

IL-16 Variability and Modulation by Antiallergic Drugs in a Murine Experimental Allergic Rhinitis Model

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Key Words

Interleukin-16 • Allergic rhinitis • Eosinophils • Nasal mucosa • Ovalbumin-sensitized mouse • Fexofenadine • Ramatoban

Abstract

Background: Interleukin-16 (IL-16) is a cytokine that induces selective migration of CD4⁺ cells and participates in inflammatory diseases including allergic rhinitis. Histamine and prostaglandin D₂ are important chemical mediators of allergic inflammation, and antiallergic drugs are commonly used for the treatment of allergic rhinitis. It remains unknown whether treatment with the drugs affects IL-16. **Objective:** We evaluated the variability of IL-16 and the effects of the antiallergic drugs fexofenadine (40 mg/kg/day) and ramatroban (30 mg/kg/day) on IL-16 in an OVA-sensitized BALB/c murine experimental allergic rhinitis model. **Methods:** We measured the expression level of IL-16 protein in the mouse nasal septal mucosa by immunohistochemistry, and the serum level of IL-16 by ELISA. Several other parameters associated with allergic rhinitis (nasal symptoms, OVA-specific IgE, eosinophil and T cell infiltration) were also measured. **Results:** Local and systemic expressions of IL-16 were significantly increased in OVA-sensitized mice when compared to the non-sensitized group. Fexofenadine and ramatroban significantly inhibited the following OVA-induced allergic features

when compared to the nontreated sensitized group: sneezing, nasal rubbing, eosinophil infiltration, IL-16 expressions in nasal tissue, and serum IL-16 level. Serum OVA-specific IgE and local T cell infiltration were reduced, but they did not reach significant values. **Conclusions:** These results suggest that IL-16 was both systemically and locally upregulated in the murine allergic rhinitis model and that IL-16 changed in parallel to allergic state by treatment with the drugs.

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Introduction

Interleukin-16 (IL-16) was initially identified in 1982 as a lymphocyte chemoattractant factor generated from mitogen- or antigen-stimulated human peripheral blood mononuclear cells [1]. Previous studies have provided evidence that CD4 serves as a receptor for IL-16 and that IL-16 has specific activities on CD4⁺ cells, including the induction of cell migration [2]. IL-16 has been reported to play important roles in several diseases characterized by CD4⁺ T cell involvement, including bronchial asthma [3], rheumatoid arthritis [4], atopic dermatitis [5], and delayed-type hypersensitivity reaction [6]. IL-16 has been recognized as a proinflammatory cytokine because it causes CD4⁺ cell migration or proliferation, participates in inflammatory persistence by inhibiting activation-in-

duced cell death at the inflammatory site and induces cytokine production, such as IL-1 β , IL-6 and TNF- α [7, 8]. On the other hand, anti-inflammatory roles have also been reported for IL-16 in recent years. It appears that the inhibitory effects of IL-16 on antigen-driven CD4+ T cell-mediated responses predominate in antigen-induced inflammation [9] and that exogenous IL-16 inhibits the production of IL-4, IL-5 and IL-13 from T cells [10, 11]. Therefore, IL-16 is recognized to have pleiotropic effects, including CD4+ cell migration (CD4+ T cells, eosinophils, mast cells, etc.) and CD4+ T cell modulation.

IL-16 has been much reported in allergic diseases, including atopic asthma and allergic rhinitis [12, 13]. IL-16 is upregulated in the bronchial mucosa and in bronchoalveolar lavage fluid after allergen challenge in atopic asthmatic patients or OVA-sensitized asthmatic model mice [3, 14]. IL-16 increases in the nasal mucosa of patients with seasonal allergic rhinitis during the pollen season or after allergen challenge, and topical steroid treatment inhibits IL-16 mRNA and protein expression in the nasal mucosa [12, 15]. Previously, we reported that IL-16 protein expression was increased in the serum and nasal mucosa of patients with perennial and seasonal allergic rhinitis when compared to healthy controls, and that the serum IL-16 level was significantly correlated with the blood eosinophil percentage [13]. These reports suggest that IL-16 is well related to the pathology of allergic rhinitis, but the detailed mechanisms remain largely unknown. Histamine, prostaglandin D₂ (PGD₂) and thromboxane A₂ (TxA₂) are important chemical mediators of allergic inflammation, and antihistamine, anti-PGD₂ or TxA₂ drugs are commonly used for the treatment of allergic rhinitis, but it remains unknown whether treatment with these nonsteroidal antiallergic drugs affects IL-16.

In this study we examined the variability of IL-16 and the effects of the antiallergic drugs, fexofenadine [a selective histamine receptor-1 (H₁) blocker] and ramatroban (a PGD₂ and TxA₂ receptor dual blocker), on IL-16 using an ovalbumin (OVA)-sensitized murine allergic rhinitis model to investigate whether IL-16 expression is correlated with the allergic states and therapeutic process in allergic rhinitis.

Materials and Methods

Animals and Reagents

Four-week-old female BALB/c mice were obtained from Japan CLEA (Hamamatsu, Japan) and housed in pathogen-free conditions in the Kagawa University Faculty of Medicine. Mice were

given standard laboratory rodent chow and water. This research was approved by the Animal Care and Use Committees of Kagawa University. The following reagents were obtained as follows: OVA grade V (Sigma, St. Louis, Mo., USA), aluminium hydroxide gel (Wako Pure Chemical Ind., Osaka, Japan), and CMC (carboxymethylcellulose)-Na (Wako Pure Chemical Ind.). The following drugs were kindly provided by the companies indicated: ramatroban (Bayer, Berlin, Germany and Nippon Shinyaku, Kyoto, Japan) and fexofenadine (Sanofi-Aventis KK, Tokyo, Japan).

Sensitization and Drug Administration

Mice were divided into five groups: (1) sensitized with OVA, (2) sensitized with OVA + vehicle, (3) no sensitization, (4) sensitized with OVA + fexofenadine (40 mg/kg/day), and (5) sensitized with OVA + ramatroban (30 mg/kg/day). The OVA sensitization method in this study was performed according to previous reports with modifications [16, 17]. Mice were sensitized by intraperitoneal injections of 0.5 ml physiological saline containing 1 mg OVA and 1 mg aluminium hydroxide gel on days 1, 5 and 9. Local allergen challenge was then performed every day from day 15 to day 21 by administering OVA in physiological saline (1 mg/ml, 20 μ l) to the bilateral nasal cavities using a micropipette. In the nonsensitized group, only physiological saline was applied according to the same schedule as in the sensitized group. Fexofenadine and ramatroban were suspended in 0.5% CMC-Na and these drugs were administered orally 30 min before daily intranasal (i.n.) allergen challenge from day 15 to day 21. In the vehicle administration group, only 0.5% CMC-Na (200 μ l) was orally administered using the same schedule as in the drug administration groups. The daily doses of the drugs were determined according to previous reports that could sufficiently inhibit symptoms of allergic rhinitis and some of allergic pathogenesis in OVA-sensitized murine models [18, 19].

Nasal Symptoms

On day 21, after the final i.n. administration of OVA or physiological saline, the mice were placed individually in an observation cage and the number of sneezes and the frequency of nasal rubbing (nasal scratching movements) were counted for 10 min.

Tissue and Serum Sampling

Twenty-four hours after the final i.n. allergen challenge, mice were deeply anesthetized in diethyl ether. Their abdomen was opened and blood samples were obtained via the inferior vena cava, and they were then killed by exsanguination. Their facial skin was stripped, their heads were severed between the upper and lower jaws, and the tip of the nose was removed. Samples were immediately fixed in 4% paraformaldehyde for several hours and decalcified in 0.12 M EDTA solution (pH 6.5) for 10 days at 4°C. The EDTA solution was changed daily. After decalcification, tissues were embedded in OCT tissue compound (Tissue-Tec, Sakura, Tokyo, Japan). Sections (7 μ m thick) were cut on a cryostat at -20°C and mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan). Blood samples were left for 30 min at room temperature, and serum was obtained after centrifugation. These samples were stored at -20°C until use.

Measurement of Serum OVA-Specific IgE and IL-16

Murine serum OVA-specific IgE and IL-16 were measured by ELISA (Shibayagi, Gunma, Japan, and R&D Systems, Minneapo-

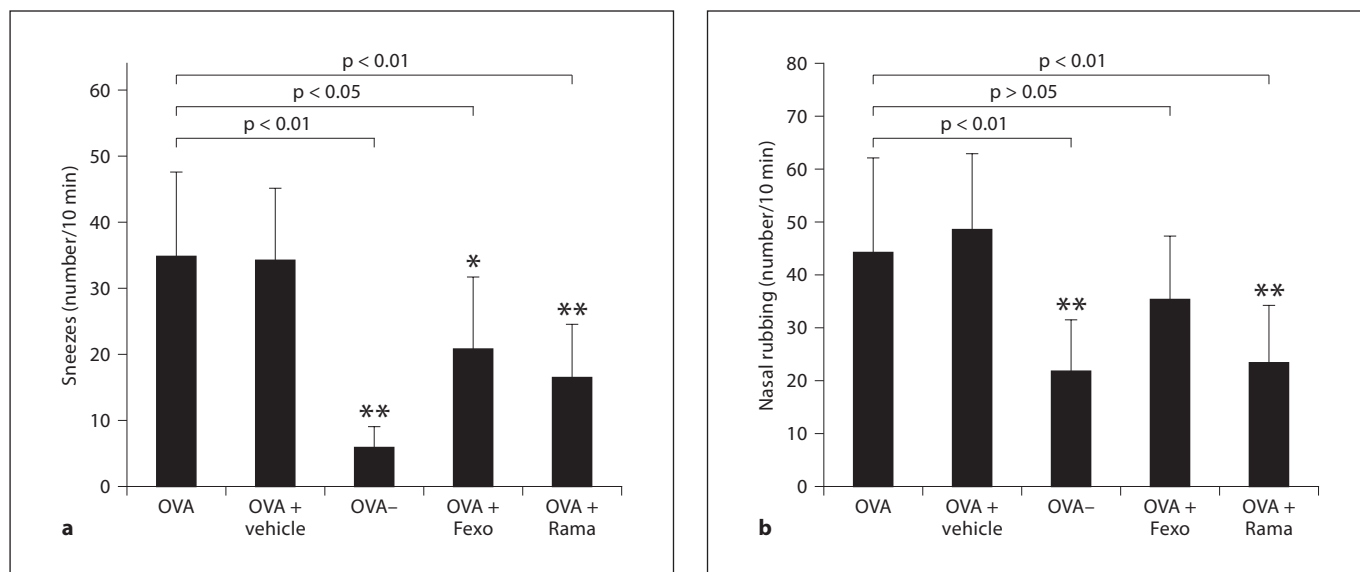


Fig. 1. Nasal symptoms after i.n. administration of saline or OVA on day 21. Number of sneezes/10 min (**a**) and incidence of nasal rubbing/10 min (**b**) for each group. Data are expressed as mean $\pm$ SD. Fexo = Fexofenadine; Rama = ramatroban. * $p < 0.05$; ** $p < 0.01$ as compared to the OVA-sensitized nontreated group.

lis, Minn., USA) according to each manufacturer's protocol. Originally, the IL-16 ELISA kit was developed for humans, but it was previously reported as acceptable for mice because of the high sequence homology and functional cross-reactivity between humans and mice [20]. The assays were sensitive above 1.88 U/ml for OVA-specific IgE and 8 pg/ml for IL-16.

Measurement of Eosinophil Infiltrations

Dried sections were stained by Diff-Quik stain (Sysmex International Reagents, Kobe, Japan) to measure the number of eosinophils that had infiltrated the nasal septal mucosa. In each specimen, three serial sections of a similar part were selected, the numbers of eosinophil in the bilateral nasal septal mucosa were counted with an Olympus BX 51 light microscope (Olympus, Tokyo, Japan), and the area was measured using ImageJ software [21]; then they were averaged and the results for each group are shown as the mean number of cells/mm².

Immunohistochemistry

For inhibition of endogenous peroxidase, slides were incubated with 2% H₂O₂ for 45 min. To block unspecific binding sites, slides were treated with a blocking reagent (Nittrei Bio, Tokyo, Japan) for 30 min. IL-16 or CD3 immunostaining were performed with a polyclonal rabbit anti-mouse IL-16 antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) or a polyclonal rat anti-mouse CD3 (1:250; Biologend, San Diego, Calif., USA) for 16 h incubation at 4°C. As negative control, slides were incubated with normal rabbit IgG (Santa Cruz) instead of primary antibody. After washing with PBS, the antibodies were visualized using Histofine simple staining (Nittrei Bio) and 3,3'-diaminobenzidine simple staining (DAB; Nittrei Bio). After immunostaining, hematoxylin staining was performed. In each specimen, three se-

rial sections of a similar part were selected, IL-16 or CD3 immunoreactive cells in the bilateral nasal septal mucosa were counted using a light microscope, and the area was measured using ImageJ; then they were averaged and the results of each group are shown as the mean number of cells/mm².

Statistical Analysis

All data are expressed as means $\pm$ SD. Significant differences between groups were determined using the Mann-Whitney U test. Significant values were defined as those with $p < 0.05$.

Results

Nasal Symptoms

The number of sneezes and the incidence of nasal rubbing after final i.n. OVA challenge were reduced by treatment with fexofenadine (20.8 ± 10.9 times/10 min, $p < 0.05$ and 35.6 ± 11.9 times/10 min, $p = 0.3072$) and ramatroban (16.5 ± 8.2 times/10 min, $p < 0.01$ and 23.4 ± 10.6 times/10 min, $p < 0.01$) when compared to the OVA-sensitized nontreated group (35.1 ± 12.7 and 44.3 ± 17.8 times/10 min). Treatment with only vehicle had no inhibitory effect (34.4 ± 10.6 times/10 min, $p = 0.8944$ and 48.7 ± 14.4 times/10 min, $p = 0.6261$). Each group included 9 mice. These results are shown in figure 1.

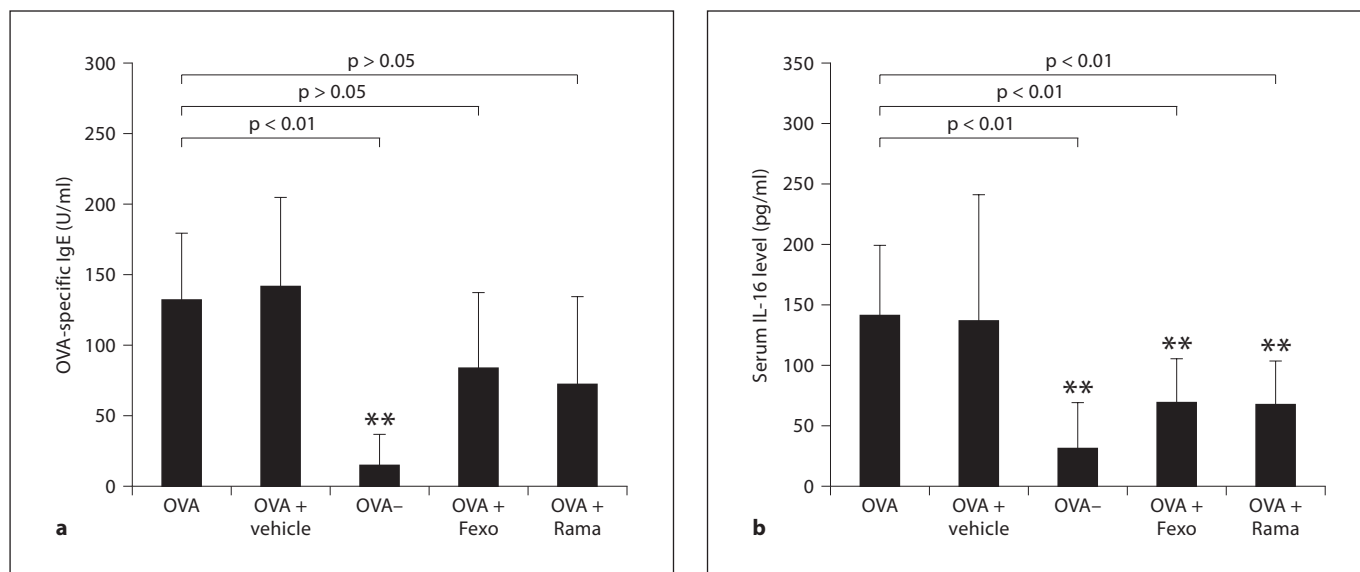


Fig. 2. OVA-specific IgE (a) and IL-16 (b) measured in serum after systemic and i.n. administration of saline or OVA. Data are expressed as mean $\pm$ SD. Fexo = Fexofenadine; Rama = ramatroban. ** $p < 0.01$ as compared to the OVA-sensitized nontreated group.

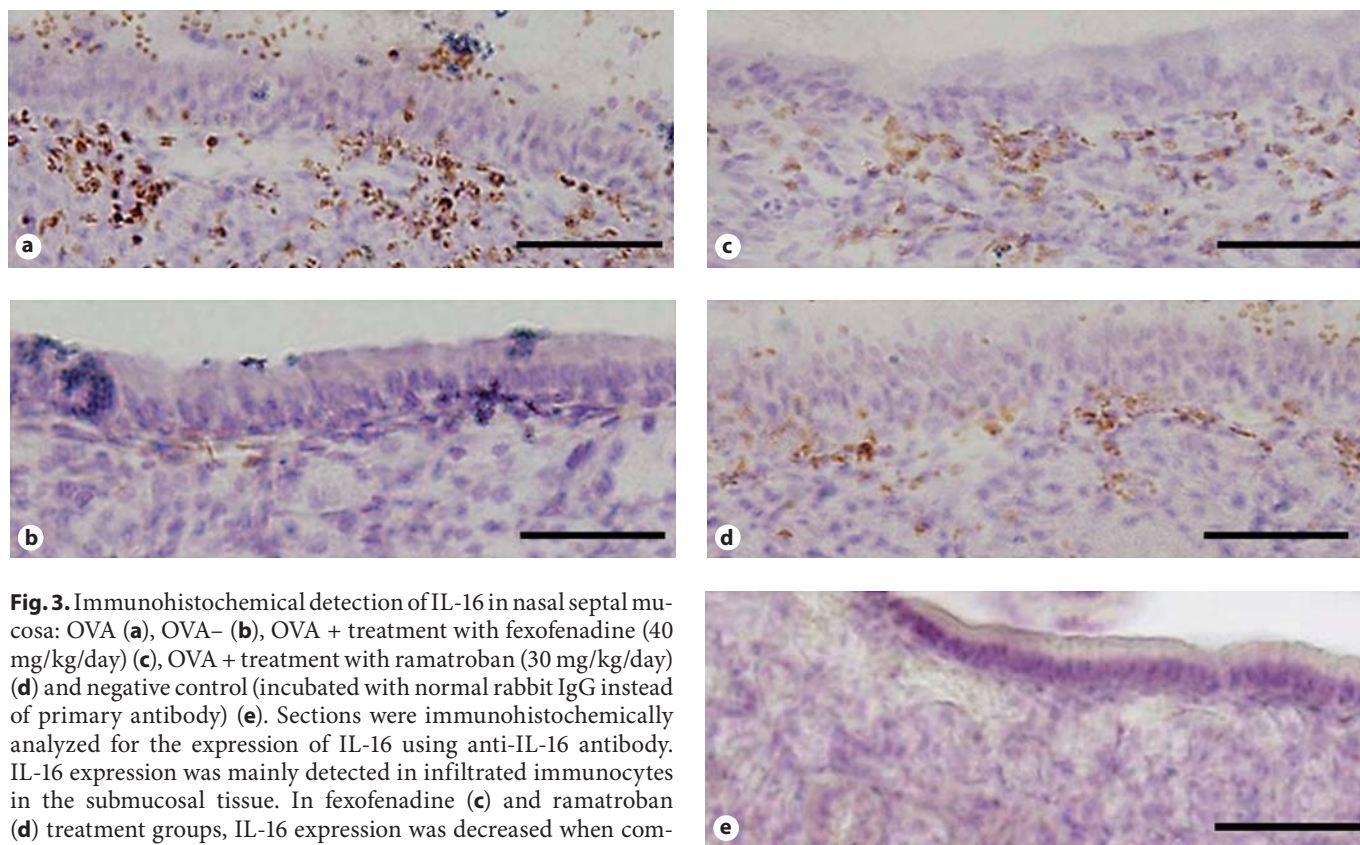
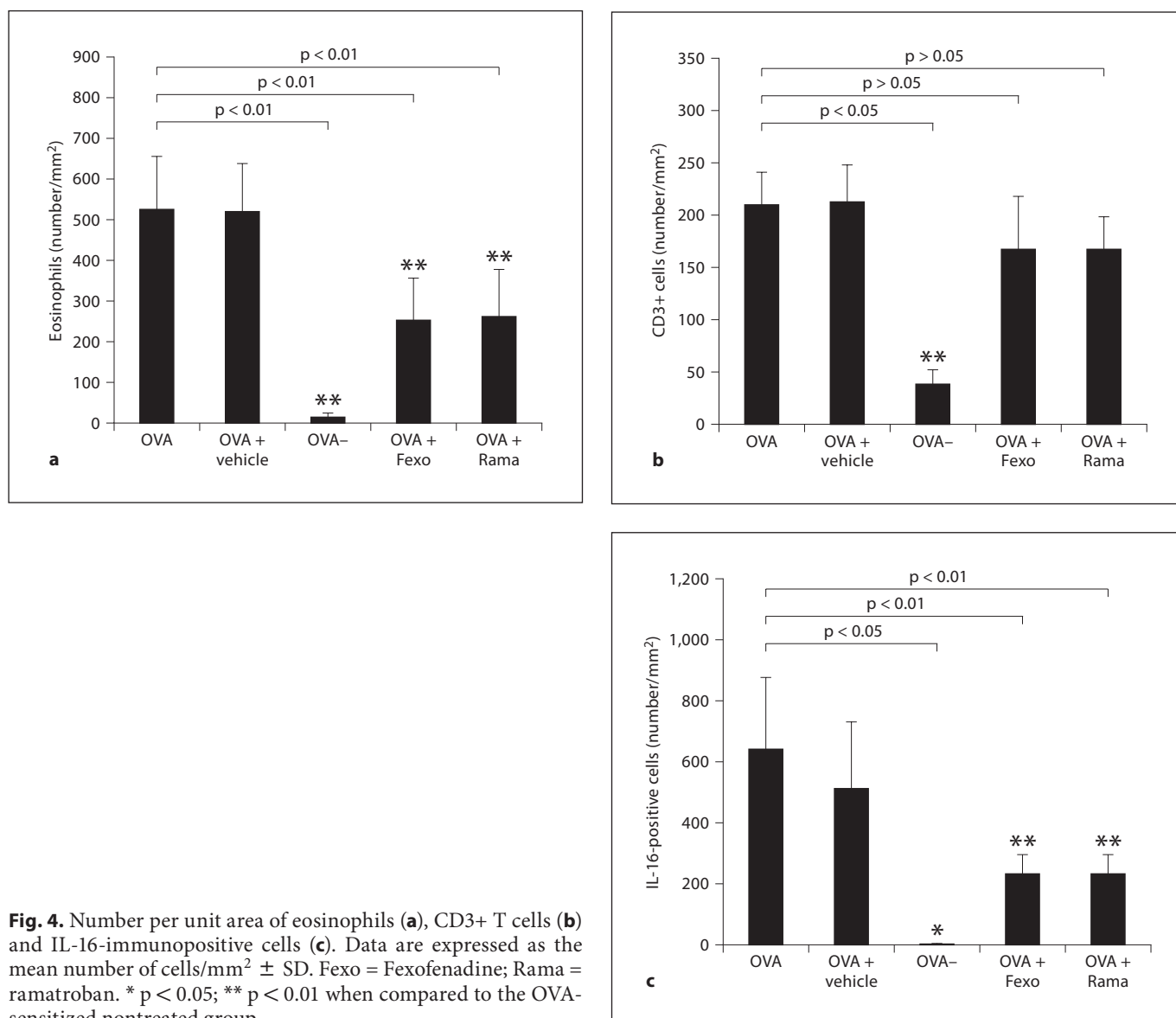


Fig. 3. Immunohistochemical detection of IL-16 in nasal septal mucosa: OVA (a), OVA- (b), OVA + treatment with fexofenadine (40 mg/kg/day) (c), OVA + treatment with ramatroban (30 mg/kg/day) (d) and negative control (incubated with normal rabbit IgG instead of primary antibody) (e). Sections were immunohistochemically analyzed for the expression of IL-16 using anti-IL-16 antibody. IL-16 expression was mainly detected in infiltrated immunocytes in the submucosal tissue. In fexofenadine (c) and ramatroban (d) treatment groups, IL-16 expression was decreased when compared to the OVA-sensitized nontreated group (a). b In OVA-, a few immunoreactive cells were detected. Scale bar = 50 μ m.



ELISA for Serum OVA-Specific IgE and IL-16
 Serum levels of OVA-specific IgE were reduced by treatment with fexofenadine (84.2 ± 53.5 U/ml, $p = 0.1275$) and ramatroban (73.7 ± 60.8 U/ml, $p = 0.1275$) when compared to the OVA-sensitized nontreated group (132.8 ± 47.3 U/ml); however, they did not reach statistically significant values. Treatment with only vehicle showed no inhibitory effect (142.4 ± 63.5 U/ml, $p = 0.6943$). In each group, 7–9 serum samples were measured. These results are shown in figure 2a.

The mean concentrations of serum IL-16 in the OVA-sensitized nontreated group and nonsensitized group

were 143.9 ± 57.1 and 32.6 ± 36.8 pg/ml, respectively ($p < 0.01$). Treatment with fexofenadine and ramatroban resulted in significant reduction of serum IL-16 levels (70.1 ± 35.6 pg/ml, $p < 0.01$ and 68.4 ± 35.9 pg/ml, $p < 0.01$). There was no inhibitory effect in the vehicle-administered group (138.5 ± 102.9 pg/ml, $p = 0.2698$). In each group, 7–9 serum samples were measured. These results are shown in figure 2b.

Eosinophil Infiltration and Immunohistochemistry

In each group, the mean number of infiltrated eosinophils per unit area, T cells per unit area and the number

of IL-16 immunoreactive cells per unit area are shown as the number of cells/mm². Treatment with fexofenadine (253.3 ± 101.9 cells/mm², $p < 0.01$) and ramatroban (263.4 ± 115.7 cells/mm², $p < 0.01$) significantly inhibited eosinophil infiltration when compared to the OVA-sensitized nontreated group (525.1 ± 131.9 cells/mm²). Infiltrations of T cells were inhibited by fexofenadine (166.8 ± 51.1 cells/mm², $p = 0.1255$) or ramatroban (167.1 ± 30.4 cells/mm², $p = 0.0519$) when compared to the OVA-sensitized nontreated group (210.2 ± 31.1 cells/mm²); however, they did not reach statistically significant values. In each group, 5–7 specimens were available.

IL-16 immunoreactivity was significantly reduced after treatment with fexofenadine (233.1 ± 60.6 cells/mm², $p < 0.01$) and ramatroban (234.4 ± 58.9 cells/mm², $p < 0.01$) when compared to the OVA-sensitized nontreated group (642.8 ± 234.4 cells/mm²). In the vehicle administration group, no inhibitory effect was observed (514.1 ± 216.3 cells/mm², $p = 0.5476$). In each group, 5–7 specimens were available. Images are shown in figure 3 and quantified results are shown in figure 4.

Discussion

Previous reports have shown that IL-16 is a specific chemotactic factor for CD4+ cells [2]. In human allergic rhinitis, Pullerits et al. [15] reported a significant correlation between CD4+ cell infiltration and IL-16 protein expression in the nasal mucosa after antigen challenge, and Lim et al. [22] reported that IL-16 was also an eosinophil chemoattractant factor and had a higher chemotactic effect than RANTES, suggesting that IL-16 may play an important role in the recruitment of CD4+ cells including eosinophils to the nasal mucosa in allergic rhinitis. Previous *in vivo* studies of IL-16 in allergic rhinitis were performed using human inferior turbinate or blood, but there have been no reports using animal allergic rhinitis models. Therefore, in the present study we first examined IL-16 variability using OVA-sensitized allergic rhinitis BALB/c mice. The serum levels of IL-16 and IL-16-positive cells in the nasal mucosa were significantly increased in the OVA-sensitized murine group when compared to the nonsensitized group. From these results, we confirmed that IL-16 was upregulated in OVA-sensitized mice in a manner similar to patients with allergic rhinitis.

Allergic rhinitis is a chronic inflammatory disease of the nasal mucosa that is characterized by lymphocyte, eosinophil and mast cell infiltrations. There, activated

CD4+ T cells produce several inflammatory cytokines, such as IL-4, IL-5 and GM-CSF. Infiltrated eosinophils are also activated, then they damage the mucosal epithelium by releasing their granules and also produce cytokines. It is suggested that IL-16 has important roles in this allergic cascade [3, 23–25]. Regarding eosinophils, IL-5 has been known as a main eosinophil chemoattractant factor, whereas it was reported that IL-16 neutralization suppressed eosinophilic chemotaxis by 60% *in vitro* [22], which indicates that IL-16 may also have a positive correlation with this matter. Our previous report confirmed that IL-16 serum level and local expressions in patients with allergic rhinitis were significantly correlated with the blood eosinophil percentage [13]. In addition, IL-16 can indirectly activate specific responses of eosinophils; in CD4-mediated cells, IL-16 elicits the extracellular release of RANTES and eotaxin, which then act in an autocrine fashion on CCR3 receptors to stimulate both enhanced leukotriene C₄ production and the release of IL-4 from within eosinophils [26]. Therefore, IL-16 may have an important effect on eosinophilic chemotaxis and activation. In the present study, the numbers of infiltrated T cells and especially eosinophils, which had been considered as main sources of IL-16 [27], were reduced by treatment with fexofenadine and ramatroban (however, T cell prevention did not reach statistical significance). We also confirmed that IL-16-positive cells were decreased by the drugs, which might result from the reduction of such IL-16-producing cells. Local IL-16 reduction and inhibition of inflammatory cell infiltration might both be affected.

It was reported that treatment with IL-16-neutralizing monoclonal antibodies or IL-16-blocking peptides suppressed OVA-specific IgE upregulation and markedly inhibited the development of airway hyperreactivity in a murine model [14, 28], and that the downregulation of IL-16 expression by glucocorticoid was involved in improving allergic rhinitis [15]. In addition, the IL-16 protein level had significant correlations with eosinophils and CD4+ T cells in bronchoalveolar lavage fluid 24 h after allergen challenge in asthmatic patients *in vivo* [29]. On the other hand, IL-16 shows immune-modulatory effects; De Bie et al. [10] investigated the ability of exogenously added IL-16 to alter the inflammatory response in a murine model of allergic asthma and observed a significant decrease in airway hyperreactivity after treatment with IL-16. In addition, it was suggested that IL-16 induced T regulatory cells *in vitro* [30]. It is apparent that IL-16 has not only proinflammatory effects but also immune-suppressive effects, but its actual roles *in vivo* have not been identified. If there are limited endogenous IL-16

effects, inhibition of endogenous IL-16 may contribute to improving the allergic state in vivo without aggravating it, or at least IL-16 reduction is parallel to an improvement in the allergic state. In this study, IL-16 in serum and nasal mucosa also varied according to allergic rhinitis conditions. Further studies are needed to elucidate the relationship between IL-16 and allergic rhinitis.

The details of IL-16 inhibition mechanisms by these drugs are unknown. Eosinophils and T cells are major IL-16 production sources in allergic disease [13, 27]; therefore, inhibition of their migration and activation induced by the drugs is one possible process. In the present study, both fexofenadine and ramatroban, which have antihistamine effects and anti-PGD2 or TxA2 effects, respectively, could inhibit IL-16; this suggested that IL-16 upregulation was induced through both histamine- and PGD2 (or TxA2)-mediated allergic pathways. In addition, the drugs have the ability to inhibit directly some particular inflammatory cytokines from inflammatory cells independent of histamine or PGD2 [31–33]. In the present study, OVA-specific IgE production was prevented by the drugs, and this result supported the fact the cytokines (such as IL-4 and IL-13) were inhibited in our model; however, whether IL-16 was involved in IgE pro-

duction was unknown. The drugs might also act on inflammatory cells to decrease IL-16 expression. Further studies are needed to confirm the detailed mechanism.

In conclusion, IL-16 was locally and systemically up-regulated in a murine allergic rhinitis model in a manner similar to that observed in patients with allergic rhinitis. The IL-16 mechanism in vivo has not been confirmed completely; however, the current study suggests that IL-16 may play an important role in the recruitment of peripheral eosinophils and CD4⁺ T cells to the inflammatory site and as a mediator of inflammation. Our study showed that local and systemic IL-16 variability was parallel to allergic rhinitis conditions. In addition, after treatment with fexofenadine or ramatroban, both IL-16 and inflammatory cell infiltration, especially eosinophils, were inhibited.

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