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Repeated instillations of *Dermatophagoides farinae* into the airways can induce Th2-dependent airway hyperresponsiveness, eosinophilia and remodeling in mice
Effect of intratracheal treatment of fluticasone propionate

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Abstract

Dermatophagoides farinae are known to be a common environmental allergen causing allergic asthma; however, little is known about their pathophysiological effect via the allergenicities in vivo. Therefore, we first established a mouse model of asthma induced by repeated instillations of *D. farinae*. Second, to investigate whether the asthmatic responses are Th2-dependent, we examined the effect of the deficiency of interleukin-4 (IL-4) receptor α chain gene. Finally, we examined the effect of fluticasone propionate on this model. Mice were instilled with *D. farinae* without additional adjuvants into the trachea 8 times. After the final allergen instillation, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage and histological examination were carried out. The instillation of the allergen-induced airway hyperresponsiveness, the accumulation of inflammatory cells and increases in the levels of Th2 cytokines and transforming growth factor-β_1 production in the bronchoalveolar lavage fluid dose dependently. The number of goblet cells in the epithelium and the extent of the fibrotic area beneath the basement membrane were also increased in the morphometric study. In contrast, the defect of IL-4/IL-13 signaling through IL-4 receptor α chain completely abrogated all these responses. Furthermore, the simultaneous instillation of fluticasone propionate with the allergen showed significant inhibition or an inhibitory tendency of these changes. These findings demonstrate that the repetitive intratracheal instillations of *D. farinae* can induce airway remodeling through Th2-type inflammation, and that fluticasone propionate inhibits *D. farinae*-induced airway remodeling in mice, and this model would be useful for studying mechanisms involved in the development of allergic asthma.

Keywords: Airway; Asthma; Corticosteroid; *Dermatophagoides farinae*; Eosinophil; Goblet cell; IL-13; Mouse; Subepithelial fibrosis

1. Introduction

Bronchial asthma is one of the most common health problems in the worldwide, especially within industrialized societies, and the prevalence rates have been increasing considerably over the last few decades (Mannino et al., 2002; Robertson et al., 2004; Verlato et al., 2003), for reason that are not yet completely understood. Changes in lifestyle and an increase in indoor allergen exposure caused by higher indoor temperature and humidity have been suggested as potential determinants, and it is reasonable to consider that environmental exposures to allergens are of primary importance for the prevalence and development of asthma, in genetically predisposed individuals, because genes

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controlling the inflammatory responses, IgE production, cytokine and chemokine production, airway remodeling as well as airway function, has not changed significantly in the last few decades. Among allergens like ragweed, pollens, house dust mite, and cockroach, house dust mites including *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* are known to be principle allergen for the induction of asthma (Huss et al., 2001; Platts-Mills et al., 1997; Sporik et al., 1990), although the precise molecular mechanisms underlying the allergenicity of the allergens is not fully understood.

In contrast to the increased prevalence of allergic airway diseases, the control of bronchial asthma is becoming easier due to the wide use of inhaled corticosteroid. However, there are still some patients that are resistant to medical treatment. Airway remodeling, which is characterized by goblet cell hyperplasia/hypertrophy, subepithelial fibrosis and smooth muscle hyperplasia/hypertrophy (Aikawa et al., 1992; Heard and Hossain, 1973; Roche et al., 1989), is one of the causes of this problem. Although these structural changes have been considered to be characteristics of chronic and severe asthma, the latest clinical studies, using bronchial biopsy sampling, have demonstrated that they may exist even in patients with mild asthma and early in the disease process. Roche et al. reported that the deposition of collagen beneath the bronchial epithelium was also observed in young patients with mild atopic asthma (Roche et al., 1989), and Boulet et al. demonstrated that the degree of subepithelial collagen deposition in patients with mild asthma recently diagnosed was not significantly different from those of long-standing mild asthma (Boulet et al., 2000). Therefore, the comprehension of the mechanisms underlying airway remodeling is becoming an increasingly important problem, and the development of new anti-remodeling agents is strongly desired.

Animal models have been used for a long time to analyze the pathophysiology of human diseases and to seek new remedies for various diseases. In the case of bronchial asthma, guinea pigs, rats and mice have mainly been used (Pauluhn and Mohr, 2005). Although each animal has some good characteristics, recently, mice have been widely used because this species allows for the application in vivo of a broad range of immunological tools, including gene deletion technology (Kips et al., 2003). To date, in typical experiments using mice, they are systemically immunized to ovalbumin with a T helper type 2 (Th2)-skewing adjuvant, such as aluminum hydroxide gel, and challenged through airway application of the antigen in the form of an aerosol or an intranasal droplet aspiration. In fact, these murine models have been useful in understanding the immune responses, such as Th2 dominant phenotypes underlying allergic sensitization and airway eosinophilic inflammation (Wills-Karp, 2000), but it is still unknown how allergic sensitization via the mucosal surfaces of airways with aeroallergens, including house dust mite, is induced. Several studies have previously investigated aeroallergen-induced airway inflammation in vivo (Johnson et al., 2004; Sadakane et al., 2002; Yu et al., 1999). These studies demonstrated that repeated instillations of *D. farinae* without additional adjuvants can induce airway eosinophilic inflammation, probably through Th2-polarized responses in mice; however, it remains to be determined whether the repeated inoculation of *D. farinae* can induce local Th2 responses and airway remodeling, as well as airway inflammation and airway hyperresponsiveness, and whether the allergic responses induced by this allergen are Th2-dependent. Moreover, the effect of fluticasone propionate on *D. farinae*-induced airway eosinophilic inflammation, hyperresponsiveness to acetylcholine and remodeling in vivo has not been elucidated.

Therefore, to address these unanswered questions, we first established a *D. farinae*-induced airway inflammation using mice, which was locally immunized without any additional adjuvants. Then, we examined the characteristics of this model regarding the local Th1/Th2 balance, the development of airway remodeling and the peculiarity of the allergen by comparing with the characteristics of the ovalbumin-instilled mice. Furthermore, to clarify whether these allergic responses are Th2-dependent, we used interleukin (IL)-4 receptor α chain gene-deficient mice because the IL-4 receptor α chain is a common receptor for IL-4 and IL-13, which are both critical for Th2 polarization and the development. Finally, we examined the effect of fluticasone propionate, one of the strongest anti-inflammatory medicines available at the moment, on this model, especially on airway remodeling.

2. Materials and methods

2.1. Animals

Seven-week-old male BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). IL-4 receptor α chain gene-deficient mice (IL-4Rα; BALB/c background) (Noben-Trauth et al., 1999; Noben-Trauth et al., 1997) were purchased from Immuno-Biological Laboratories, Co. Ltd. (Takasaki, Japan). The animals were housed in plastic cages in an air-conditioned room at 22±1 °C with a relative humidity of 60±5%, fed a standard laboratory diet and given water *ad libitum*. Experiments were undertaken following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animals Science in 1987.

2.2. Agents

The following drugs and chemicals were purchased commercially and used: crude extract of *D. farinae* (LSL Co., Tokyo, Japan), ovalbumin (chicken egg white, grade V, Sigma, St. Louis, MO., USA), dimethyl sulfoxide (DMSO, Nacalai Tesque, Inc., Kyoto, Japan), phosphate-buffered saline (PBS, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), halothane (Takeda Chemical Industries, Ltd, Osaka, Japan), acetylcholine chloride (Nacalai Tesque, Inc.), bovine serum albumin (Seikagaku Kogyo, Tokyo, Japan), Türk solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), pancuronium bromide (Sigma), sodium pentobarbitone (Abbott Lab., Chicago, IL, USA), disodium ethylenediaminetetraacetic acid (EDTA-2Na; Nacalai Tesque) and Diff-Quick solution (International Reagent Corp., Ltd., Kobe, Japan). Fluticasone propionate was kindly given to us by GlaxoSmithKline, Japan.

2.3. Experimental protocol

Mice were instilled 8 times with *D. farinae* (4 μg or 20 μg of protein in 100 μl of PBS) or PBS alone into the trachea using a polyethylene cannula under halothane anesthesia (Fig. 1A). In the ovalbumin-induced airway inflammation model, mice were instilled with ovalbumin in the same way. Forty-eight hours after the final antigen challenge, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage and histological study were performed.

2.4. Treatment with fluticasone propionate

Fluticasone propionate was dissolved in DMSO and diluted in PBS to a final concentration of 0.01%. Fluticasone propionate was instilled with *D. farinae* on day 14, 15, 21, 22, 28 and 29, and without the allergen on days 13, 20 and 27 according to previously described methods (Fig. 1B). The *D. farinae*-instilled group mice, which were the control group mice, were administrated 20 μg of the allergen with 0.01% DMSO.

2.5. Bronchoalveolar lavage

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in bronchoalveolar lavage fluid. Experiments were performed according to previously described methods (Tanaka et al., 2001). Animals were killed with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and the left bronchi were tied for histological examination. Then, the right air lumen was washed 4 times with 0.5-ml calcium- and magnesium-free PBS containing 0.1% bovine serum albumin and 0.05 mM EDTA-2Na. This procedure was repeated three times (total volume; 1.3 ml, recovery >85%). The bronchoalveolar lavage fluid from each animal was pooled in a plastic tube, cooled on ice and centrifuged (150 ×g) at 4 °C for 10 min. Cell pellets were resuspended in the same buffer (0.5 ml). Bronchoalveolar lavage fluid was stained with Türk solution and the number of nucleated cells was counted in a Burker chamber. A differential count was made on a smear prepared with a cytocentrifuge

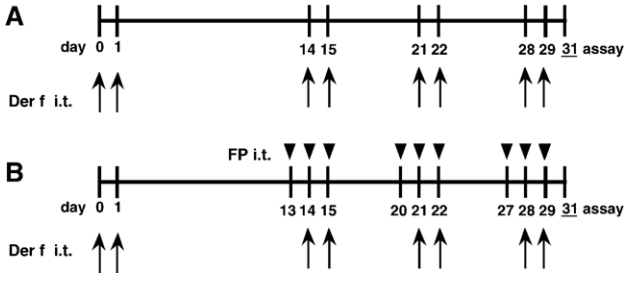

Fig. 1. Experimental protocol for *Dermatophagoides farinae* (Der f)-induced airway inflammation in BALB/c mice. A: *D. farinae* was instilled 8 times into the airways of BALB/c mice. B: Fluticasone propionate (FP) was administrated via the trachea with or without *D. farinae*, starting from the day before the third instillation of the allergen. i.t., intratracheal injection.

(Cytospin II, Shandon, Cheshire, England) and stained with Diff-Quick solution (based on standard morphological criteria) of at least 300 cells (magnification ×500). The supernatant of the bronchoalveolar lavage fluid was stored at −80 °C for the determination of cytokine production.

2.6. Cytokine levels in the bronchoalveolar lavage fluid

The amount of cytokine in the supernatant of the bronchoalveolar lavage fluid was measured using the enzyme-linked immunosorbent assay (ELISA) (Endogen Inc., Woburn, MA, USA for IL-4, IL-5 and interferon (IFN)-γ; R&D Systems Inc., Minneapolis, MN, USA for IL-13). The transforming growth factor (TGF)-β_1 content in the bronchoalveolar lavage fluid was also measured using ELISA (Genzyme Tecne, Minneapolis, MN, USA), which can detect mouse TGF-β_1 protein, because of the high homology of TGF-β_1 across species. The assay detects only the active form of TGF-β_1. Each sample was activated before measuring, according to the manufacturer's recommendations. The detection limit of each kit was 5 pg/ml for IL-4 and IL-5, 10 pg/ml for IFN- γ, 1.5 pg/ml for IL-13, or 7 pg/ml for TGF-β_1, respectively.

2.7. Measurement of airway function

Measurement of the bronchial responsiveness to intravenous acetylcholine was performed as previously described (Komai et al., 2003; Tanaka et al., 2001). Briefly, to measure the airway responsiveness to acetylcholine, mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the jugular vein was cannulated for the intravenous injection of acetylcholine. Mice were injected with pancuronium bromide (0.1 mg/kg, i.v.) to stop spontaneous respiration, and animals were ventilated with a rodent ventilator (New England Medical Instruments Inc., Medway, MA, USA). Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (Ugo Basil 7020, Milan, Italy) connected to the tracheal cannula. To measure airway responsiveness to acetylcholine, changes in the respiratory overflow volume were measured using increasing doses of acetylcholine. The increase in respiratory overflow volume induced by acetylcholine was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The area under the curve (AUC) calculated from dose–response curves for acetylcholine is expressed as the magnitude of airway hyperresponsiveness. Briefly, each dose was converted logarithmically, and then AUC was calculated and represented as arbitrary units (Tanaka et al., 2001).

2.8. Histological study

The left lungs were distended by the injection of 10% buffered formalin via the trachea, excised 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 periodic acid-Schiff (PAS) and Masson-trichrome for light microscopy examination.

Examination of goblet cell hyperplasia was carried out with the PAS-stained histological preparations of the left lobe using a Leica image analysis system (Leica, Cambridge, UK). Using a ×10 objective, 4 typical areas were chosen from the largest visible airway, which, in these horizontal sections through the hilus, was the left main bronchus. Changing to a ×40 objective, in each chosen area, which corresponded to one microscopic field, the hyperplasia of the goblet cells in the epithelial lining was expressed by a score according to the percentage of the goblet cells in the epithelial cells described below. The length of the epithelial basement membrane of the bronchus of one area was 500 μm and over. To minimize the sampling errors, the 5-point scoring system (grade 0–4) (Tanaka et al., 2001) was adopted: grade 0, no goblet cells; grade 1, <25%; grade 2, 25–50%; grade 3, 50–75%; grade 4, ≥75%. The mean score of the total epithelial cells in the 4 areas of one mouse were counted. The mean scores of hyperplasia of the goblet cells were calculated in 5–9 animals.

Masson-trichrome stained sections were used for the assessment of the thickness of the epithelial layer and the detection of subepithelial fibrosis using a Leica image analysis system (Leica). As described above, using a ×10 objective, 3 representative areas were chosen, avoiding the selection of the furcation of the bronchus and the surrounding blood vessels in the largest airway. Changing a ×40 objective, epithelial basement membrane areas 250 μm and over were selected, and the thickness of the epithelial layer and the fibrotic area (stained in blue) 30 μm beneath the basement membrane of the standardized sampling points were measured. The mean of the thickness of the epithelial layer and the fibrotic area divided by the basement membrane length were calculated in 5–9 animals.

2.9. Statistical analysis

Values are presented as the mean with standard error. Statistical significance between two groups was estimated using the two-tailed Student's *t*-test or the Mann–Whitney's *U*-test after the variances of the data were evaluated with the *F*-test. To define significant differences among PBS-instilled animals and *D. farinae*-instilled animals, and among the control animals and fluticasone propionate-treated animals, the data were subjected to Bartlett's analysis, followed by a parametric or a non-parametric Dunnett's multiple range test. A *P* value less than 0.05 was considered to be significant.

3. Results

3.1. The effect of repeated instillations of D. farinae into the mouse trachea on airway responsiveness to acetylcholine, Th1/Th2 immune responses and inflammatory infiltrates in the airways

To examine the effect of repeated instillation of *D. farinae* into the mouse trachea on Th1/Th2 immune responses, airway inflammation and airway responsiveness to acetylcholine, we examined the airway responsiveness to acetylcholine, inflammatory infiltrates and cytokine production in the bronchoalveolar lavage fluid, and the histopathological changes in the airways. Repeated allergen instillations induced increases in the airway responsiveness to acetylcholine (Fig. 2A and B) and the numbers of inflammatory cells, including eosinophils, lymphocytes and neutrophils in the bronchoalveolar lavage fluid in a dose-dependent manner (Fig. 2C). Furthermore, as shown in Table 1, the levels of Th2 cytokines, IL-5 and IL-13, in the bronchoalveolar lavage fluid were also significantly increased; whereas, the level of Th1 cytokine, IFN-γ, in the bronchoalveolar lavage fluid was decreased by the allergen instillations in a dose-dependent fashion. However, there were no

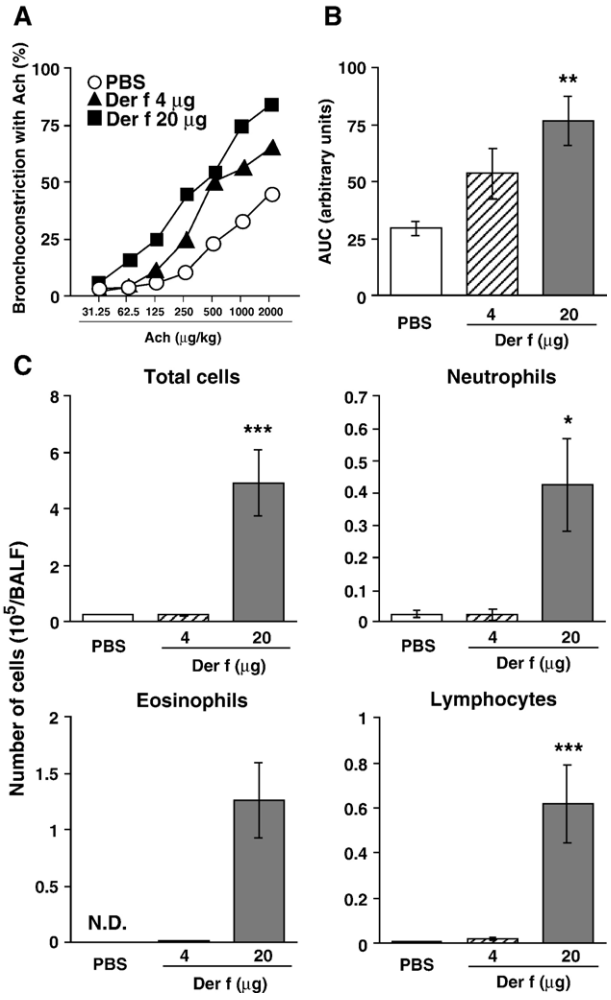

Fig. 2. Effect of repeated allergen instillation on airway responsiveness to acetylcholine and inflammatory infiltrates in the bronchoalveolar lavage fluid in BALB/c mice. A: Airway responsiveness to acetylcholine 48 h after the final allergen challenge. B: Area under the curve (AUC) calculated from the dose–response curves of bronchoconstriction for acetylcholine (range: 31.25–2000 μg/kg). C: Number of leukocytes in the bronchoalveolar lavage fluid 48 h after the final allergen challenge. Values represent the mean±S.E.M. of seven to eight mice in each group. PBS, phosphate-buffered saline-instilled; Der f, *Dermatophagoides farinae*-instilled; N.D., not detected; **P*<0.05, ***P*<0.01, ****P*<0.001 (vs PBS).

Table 1
Cytokine production in bronchoalveolar lavage fluid and structural changes in the airways 48 h after the final allergen challenge in BALB/c mice

Treatment	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFN γ (pg/ml)	Goblet cell hyperplasia (arbitrary unit)	Goblet cell hypertrophy (μm)	Fibrotic area (Area/BM)	TGF-β_1 (pg/ml)
PBS	20.7±6.1	N.D.	0.8±0.4	164.5±10.3	0.08±0.05	32.3±1.7	5.5±0.7	30.6±6.2
Der f 4 μg	20.4±8.4	0.9±0.7	1.6±0.4	149.8±10.7	2.82±0.22	53.5±5.5 b	10.4±1.0 b	42.8±15.5
Der f 20 μg	25.0±5.1	7.1±2.5	10.6±2.5 c	131.9±2.5 a	3.83±0.17 c	77.3±7.9 c	15.6±0.9 c	217.3±38.9 b

Values represent the means±S.E.M. of 7–8 animals in each group. BM, Basement membrane; Der f, *Dermatophagoides farinae*; N.D., not detected.
a $P<0.05$; b $P<0.01$; c $P<0.001$ (vs PBS).

significant differences in the levels of IL-4 in the bronchoalveolar lavage fluid between the PBS group and the allergen group (Table 1).

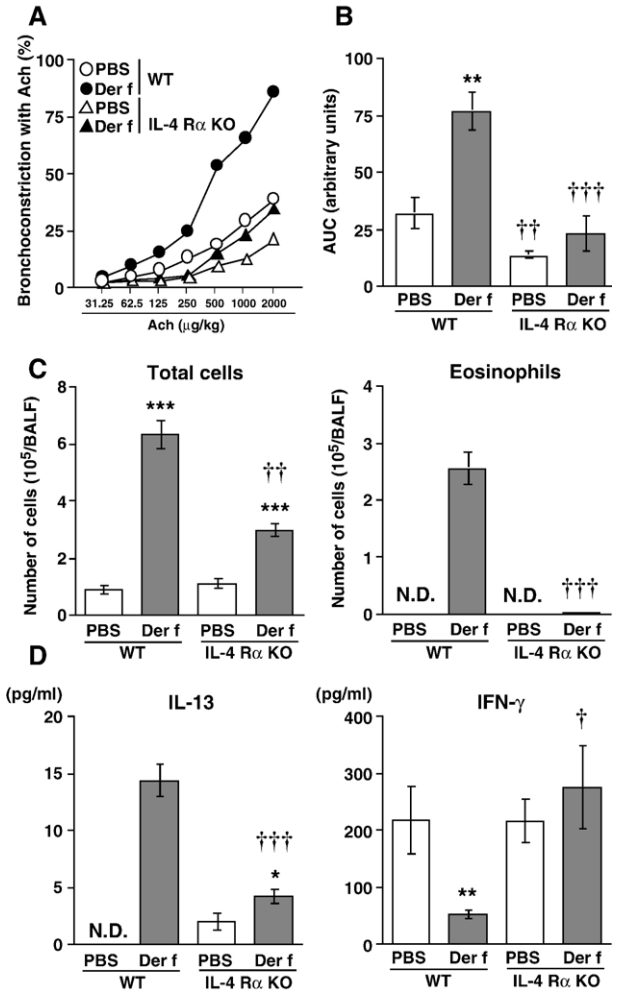

Fig. 3. Effect of IL-4 receptor α chain gene deficiency on the allergen-induced airway hyperresponsiveness to acetylcholine, increases in the numbers of total leukocytes and eosinophils and the production of IL-13 in the bronchoalveolar lavage fluid, and decreases in IFN-γ production in the bronchoalveolar lavage fluid in BALB/c mice. Forty-eight hours after the final allergen instillation, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage and histological examination were carried out. Values represent the mean± S.E.M. of seven to nine mice in each group. PBS, phosphate-buffered saline-instilled; Der f, *Dermatophagoides farinae*-instilled; N.D., not detected; *$P<0.05$, **$P<0.01$, ***$P<0.001$ (vs PBS, Student's *t*-test or Mann–Whitney's *U*-test); †$P<0.05$, ††$P<0.01$, †††$P<0.001$ (vs WT, Dunnett's test).

Next, to clarify whether allergic responses induced by *D. farinae* are Th2-dependent or not, we examined the effect of IL-4 receptor α chain gene deficiency using the gene-knockout mice compared with wild-type (BALB/c) mice. As shown in Fig. 3, the defect of IL-4/IL-13 signaling through IL-4 receptor α chain clearly abrogated the airway hyperresponsiveness ($P<0.001$, Fig. 3A and B), airway eosinophilia ($P<0.001$, Fig. 3C), and imbalance in the IL-13/IFN-γ production in the bronchoalveolar lavage fluid ($P<0.001$ for IL-13 or $P<0.05$ for IFN-γ, Fig. 3D) induced by the allergen administration.

Then, to investigate the peculiarity of *D. farinae* in this model, mice were instilled with the same amount of ovalbumin as protein content instead of the allergen in a similar manner. Neither airway hyperresponsiveness, the accumulation of inflammatory cells into the airways or increases in the Th2 cytokine level in the bronchoalveolar lavage fluid was observed in the ovalbumin-instilled group (data not shown).

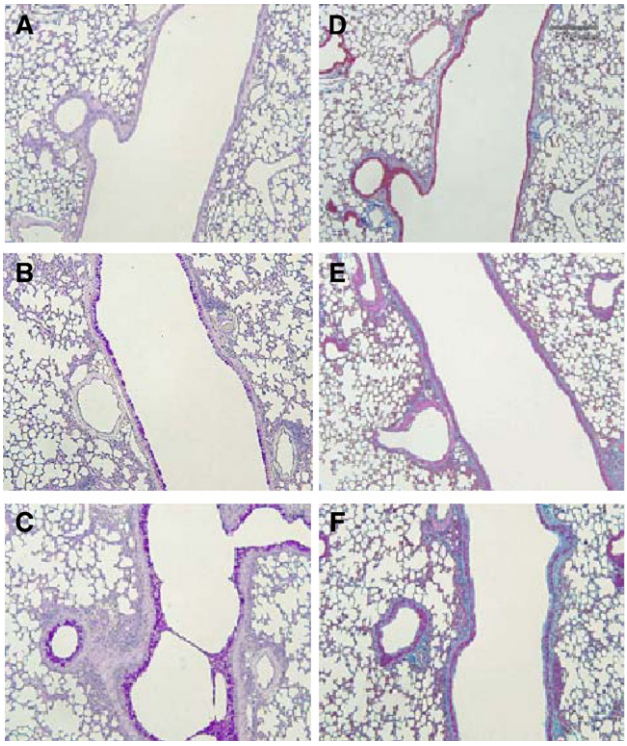

Fig. 4. Histological analysis of lung section with periodic acid-Schiff (A–C) and with Masson-trichrome (D–F) 48 h after the final allergen challenge in BALB/c mice. (A and D) PBS-instilled animal; (B and E) 4 μg of *D. farinae*-instilled animal; (C and F) 20 μg of *D. farinae*-instilled animal. Scale bar 200 μm.

Table 2
Effect of IL-4 receptor α chain gene deficiency on *Dermatophagoides farinae* (Der f)-induced airway remodeling in BALB/c mice

Genotype	Treatment	Goblet cell hyperplasia (arbitrary units)	Goblet cell hypertrophy (μm)	Fibrotic area (Area/BM)	TGF-β_1 (pg/ml)
Wild-type	PBS	0.43±0.12	0.43±0.12	5.41±0.44	55.5±12.4
	Der f 20 μg	3.92±0.04 b	3.92±0.04 b	18.62±2.10 b	334.8±23.9 b
IL-4 α chain gene deficiency	PBS	0.44±0.09	0.44±0.09	5.62±0.52	46.4±3.0
	Der f 20 g	0.57±0.12 c	0.57±0.12 c	5.73±0.36 c	81.8±7.8 a, c

Values represent the means±S.E.M. of 7–9 animals in each group. BM, Basement membrane; TGF, transforming growth factor.
a $P<0.01$; b $P<0.001$ (vs PBS group); c $P<0.001$ (vs Wild-type).

3.2. The effect of repeated instillations of *D. farinae* into the mouse trachea on airway remodeling

Fig. 4 shows the representative sections of each group stained with PAS for the detection of goblet cells and with Masson-trichrome for the detection of the fibrotic area. Repeated instillations of *D. farinae* caused goblet cell hyperplasia/hypertrophy in the epithelium and enlarged the area of subepithelial fibrosis dose dependently. These structural changes were mainly observed in the central large airways although the peripheral airways were mostly in tact.

To examine these histological changes quantitatively, goblet cell hyperplasia/hypertrophy and subepithelial fibrosis were evaluated by a score of PAS positive cells (stained in purple), the thickness of the epithelial layer and the area of fibrosis (stained in blue). As shown in Table 1, each score was significantly increased by repeated allergen inoculations in a dose-dependent manner. Furthermore, the level of profibrotic cytokine, TGF-β_1, in the bronchoalveolar lavage fluid was also dramatically increased by the instillation of *D. farinae* (Table 1). In contrast, these histopathological changes in the airways and increased TGF-β_1 production in the bronchoalveolar lavage fluid were completely diminished in IL-4 receptor α chain gene-deficient mice ($P<0.001$, Table 2).

3.3. The effect of fluticasone propionate on D. farinae-induced airway hyperresponsiveness, eosinophilic inflammation and Th1/Th2 immune responses

To investigate the effect of fluticasone propionate on the *D. farinae*-induced asthma-like phenotypes, mice were given 100–1000 μg/kg fluticasone propionate via an intratracheal route with the allergen on days 14, 15, 21, 22, 28 and 29, and without the allergen on days 13, 20 and 27 (Fig. 1B). To determine the initial timing of fluticasone propionate treatment, we examined the time course study for repeated allergen instillation in this protocol, and observed increases in the number of eosinophils and the level of IL-13 in the bronchoalveolar lavage fluid on day 17, 48 h after the fourth allergen instillation (data not shown), suggesting that sensitization with *D. farinae* was established and allergic responses in the airways were ongoing at that time.

Fig. 5 shows the effect of fluticasone propionate on allergen-induced airway hyperresponsiveness and leukocyte infiltrates in the bronchoalveolar lavage fluid. Repeated instillations of *D. farinae* significantly increased the airway responsiveness to acetylcholine and the number of inflammatory cells in the bronchoalveolar lavage fluid. Treatment with fluticasone propionate, at doses of 300 and 1000 μg/kg, significantly inhibited the increases in the numbers of neutrophils,

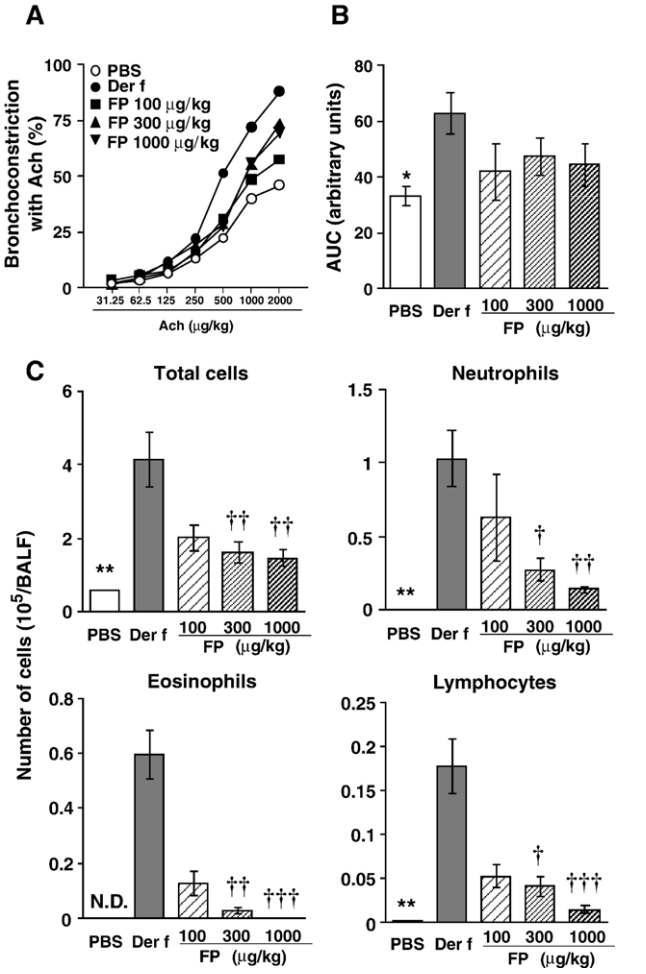

Fig. 5. The effect of fluticasone propionate (FP) on the allergen-induced airway hyperresponsiveness to acetylcholine and on the number of leukocytes in the bronchoalveolar lavage fluid. Forty-eight hours after the final allergen instillation, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage was carried out. A: Airway responsiveness to acetylcholine 48 h after the final allergen challenge. B: Area under the curve (AUC) calculated from the dose–response curves of bronchoconstriction for acetylcholine (range: 31.25–2000 g/kg). C: Number of leukocytes in the bronchoalveolar lavage fluid 48 h after the final allergen challenge. Values represent the mean±S.E.M. of five to seven mice in each group. Der f, *Dermatophagoides farinae*; PBS, phosphate-buffered saline-instilled; Der f, 20 μg of *D. farinae*-instilled; FP, Der f and fluticasone propionate-instilled; N.D., not detected; *$P<0.05$, **$P<0.01$, *** $P<0.001$ (vs Der f, Student's *t*-test or Mann–Whitney's *U*-test); †$P<0.05$, ††$P<0.01$, †††$P<0.001$ (vs WT, Dunnett's test).

Table 3
Effect of fluticasone propionate (FP) on *Dermatophagoides farinae* (Der f)-induced Th2-polarized cytokine production and airway remodeling in BALB/c mice

Treatment	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFNγ (pg/ml)	Goblet cell hyperplasia (arbitrary unit)	Goblet cell hypertrophy (μm)	Fibrotic area (Area/BM)	TGF-β_1 (pg/ml)
PBS	6.1±3.2	1.2±0.7a	0.4±0.4 b	168.6±7.1	0.17±0.05 b	36.3±1.6 a	5.1±0.6 b	21.6±3.9 b
Der f	7.2±4.6	13.1±3.6	6.6±1.3	149.8±15.2	3.54±0.07	58.4±5.8	12.6±0.5	253.7±46.3
FP 100 μg/kg	1.2±1.2	10.9±2.3	3.4±1.7	139.5±9.2	3.36±0.20	52.5±1.3	8.7±0.6 e	133.8±35.9
FP 300 μg/kg	8.8±4.4	5.2±2.1	2.3±0.8	171.8±10.1	2.82±0.22	47.9±3.3	7.9±0.3 e	56.7±9.3 d
FP 1000 μg/kg	5.3±3.4	2.5±0.9 d	0.7±0.3 d	173.1±13.7	1.35±0.37 d	41.7±2.7 c	5.3±0.5 e	45.8±8.8 e

Values represent the means±S.E.M. of 5–7 animals in each group. BM, Basement membrane; TGF, transforming growth factor.
a $P<0.05$; b $P<0.01$ (vs Der f group, Mann–Whitney's *U*-test); c $P<0.05$; d $P<0.01$; e $P<0.001$ (vs Der f group, Dunnett's test).

eosinophils and lymphocytes in the bronchoalveolar lavage fluid; whereas, the drug showed a tendency to inhibit airway hyperresponsiveness (Fig. 5). Furthermore, in parallel with the effect on airway eosinophilic inflammation, the increased levels of Th2 cytokines, IL-5 and IL-13, in the bronchoalveolar lavage fluid was almost reversed by fluticasone propionate treatment in a dose-dependent fashion (Table 3).

3.4. The effect of fluticasone propionate on the development of airway structural changes induced by D. farinae

Fig. 6 shows the representative lung specimens of the *D. farinae*-instilled group and the fluticasone propionate-treated group. The quantitative findings of histopathological examination and TGF-β_1 production in each group are shown in Table 3.

As shown in Fig. 6 and Table 3, severe goblet cell hyperplasia/hypertrophy, marked subepithelial fibrosis and the increased production of TGF-β_1 in the bronchoalveolar lavage fluid were all observed in the allergen-instilled group compared with those in the PBS-instilled group. In contrast, the treatment with fluticasone propionate, at a dose of 1000 μg/kg, during allergen challenge significantly inhibited these quantitative changes.

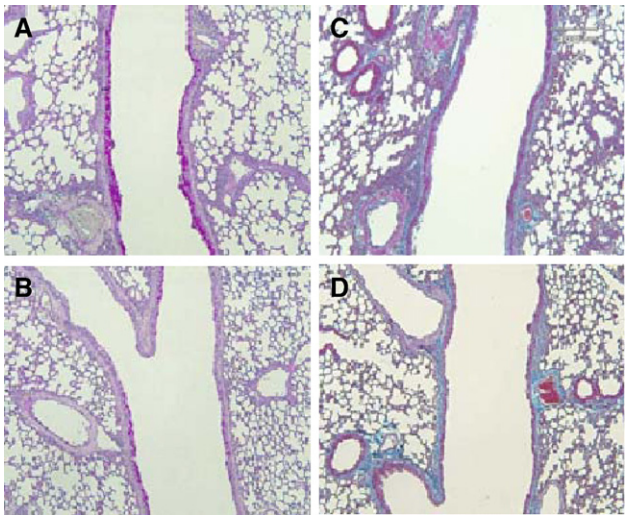

Fig. 6. Histological analysis of a lung section with periodic acid-Schiff (A and B) and with Masson-trichrome (C and D) 48 h after the final allergen challenge in fluticasone propionate-treated mice. (A and C) 20 μg of *Dermatophagoides farinae*-instilled animals; (B and D) fluticasone propionate-treated animals. Scale bar 200 μm.

4. Discussion

Bronchial asthma is a chronic inflammatory airway disease that is characterized by eosinophil inflammation, bronchial hyperresponsiveness and airway remodeling in pathophysiological studies (Cohn et al., 2004). In addition, many clinical and experimental studies suggest that Th2 cells that secrete IL-4, IL-5 and IL-13 have important functions in directing and maintaining the airway inflammatory processes (Robinson et al., 1992; Temann et al., 1997; Tomkinson et al., 2001). To understand the basic cellular and molecular mechanisms of allergic sensitization and airway inflammation, various animal models that possess these characteristics have provided valuable information on several aspects of asthma, and we reported the ovalbumin-induced allergic asthma model in mice by systemic sensitization and repeated allergen exposure (Nagai et al., 1997; Tanaka et al., 2001). Although this model is useful for the analysis of specific IgE-dependent airway eosinophilic inflammation, it is difficult to investigate the onset of the disease (especially local sensitization and challenge) and the specificity of relevant allergens that trigger human asthma. Therefore, we established a mouse model of allergic asthma by the intratracheal instillation of *D. farinae* without additional adjuvants.

In the present study, we showed *D. farinae*-induced airway eosinophilic inflammation in mice by the local instillation. Our mouse model is characterized by eosinophil infiltrates into the airways, increases in the levels of IL-5 and IL-13 in the bronchoalveolar lavage fluid, a decrease in the level of IFN-γ in the bronchoalveolar lavage fluid, and airway hyperresponsiveness to acetylcholine in a dose-dependent manner. Furthermore, these asthma-like phenotypes were completely abrogated in IL-4 receptor α chain gene-deficient mice, suggesting that IL-4 and/or IL-13 play a predominant role in the induction and development of *D. farinae*-induced Th2 responses. In addition, another foreign protein, ovalbumin, could not induce asthmatic responses at all in the same protocol. Indeed, it has been reported that the repeated inoculation of *D. farinae* into the trachea (Yu et al., 1999) or the nose (Johnson et al., 2004) of mice induces airway eosinophilia, but these reports are limited to the increases in Th1/Th2 cytokines in the bronchoalveolar lavage fluid or in Th2 cytokines in the allergen-stimulated splenocytes, respectively. Thus, this is the first report to prove directly that *D. farinae* by itself has the capacity to induce the imbalance of Th2/Th1 responses in the airways and to promote local Th2 type immune responses through IL-4 receptor α chain.

House dust mite is known to be composed of mixed materials, including *D. farinae* and pteronyssinus. Moreover, *D. farinae* contains at least 2 major allergens (*D. farinae* 1 and 2) (King et al., 1994). As described above, it was reported that repeated ovalbumin inhalation to sensitized mice induced tolerance (Holt et al., 1981; Sakai et al., 2001; Swirski et al., 2002); however, in the case of *D. farinae*, the number of eosinophils gradually increased, even after 24 instillations (unpublished data), suggesting that *D. farinae* does not easily induce tolerogenic responses. Thus, there seems to be some different characteristics in the allergen compared to ovalbumin, including their proteolytic activities. More importantly, the molecular mechanisms underlying in mucosal sensitization, airway inflammation and airway remodeling by repeated allergen instillation might be different even though each phenotype like Th2-polarized immune responses is quite similar. Therefore, further experiments are needed to clarify which types of allergen and/or what kinds of components are responsible for these asthmatic phenotypes in this model.

The present data demonstrated that no increase of the IL-4 level was observed 48 h after the final allergen challenge; whereas, there were significant increases in the levels of Th2 cytokines and a decrease in Th1 cytokine production in the bronchoalveolar lavage fluid, respectively. It may be due to the difference of kinetics in the cytokine production after allergen challenge. For instance, Ohkawara et al. demonstrated that the peak level of IL-4 and IL-5 in the fluid occurred 24 h after antigen challenge in the ovalbumin-sensitized mice (Ohkawara et al., 1997). While a remarkable increase in the IL-5 level in the fluid was observed from 3 h after the challenge and continued for 5 days, the kinetics of the IL-4 level in the fluid were much shorter than those of IL-5. In our study, we measured the cytokine levels 48 h after the last antigen challenge; therefore, the peak level of IL-4 in the BALF might have passed.

In contrast to the level of IL-4 in the airways, we detected increased levels of *D. farinae*-specific IgG1 on day 13, and the levels were much increased on day 27 and 31 using an allergen-coated ELISA system (data not shown). Serum allergen-specific IgE was also detected on day 27 by passive cutaneous anaphylaxis in rats (data not shown). Therefore, we provide evidence of Th2-polarized systemic immune responses in the allergen-instilled mice.

Mucus hypersecretion from hyperplastic goblet cells causes airway mucous plugging, especially in the peripheral airways of asthmatics. Mucous plugging has been reported to be an important factor in the mortality rates associated with severe acute asthma (Aikawa et al., 1992; Saetta et al., 1991). Recently, several observations have implicated Th2 cytokines, IL-4, IL-9 and IL-13, in goblet cell metaplasia of mice (Grunig et al., 1998; Louahed et al., 2000; Temann et al., 1997; Wills-Karp et al., 1998). In addition, IL-4 and IL-13 have been shown to be major contributing factors for allergen (ovalbumin)-induced mucus production via IL-4 receptor α chain in an allergic mouse model (Cohn et al., 1999; Kuperman et al., 2005). However, it is not clear whether *D. farinae*-induced mucous production is dependent on IL-4 receptor α chain in vivo. Therefore, we investigated the dependency using IL-4 receptor α chain gene-deficient mice. As a result, goblet cell hyperplasia/hypertrophy caused by the repeated allergen challenge was completely dependent on IL-4 receptor α chain. It is difficult to determine whether the allergen-induced mucous metaplasia is due to a Th2-polarized immune response in the airways, or to the direct effects of *D. farinae*. In the present study, marked increases in goblet cells and goblet cell hypertrophy in the epithelium were observed in both the low dose (4 μg) and high dose (20 μg) *D. farinae*-instilled groups, and their extents were comparable; whereas, Th2 cytokine production and the imbalance of the Th1/Th2 responses in the airways were more prominent in the high dose group. Therefore, it is possible that *D. farinae* directly affected the ciliated epithelial cells to transform/differentiate into goblet cells, probably through their protease activity, and that their protease activity influenced the allergenicity, which can induce Th2-polarized immune responses dependent on IL-4 receptor α chain, although further experiments are needed to clarify whether these protease activities are involved in their transformation/differentiation.

In histological analysis, the fibrotic areas under the basal lamina of the central airways were quantitatively evaluated, and increases in the areas were observed in a dose-dependent fashion. Furthermore, an increase in the TGF-β_1 level in the bronchoalveolar lavage fluid was observed in a dose-dependent fashion, and the increased production after the repeated instillation of *D. farinae* was IL-4/IL-13 dependent through IL-4 receptor α chain. TGF-β_1 plays a central role in the pathogenesis of a variety of fibrotic disorders. It stimulates the production of extracellular matrix proteins and inhibits the formation of extracellular proteases. An increase in the TGF-β_1 levels was observed in the clinical specimens of fibrotic kidney disease, hepatic fibrosis and pulmonary fibrosis (Blobe et al., 2000). In asthmatic patients, the bronchoalveolar lavage study or biopsy specimens indicated an increase in the TGF-β_1 levels, and its expression levels correlated with the severity of the disease and the degree of subepithelial fibrosis (Redington et al., 1997; Vignola et al., 1997). We previously reported that the increased levels of TGF-β_1 in the bronchoalveolar lavage fluid were significantly correlated with fibrotic changes in the morphometric studies following ovalbumin sensitization and challenge in mice (Komai et al., 2003; Tanaka et al., 2001), and that eosinophils and myofibroblasts play a critical role in the development of subepithelial fibrosis in mice (Tanaka et al., 2004). Therefore, it is also possible that these cells that infiltrated around the airways can produce the fibrogenic factor in this model.

Now, inhaled corticosteroid maintains its position as a first-line therapy for bronchial asthma. Several clinical studies have suggested that inhaled corticosteroid is effective for airway inflammation and structural changes, such as the thickness of the subepithelial basement membrane (Olivieri et al., 1997; Sont et al., 1999). However, there are conflicting findings about the effect on airway remodeling (Jeffery et al., 1992; Lundgren et al., 1988). Therefore, we investigated the effect of the corticosteroid, fluticasone propionate, on this model. As a result, it was found that fluticasone propionate dramatically inhibited airway eosinophilic inflammation and increased Th2

cytokines and TGF-β_1 productions in the bronchoalveolar lavage fluid, and that fluticasone propionate showed a tendency to inhibit the airway hyperresponsiveness to acetylcholine. Furthermore, goblet cell hyperplasia in the airways and the fibrotic area beneath the basal lamina of the central airways were significantly improved by fluticasone propionate treatment in a dose-dependent manner. These findings suggest that inhaled corticosteroid can inhibit *D. farinae*-induced airway remodeling, probably through inhibiting Th2-mediated airway eosinophilic inflammation. However, there is a discrepancy in the fluticasone propionate doses between the effects on inflammatory infiltrates and airway remodeling/airway hyperresponsiveness in the present study. Vanacker et al. also demonstrated that fluticasone propionate prevented antigen-induced airway inflammation and structural changes using a rat asthma model, and they also argued that a higher dose of inhaled corticosteroid is needed to prevent allergen-induced structural changes than that required to inhibit eosinophil recruitment (Vanacker et al., 2002). Furthermore, Boulet et al. recently demonstrated that there is no significant difference in airway inflammation and subepithelial collagen deposition in steroid-naïve patients with mild recently diagnosed vs long-standing asthma, and that there was no significant improvement in the airway hyperresponsiveness after high doses of inhaled corticosteroids between these two groups (Boulet et al., 2000). These findings suggest that there are irreversible airway structural and/or physiological changes, despite appropriate and aggressive anti-inflammatory therapies, which may explain the reason why airway remodeling and airway hyperresponsiveness could not be normalized in some patients.

Another question to be addressed is whether fluticasone propionate is effective for airway hyperresponsiveness in this model. We observed that fluticasone propionate showed a tendency to inhibit allergen-induced airway hyperresponsiveness, but it was not significant. Many clinical studies and a few animal studies suggested that inhaled corticosteroid improved airway hyperresponsiveness (Olivieri et al., 1997; Sont et al., 1999); however, these improvements mainly resulted from the strong anti-inflammatory effects of the steroids. The relevance between airway hyperresponsiveness and airway remodeling is always a matter of concern, and undoubtedly, clinical biopsy specimens cannot assess the airways as a whole. Moreover, airway hyperresponsiveness did not return to within normal limits in most long-term studies, although it was improved (van Essen-Zandvliet et al., 1992). Therefore, our result may be due to the remaining airway structural changes, especially goblet cell hyperplasia or other factors; however, little is known about the effect of steroids on airway remodeling, especially in vivo, and further experiments will be needed.

In conclusion, we established a mouse model of atopic asthma, which was locally immunized with a major indoor allergen, *D. farinae*, without additional adjuvants. This model clearly demonstrated the local Th2-dominant inflammation and airway remodeling feature, and those parameters were improved by inhaled corticosteroid. We believe that this model is a useful tool for the investigation of mechanisms and the development of new remedies for airway remodeling in atopic asthma.

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