective increase in the number of eosinophils. The number of total leukocytes and lymphocytes also increased or tended to increase.

Histologic analysis of lungs, taken from saline-injected wild-type and IL-5 Tg mice, showed normal lung histology with no inflammation and no OA-inhaled wild-type mice exhibited pulmonary eosinophilic inflammation. Infiltrating eosinophils were mainly seen in the peribronchial and perivascular regions of the lungs (Fig. 2b,f). Furthermore, after aeroantigen challenge of IL-5 Tg mice, a marked increase in the number of eosinophils was observed, especially in the peribronchial and perivascular regions (Fig. 2d,h). There were no significant differences in histologic analyses among the OA-inhaled groups, except for the increased number of eosinophils and lymphocytes in IL-5 Tg animals.

The amount of IL-5 in BALF after antigen challenge is shown in Fig. 3. In wild-type mice, the level of IL-5 in BALF was significantly elevated 24 h after aeroantigen challenge, although the amount of IL-5 in BALF from C3H/HeN mice was less than that of BALB/c mice. Furthermore, there were no significant differences in IL-5
production after antigen challenge between wild-type and IL-5 Tg mice (Fig. 3).

**Effect of IL-5 transgene on serum IgE levels**

To study the role of the IL-5 transgene in antigen-induced IgE production, total serum IgE and antigen-specific IgE levels were measured. Serum was obtained from mice on day 30, immediately before the last antigen provocation. Sensitization and repeated antigen provocation caused significant increases in the level of total serum IgE and antigen-specific IgE when compared with the saline-injected groups; however, there were no significant differences between wild-type and IL-5 Tg mice (Fig. 4). In contrast with the OA-inhaled groups, total IgE did not increase and also OA-specific IgE remained below the detection limit (<10 ng/mL) in the serum of saline-injected groups.
Effect of the IL-5 transgene on airway responsiveness to ACh

To clarify the effect of systemic IL-5 overproduction on airway function, airway responsiveness to ACh was examined. Figure 5 shows the AUC of the dose–response to ACh in all groups.

In C3H/HeN mice, BHR to ACh was not observed in either wild-type or IL-5 Tg mice, even after repeated antigen challenge. In contrast, repeated aeroantigen provocation caused a significant increase in the airway responsiveness to ACh in both wild-type and IL-5 Tg mice of the BALB/c strain, although there were no significant differences between wild-type and IL-5 Tg mice.

DISCUSSION

In the present study, we demonstrated that repeated antigen inhalation caused a marked increase in the number of eosinophils in BALF and in the airways of sensitized IL-5 Tg mice. Furthermore, antigen-induced IL-5 production in BALF was observed in both strains, but showed a similar level in wild-type and IL-5 Tg mice. In addition, antigen-induced BHR was observed in BALB/c mice, whereas the BHR did not develop in C3H/HeN mice, even in IL-5 Tg mice. Thus, the present findings suggest that systemic overproduction of IL-5 or airway eosinophilia is not, by itself, a causative factor in the development or aggravation of BHR.

Clinical studies have demonstrated that the number of CD4+ T cells and eosinophils often correlates with disease severity. As described above, it has been reported that IL-5 regulates eosinopoiesis, eosinophil survival and activation. In both IL-5 Tg mice strains, repeated antigen challenge resulted in massive airway eosinophilia, compared with wild-type mice, whereas antigen-induced IL-5 production in BALF was observed in both strains and showed a similar level in wild-type and IL-5 Tg mice, suggesting that IL-5 plays an important role in eosinophil differentiation rather than as chemotactic factor into the airways. These findings are in agreement with reports of differentiation of in vitro eosinophils by IL-5 and suppression of in vivo eosinophilia by mAb to IL-5 in mice. Thus, cytologic and histologic examination of antigen-inhaled IL-5 Tg mice revealed a crucial role for IL-5 in the induction of airway eosinophilia.

Second, we examined whether the IL-5 transgene influences IgE production provoked by repeated antigen provocation in sensitized mice. It has been reported that IL-5 potentiates the IL-4-induced IgE response in vitro; however, the present findings showed no significant differences in antigen-induced IgE production between wild-type and IL-5 Tg mice. Thus, antigen-induced IgE response in this model may not be influenced by IL-5.

The main question to be discussed in the present study is whether systemic overproduction of IL-5 by itself induces BHR and whether it augments or aggravates antigen-induced BHR, which was observed in wild-type mice. Antigen provocation caused an increase in the
number of eosinophils and lymphocytes around vessels and bronchi in humans and, as shown here, in wild-type and IL-5 Tg mice strains. The present findings show that systemic overproduction of IL-5 caused an increase in the number of eosinophils without either immunization or antigen challenge in IL-5 Tg mice and that repeated antigen challenge caused massive airway eosinophilia in IL-5 Tg mice of both strains. In contrast with the role of IL-5 in airway eosinophilia, saline-injected IL-5 Tg mice with slight airway eosinophilia did not exhibit BHR for both strains. In addition, in OA-inhaled C3H/HeN-background mice, antigen challenge did not induce BHR, even in IL-5 Tg mice, which revealed massive airway eosinophilia. Furthermore, antigen-induced BHR was observed in both BALB/c strains, but the exaggeration of BHR was not observed in IL-5 Tg mice. These findings suggest that neither systemic IL-5 overproduction nor airway eosinophilia is, by itself, important for the development or augmentation of BHR. These observations are in agreement with recent clinical studies, in which administration of a humanized blocking mAb to IL-5 did not improve the airway function of asthmatics, whereas it clearly decreased the number of eosinophils in sputum and peripheral blood.

In the present study, BHR was not observed after aeroallergen challenge in either wild-type or IL-5 Tg
In contrast, BHR was not augmented after aeroallergen challenge even in IL-5 Tg BALB/c mice, whereas striking airway eosinophilia was observed. The reason why the aggravation of BHR was not detected in the IL-5 Tg mice is unknown; however, some possibilities exist. The degree of BHR observed in wild-type mice was already maximal; thus, we could not observe exaggeration of BHR in IL-5 Tg mice. However, we recently established another chronic asthma model in BALB/c mice. In this model, mice were sensitized using an identical method and then challenged with aeroallergen (1%) for 3 consecutive weeks, instead of three times every 4th day. As a result, the degree of BHR was stronger and more severe than that observed in the present study (data not shown). The other possibility is that infiltrated eosinophils are not fully activated, although the number of eosinophils in BALF or lung tissue was significantly enhanced in IL-5 Tg mice. Thus, further experiments will be needed to clarify whether eosinophils are activated.

Recently, the use of IL-5 transgenic animals in biologic and pathologic studies has become a standard investigative approach; however, the correlation between airway eosinophilia and airway function remains controversial. Lefort et al. have demonstrated that systemic IL-5 production influences antigen-induced airway eosinophilia, but not airway function, in CBA background mice, which did not exhibit antigen-induced BHR even in wild-type mice. In contrast, Lee et al. have reported that a lung-specific IL-5 transgene could induce BHR without immunization or antigen challenge. In addition, Iwamoto and Takatsu investigated the role of IL-5 in antigen-induced BHR using the same mice strains used in the present study, suggesting IL-5 is an important factor in the onset of BHR. These conflicting findings, including the present observations, may be due to either lung-specific effects, different protocols or strain gene backgrounds.

In conclusion, the present study indicated that systemic overproduction of IL-5 or airway eosinophilia is not, by itself, required in the development and aggravation of BHR.

REFERENCES


