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No involvement of interleukin-5 or eosinophils in experimental allergic rhinitis in guinea pigs

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Abstract

The aim of this study is to evaluate whether nasal airway eosinophilia is a true pathogenetic component of allergic rhinitis. We investigated the effects of TRFK5, an anti-interleukin-5 antibody, not only on leukocyte mobilization from the bone marrow, but also on the development of nasal symptoms and hyperresponsiveness in a guinea pig model of allergic rhinitis. Intranasally sensitized animals were repetitively challenged by exposure to Japanese cedar pollen as antigen. TRFK5 (100 μg/kg, i.p.) given 12 h before the final antigen challenge selectively prevented the antigen-induced eosinophilia in blood and the nasal airway, and suppressed the corresponding decrease in the number of cells in bone marrow; however, it failed to inhibit the immediate development of sneezing, early and late nasal blockage responses, goblet cell degranulation and nasal hyperresponsiveness to histamine. Furthermore, TRFK5 did not significantly affect the production of thromboxane A_2 and cysteinyl leukotrienes in the nasal airway during the late response. These results strongly suggest that while interleukin-5 is essential for eosinophil migration from the bone marrow to the nasal airway, neither interleukin-5 nor eosinophils are required for the development of the nasal symptoms and nasal hyperresponsiveness of allergic rhinitis.

Keywords: Allergic rhinitis; Interleukin-5; Eosinophil; TRFK5

1. Introduction

Allergic rhinitis affects approximately 20% of the world's population (Bellanti and Wallerstedt, 2000) and is characterized by three major symptoms, namely, sneezing, hypersecretion and nasal blockage (Naclerio, 1991). When allergic rhinitis patients are exposed to specific allergens, almost all patients show an early response with the development of the three major symptoms, and approximately 50% also develop a late response, with the predominant symptom of nasal blockage (Dvoracek et al., 1984). In addition, patients develop marked nasal hyperresponsiveness, which is defined as an increased nasal response to nonspecific stimuli such as histamine (Borum et al., 1983).

It is generally accepted that the early response is mainly mediated by the actions of preformed and newly generated chemical mediators released after immunoglobulin (Ig) E-dependent activation of mast cells in the nasal mucosa (Baraniuk, 1997). In contrast, the development of the late response and nasal hyperresponsiveness after antigen challenge is associated with eosinophilia in the nasal airway and blood, which are the hallmarks of allergic rhinitis, as suggested by the following indirect evidence. First, eosinophils have the potential to release lipid mediators, cytokines and toxic granule proteins (Bousquet et al., 1996; Gleich, 2000). Second, the number of accumulated eosinophils and/or the level of toxic granule proteins in the nasal airway correlate with the magnitude of the late response and/or nasal hyperresponsiveness to some stimuli in patients suffering from allergic rhinitis (Pastorello et al., 1994; De Graaf-in't Veld et al., 1996; Kita et al., 2000). However, direct evidence is needed before it can be claimed that eosinophils constitute an essential pathogenetic component of allergic rhinitis, and many conflicting data have been reported (Klementsson et al., 1991; Godthelp et al., 1996).

The most direct method to assess the role of eosinophils in allergic rhinitis is to selectively attenuate the nasal airway eosinophilic response to antigen challenge. If the infiltrated

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eosinophils in the nasal airway are the cause or contribute significantly to the development of the nasal symptoms and nasal hyperresponsiveness of allergic rhinitis, then suppression of the eosinophilia should prevent the development of these nasal disorders. It is well recognized that eosinophils are sequentially mobilized from the bone marrow pool into tissues through the blood stream in allergic inflammatory reactions. To date, several cytokines and chemokines have been implicated in this sequential process of eosinophil trafficking (Rothenberg, 1998). Of these factors, interleukin-5 is the one most often associated with significant eosinophil accumulation in the target tissue exhibiting an allergic response, with its highly specific effects on eosinophil proliferation, migration, activation and survival (Lopez et al., 1988; Yamaguchi et al., 1988; Clutterbuck et al., 1989). Therefore, we hypothesize that inhibition of interleukin-5 activity could attenuate nasal airway eosinophilia, and that the anti-interleukin-5 monoclonal antibody TRFK5 could be useful for investigating the roles of not only interleukin-5, but also of eosinophils in the pathogenesis of allergic rhinitis.

We previously established an allergic rhinitis model in which intranasally sensitized guinea pigs are given repetitive inhalation challenges of Japanese cedar pollen as antigen (Nabe et al., 1998; Mizutani et al., 1999). The animals reproducibly develop not only immediate sneezing but also early and late nasal blockage responses and potent nasal hyperresponsiveness to histamine after each intranasal antigen challenge. In addition, the pollen inhalational challenge in the sensitized animals induces both blood and nasal airway eosinophilia, in association with the development of late nasal blockage (Yamasaki et al., 2001b). Furthermore, we noted that thromboxane A_2 and cysteinyl leukotrienes, which are the major products of arachidonic acid metabolism in eosinophils (Sun et al., 1991), are the chemical mediators responsible for the development of late nasal blockage in this model (Mizutani et al., 2001; Yamasaki et al., 2001a). These findings are quite similar to those of clinical studies. Thus, this model was considered useful for analyzing the pathophysiology of human allergic rhinitis.

In this study, to examine whether interleukin-5 and/or eosinophils are true pathogenetic factors in allergic rhinitis, we investigated the effects of TRFK5 on (1) the mobilization of eosinophils from the femoral bone marrow to the nasal airway, and (2) the development of nasal symptoms and nasal hyperresponsiveness to histamine, in this guinea pig model of allergic rhinitis. In addition, we histopathologically evaluated the antigen-induced degranulation of goblet cells in the nasal epithelium, which has been shown to contribute, at least partly, to increased nasal secretion (hypersecretion) (Berger et al., 1999), and measured the levels of thromboxane B_2, a stable breakdown product of thromboxane A_2, and the levels of cysteinyl leukotrienes in nasal cavity lavage fluid collected during the late response.

2. Materials and methods

2.1. Animals

Male, 3-week-old Hartley guinea pigs weighing 201–250 g were purchased from Japan SLC (Hamamatsu, Japan). The animals were housed in an air-conditioned room illuminated from 8:00 a.m. to 8:00 p.m., at a temperature of 23 ± 1 °C and $60 \pm 10\%$ humidity; they were fed on a standard laboratory diet and given water ad libitum. Sensitization of the animals was started 1 week after the animals were purchased. The present animal experiment was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

2.2. Materials

Purified rat anti-mouse/human interleukin-5 monoclonal antibody (TRFK5) and isotype-matched control rat IgG_1 (R3-34) were purchased from PharMingen (San Diego, CA, USA). Histamine dihydrochloride, pentobarbital sodium and heparin were procured from Wako Pure Chemical Industries (Osaka, Japan), Abbott Laboratories (North Chicago, IL, USA) and Takeda Chemical Industries (Osaka, Japan), respectively. Phosphate-buffered saline was purchased from Sigma (St. Louis, MO, USA), Hank's balanced salt solution from Life Technologies (NY, USA), and HEPES from Dojindo Laboratories (Kumamoto, Japan). Japanese cedar pollen was obtained from the 1998 crop of Japanese cedar. The $Al(OH)_3$ gels and cedar pollen extracts used for sensitization were prepared as described previously (Nabe et al., 1997b). The other reagents used were of the highest grade available commercially.

2.3. Sensitization and challenge

Sensitization and challenge were performed according to previously described methods (Nabe et al., 1998). In brief, guinea pigs were sensitized by intranasal instillation of cedar pollen extracts adsorbed on $Al(OH)_3$ gel, twice a day for 7 days. One week after the last sensitization treatment, the sensitized animal was intranasally challenged once every week for 18 weeks, by inhalation of cedar pollen using a handmade inhalation apparatus (sensitized–challenged animals). We had already confirmed that this inhalation technique allowed animals to quantitatively inhale pollen (1.8 mg/each nostril) and that almost all of the inhaled pollen was trapped in the upper airways, and that less than 0.001% reached the lower airways (Nabe et al., 1997a). As negative controls, two groups of animals were used: (1) age-matched normal guinea pigs (non-sensitized, non-challenged animals) and (2) guinea pigs sensitized and then administered an inhalation challenge of cedar pollen for 17 weeks as described above, but not administered the pollen challenge in the 18th week (sensitized, non-challenged animals).

2.4. Treatment with antibody

The anti-interleukin-5 antibody, TRFK5, or control antibody, R3-34, both of which were diluted with sterile phosphate-buffered saline (pH 7.4), was administered i.p. 12 h before the 18th pollen inhalation challenge at the dose of 100 μg/kg body weight.

2.5. Nasal cavity lavage and counting of leukocytes

We previously noted that the pollen inhalation challenge in this model induced marked eosinophilia in the nasal cavity, which peaked at 5 h after the challenge (Yamasaki et al., 2001b). Thus, nasal cavity lavage was performed 5 h after the 18th pollen inhalation challenge, by a previously described method (Nabe et al., 1997a) with a slight modification. Namely, guinea pigs were anesthetized i.p. with pentobarbital sodium (30 mg/kg) and silicon tubing connected to an air pump was positioned in the left nostril and was then kept under a slightly reduced pressure. The nasal cavity was then washed from the right nostril to the left nostril with 1 ml of physiologic saline prewarmed to 37 °C. The recovered nasal cavity lavage fluid was centrifuged at 120 × *g* for 5 min at 4 °C. The resultant supernatant was stored at −80 °C until assayed for thromboxane B_2 and cysteinyl leukotrienes, and the cell pellet was suspended with a defined volume (200–800 μl/sample) of physiologic saline. The total leukocyte count in the nasal cavity lavage fluid was determined by staining with Turk's solution (Nacalai Tesque, Kyoto, Japan). For determining the differential leukocyte count, a 50-μl aliquot of the cell suspension was centrifuged on a Settling chamber (Neuro Probe, Cabin John, MD) at 50 × *g* for 30 s at 4 °C, and the settled leukocytes were stained with Diff-Quik® solution (International Reagents, Kobe, Japan). A minimum of 140 cells was counted under the microscope and classified based on morphologic criteria as mononuclear cells, eosinophils and neutrophils.

2.6. Peripheral blood sample collection and cell counts

Following the collection of nasal cavity lavage fluid, peripheral blood samples were collected from a vein in the forefoot into test tubes containing 100 U/ml of heparin. The total leukocyte count in the peripheral blood samples was determined as described above. The differential cell counts were also determined as described above after hypotonic treatment of the samples with 0.2% NaCl solution. A minimum of 400 cells was counted under the microscope and classified based on morphologic criteria as monocytes, lymphocytes, eosinophils and neutrophils.

2.7. Collection of bone marrow specimens and cell counts

After blood sample collection, the guinea pigs were additionally anesthetized i.p. with pentobarbital sodium (about 100 mg/kg) and exsanguinated from the abdominal aorta. Then, their left femurs were exposed. The femoral head and condyles were removed, and the marrow cavity of the femoral shaft was flushed with 20 ml of Hank's balanced salt solution containing 10 mM of HEPES and 10 U/ml of heparin. The displaced cells were gently suspended using a pipette, and the suspension was filtered through a nylon mesh with a pore size of 100 μm. The filtered cell suspension was centrifuged at 400 × *g* for 5 min at 4 °C, and the resultant cell pellet was resuspended in 5 ml of Hank's balanced salt solution not containing Ca^{2+} and Mg^{2+}. The total and differential leukocyte (eosinophils, neutrophils and mononuclear cells) counts were determined as described above.

2.8. Histopathological examination

Immediately after extraction of the femur, the head of each guinea pig was separated from the carcass, and the skull was subsequently fixed in 10% neutral buffered formalin and decalcified in 5% formic acid solution. After decalcification, a transverse tissue block containing the nasal airway, which was trimmed at the incisive papilla, was embedded in paraffin wax and cut into 4-μm-thick sections. The sections were stained with Luna for the identification of eosinophils, or with Alcian blue and periodic acid-Schiff (AB–PAS) for the detection of acidic and neutral mucosubstances and for the identification of goblet cells. The accumulation of eosinophils in the nasal mucosa was examined in Luna-stained sections under light microscopy and graded using a semi-quantitative, nonlinear, 4-point grading system: Grade 0; not present or very slight: Grade 1; mild: Grade 2; moderate: Grade 3; severe. In the AB–PAS-stained sections, the total area of AB–PAS-stained mucosubstances in the mucosal surface epithelium was graded in the same manner as for the evaluation of eosinophil accumulation.

2.9. Determination of sneezing frequency

The severity of sneezing, as well as the nasal blockage and nasal responsiveness to histamine, was assessed in another set of animals. The sneezing frequency during the first 10 min after the 18th pollen inhalation challenge was determined by direct observation of the animals.

2.10. Measurement of specific airway resistance

To evaluate the degree of nasal blockage, specific airway resistance was measured in conscious guinea pigs before and 1–10 h after the 18th antigen challenge using a two-chambered, double-flow plethysmograph system, according to the method of Pennock et al. (1979). In brief, an animal was placed with its neck extending through the partition of a two-chambered box, and specific airway resistance was measured using a Pulmos-I data analyzer (MIPS, Osaka, Japan) and a PC 9801 FA computer (NEC, Tokyo, Japan) after airflow was monitored via sensors attached to both the front and the rear chamber. The animal was removed from the plethysmograph

Table 1
Effect of anti-interleukin-5 antibody on the number of leukocytes in nasal cavity lavage fluid 5 h after the 18th pollen inhalation challenge in sensitized guinea pigs

Animals	Antibody	Cell number ($\times 10^4$ cells/nasal cavity lavage fluid)			
		Total cells	Eosinophils	Mononuclear cells	Neutrophils
Sensitized, non-challenged	R3-34	1.5 ± 0.4	0.6 ± 0.3	0.7 ± 0.2	0.3 ± 0.1
Sensitized–challenged	R3-34	27.2 ± 8.9[a]	21.8 ± 7.4[a]	3.8 ± 1.2[a]	1.6 ± 0.5[a]
Sensitized–challenged	TRFK5	17.1 ± 3.4	3.5 ± 1.1[b]	7.9 ± 1.9	5.7 ± 1.9

Either TRFK5 (anti-interleukin-5 antibody) or R3-34 (control antibody) was administered i.p. 12 h before the 18th pollen inhalation challenge at the dose of 100 μg/kg. Data represent the means ± S.E.M. for 10–13 animals/group.
[a] $P<0.05$, compared with the R3-34-treated sensitized, non-challenged animals.
[b] $P<0.01$, compared with the R3-34-treated sensitized–challenged animals.

between measurements at each time point. The change in specific airway resistance is expressed as the measured value after the challenge minus the prechallenge baseline value (before).

Because the guinea pig functionally respires through the nose and not through the mouth, specific airway resistance can be taken as the total resistance of the upper and lower airways in the animal. However, the pollen-inhalation challenge-induced elevation of specific airway resistance correlated well with the decrease in respiratory frequency in the present experimental allergic rhinitis model (Nabe et al., 1998), whereas the antigen inhalation-induced early bronchoconstrictor response has been characterized by rapid and shallow breathing in a guinea pig model of asthma (Iijima et al., 1987). In addition, eosinophil accumulation in the lung, which is a characteristic feature of the asthmatic response, was not observed at 5 h after the pollen inhalation challenge in this model (M. Yamasaki, personal observations). Thus, the change in specific airway resistance induced by the pollen challenge can be considered to reflect upper airway obstruction in our model.

2.11. Measurement of nasal responsiveness to histamine

To estimate the nasal responsiveness to histamine, specific airway resistance was measured before and after intranasal instillation of histamine. Briefly, 2 days after the 18th inhalational pollen challenge, 10 μl of increasing concentrations of histamine solution was consecutively applied to each nasal cavity of guinea pigs at 20-min intervals. Specific airway resistance was measured before the first instillation of histamine (10^{-4} M) and 10 min after the instillation of each dose of histamine. The change in specific airway resistance induced by the intranasal instillation of histamine solution can also be considered to reflect upper airway obstruction, because the histamine challenge decreases respiratory frequency in our allergic rhinitis model (Mizutani et al., 1999).

2.12. Measurement of thromboxane B_2 and cysteinyl leukotriene concentrations

The concentrations of thromboxane B_2 and cysteinyl leukotrienes in the nasal cavity lavage fluid were measured after extraction of the sample, using a thromboxane B_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) and leukotriene $C_4/D_4/E_4$ enzyme immunoassay system (Amersham International, Buckinghamshire, UK), respectively, according to a previously described method (Yamasaki et al., 2001a).

2.13. Statistical analyses

Data are presented as means ± S.E.M. Statistical analyses were performed with Student's *t*-test or Wilcoxon's test with Bonferroni correction where appropriate. A probability value (P) of less than 0.05 was considered to denote statistical significance. All statistical calculations were performed using a SAS statistical package (SAS Institute, Cary, NC, USA).

Table 2
Effect of anti-interleukin-5 antibody on the number of leukocytes in peripheral blood 5 h after the 18th pollen inhalation challenge in sensitized guinea pigs

Animal	Antibody	Cell number ($\times 10^4$ cells/ml blood)				
		Total cells	Eosinophils	Monocytes	Lymphocytes	Neutrophils
Sensitized, non-challenged	R3-34	470.3 ± 26.8	10.2 ± 1.8	98.6 ± 4.4	132.1 ± 7.9	229.3 ± 23.2
Sensitized–challenged	R3-34	709.3 ± 51.6[a]	40.8 ± 6.5[a]	113.3 ± 9.0	133.8 ± 16.9	421.4 ± 38.1[a]
Sensitized–challenged	TRFK5	585.8 ± 49.1	1.1 ± 0.4[b]	83.7 ± 10.1[c]	102.0 ± 10.9	399.0 ± 37.3

Either TRFK5 (anti-interleukin-5 antibody) or R3-34 (control antibody) was administered i.p. 12 h before the 18th pollen inhalation challenge at the dose of 100 μg/kg. Data represent the means ± S.E.M. for 10–13 animals/group.
[a] $P<0.01$, compared with the R3-34-treated sensitized, non-challenged animals.
[b] $P<0.01$, compared with the R-34-treated sensitized–challenged animals.
[c] $P<0.05$, compared with the R-34-treated sensitized–challenged animals.

3. Results

3.1. Numbers of leukocytes in nasal cavity lavage fluid

Table 1 shows the effect of TRFK5 on the number of leukocytes in nasal cavity lavage fluid 5 h after the 18th pollen inhalation challenge. A significant increase in the total leukocyte count in the nasal cavity lavage fluid was observed in control antibody (R3-34)-treated sensitized–challenged animals, as compared with that in the control antibody-treated sensitized, non-challenged animals. This antigen-induced increase in the total leukocyte count in the nasal cavity lavage fluid mainly resulted from an increase in eosinophil numbers. The numbers of mononuclear cells and neutrophils in the nasal cavity lavage fluid also increased following the antigen challenge; however, these increases were considerably less significant than the increase in eosinophil numbers. Pretreatment of the sensitized–challenged animals with TRFK5 significantly inhibited the antigen-induced increase in the number of eosinophils by 86%, while it had no significant effects on the antigen-induced increases in the numbers of mononuclear cells and neutrophils in the nasal cavity lavage fluid.

3.2. Numbers of circulating leukocytes

As shown in Table 2, a significant increase in the total leukocyte count in the peripheral blood was observed 5 h after the pollen inhalation challenge in the control antibody-treated sensitized–challenged animals. Although this antigen-induced increase in the number of circulating leukocytes mainly reflected an increase in the number of neutrophils, the eosinophil count in the blood also increased after the antigen challenge. In contrast, no significant changes in the numbers of circulating monocytes and lymphocytes were noted after the antigen challenge. Pretreatment of the sensitized–challenged animals with TRFK5 significantly and completely inhibited the antigen-induced increase in the number of circulating eosinophils, and the eosinophil number was lower than that in the control antibody-treated sensitized, non-challenged animals. While the TRFK5 pretreatment had no apparent effects on the antigen-induced increase in the number of circulating neutrophils and on the number of circulating lymphocytes, it significantly reduced the number of monocytes.

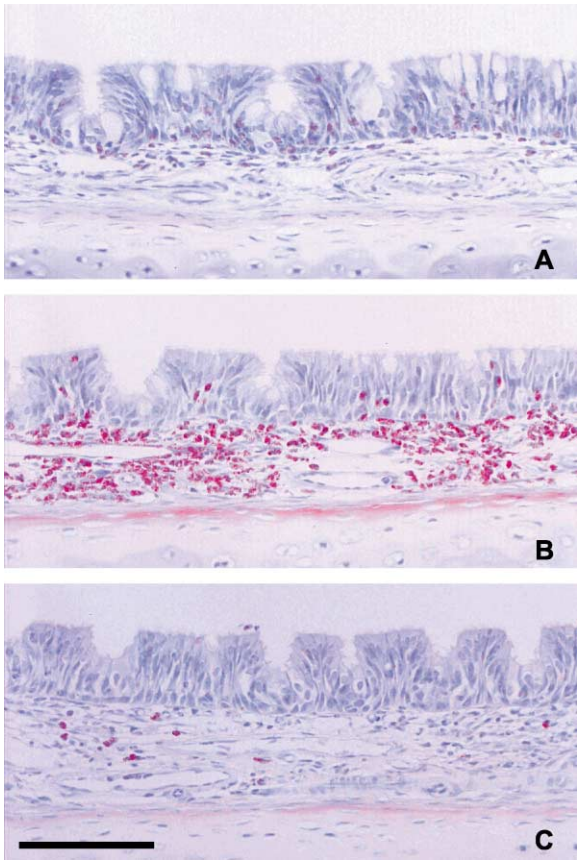

Fig. 1. Representative light photomicrographs of Luna-stained sections of the nasal mucosa from control antibody- or TRFK5-treated guinea pigs. Few eosinophils (in red) were observed in the specimens from the control antibody-treated sensitized, non-challenged guinea pigs (A). In contrast, numerous eosinophils were found to be accumulated 5 h after the 18th pollen inhalation challenge in the nasal mucosa specimens obtained from control antibody-treated sensitized–challenged animals (B). Pretreatment of the sensitized–challenged animals with TRFK5 completely inhibited the eosinophil accumulation in the nasal mucosa (C). Scale bar = 100 μm.

3.3. Numbers of leukocytes in the bone marrow

To evaluate the effect of TRFK5 on the number of leukocytes in the bone marrow following the pollen inha-

Table 3
Effect of anti-interleukin-5 antibody on the number of leukocytes in bone marrow 5 h after the 18th pollen inhalation challenge in sensitized guinea pigs

Animal	Antibody	Cell number ($\times 10^7$ cells/femur)			
		Total cells	Eosinophils	Mononuclear cells	Neutrophils
Sensitized, non-challenged	R3-34	17.1 ± 1.7	1.2 ± 0.1	8.8 ± 0.9	4.4 ± 0.4
Sensitized–challenged	R3-34	14.7 ± 1.3	0.7 ± 0.1[a]	8.5 ± 0.7	3.1 ± 0.3[b]
Sensitized–challenged	TRFK5	14.3 ± 0.7	1.1 ± 0.1[c]	8.2 ± 0.4	2.9 ± 0.2

Either TRFK5 (anti-interleukin-5 antibody) or R3-34 (control antibody) was administered i.p. 12 h before the 18th pollen inhalation challenge at the dose of 100 μg/kg. Data represent the means ± S.E.M. for 10–13 animals/group.

[a] $P < 0.01$, compared with the R3-34-treated sensitized, non-challenged group.

[b] $P < 0.05$, compared with the R3-34-treated sensitized, non-challenged group.

[c] $P < 0.01$, compared with the R3-34-treated sensitized–challenged group.

lation challenge in this model, we measured the number of leukocytes in bone marrow specimens collected from the left femur at 5 h after the antigen challenge (Table 3). The number of eosinophils in the bone marrow in the control antibody-treated sensitized–challenged animals was significantly decreased after the antigen challenge, as compared with that in the control antibody-treated sensitized, non-challenged animals. A significant decrease in the number of neutrophils was also observed in the control antibody-treated sensitized–challenged animals. In contrast, there were no significant changes in the numbers of total leukocytes and mononuclear cells. Pretreatment of the sensitized–challenged animals with TRFK5 significantly suppressed the antigen-induced decrease in eosinophil numbers in bone marrow by 75%. However, it did not have a significant effect on the antigen-induced decrease in the total number of leukocytes, neutrophils or mononuclear cells.

3.4. Eosinophil accumulation in the nasal mucosa

The effect of TRFK5 on the antigen-induced eosinophil accumulation in the nasal mucosa was examined in Luna-stained preparations obtained 5 h after the pollen inhalation challenge. Light-microscopic examination of Luna-stained preparations revealed that the antigen challenge in the control antibody-treated sensitized animal induced a marked eosinophil accumulation in the nasal mucosa, which was scarcely seen in the control antibody-treated sensitized, non-challenged animal (Fig. 1A and B). The mean eosinophil accumulation in the control antibody-treated sensitized–challenged animals was significantly greater than that in the control antibody-treated sensitized, non-challenged animals (Table 4). Pretreatment of the sensitized–challenged animals with TRFK5 completely inhibited the antigen-induced eosinophil accumulation in the nasal mucosa (Fig. 1C), and the mean score was significantly reduced to 0.4 ± 0.1 (Table 4). In contrast, the treatment did not affect the accumulation of the other leukocytes, including neutrophils and mononuclear cells, in the nasal mucosa (Fig. 1).

Table 4
Semiquantitative analysis of the effects of anti-interleukin-5 antibody on eosinophil accumulation in nasal mucosa and on goblet cell degranulation 5 h after the 18th pollen inhalation challenge in sensitized guinea pigs

Animal	Antibody	Mean score	
		Eosinophil accumulation	AB–PAS-stained area in the nasal epithelium
Sensitized, non-challenged	R3-34	0.7 ± 0.2	2.7 ± 0.2
Sensitized–challenged	R3-34	1.4 ± 0.2[a]	1.4 ± 0.2[b]
Sensitized–challenged	TRFK5	0.4 ± 0.1[c]	1.2 ± 0.1

Either TRFK5 (anti-interleukin-5 antibody) or R3-34 (control antibody) was administered i.p. 12 h before the 18th pollen inhalation challenge at the dose of 100 μg/kg. The eosinophil accumulation in the nasal mucosa and the Alcian blue and periodic acid Schiff (AB–PAS)-stained area in the mucosal surface epithelium were examined in Luna- and AB–PAS-stained sections, respectively, and graded as described in Materials and methods. Data represent the means ± S.E.M. for 10–13 animals/group.

[a] $P<0.05$, compared with R3-34-treated sensitized, non-challenged animals.

[b] $P<0.01$, compared with R3-34-treated sensitized, non-challenged animals.

[c] $P<0.01$, compared with R3-34-treated sensitized–challenged animals.

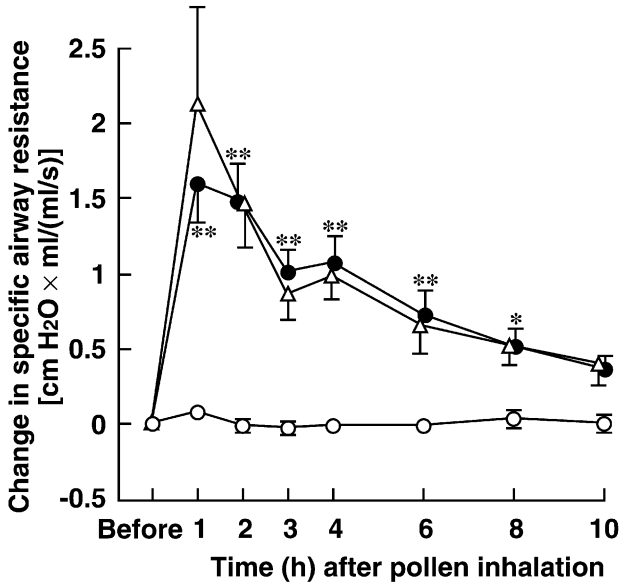

Fig. 2. Influence of pretreatment with TRFK5 on nasal blockage induced by the 18th pollen inhalation challenge in sensitized guinea pigs. In the control antibody-treated sensitized–challenged guinea pigs (closed circles), the pollen inhalation challenge induced a significant increase in the specific airway resistance (reflecting the early and late nasal blockage response), with peaks at 1 and 4 h after the challenge. Pretreatment of the sensitized–challenged guinea pigs with TRFK5 (open triangles) had no significant effect on the antigen-induced early and late nasal blockage response. The results represent the means ± S.E.M. for 10 animals/group. * $P<0.05$ and ** $P<0.01$, compared with non-sensitized, non-challenged guinea pigs (open circles).

3.5. Sneezing and nasal blockage

The control antibody-treated sensitized–challenged guinea pigs sneezed within 10 min after the 18th pollen inhalation challenge (10.3 ± 0.8 times/10 min, $P<0.01$), while the non-sensitized, non-challenged animals did not (0.0 ± 0.0 times/10 min). Pretreatment of the sensitized–challenged animals with TRFK5 had scarcely any effect on the antigen-induced sneezing, and the frequency of sneezing in this group was 9.7 ± 1.0 times/10 min. In addition to the occurrence of sneezing, a marked and prolonged elevation of specific airway resistance (an index of nasal blockage) was observed in the control antibody-treated sensitized–challenged animals (Fig. 2). There were two peaks (1 and 4 h after the antigen challenge) in the time course of the change in specific airway resistance, indicating that the antigen-induced increase in specific airway resistance consisted of early (0–3

h) and late (3–10 h) nasal blockage responses, although these responses partially overlapped each other. Pretreatment of the sensitized–challenged animals with TRFK5 did not affect either antigen-induced nasal blockage response.

3.6. Goblet cell degranulation

Goblet cell degranulation after the pollen inhalation challenge was histopathologically assessed in AB–PAS-stained preparations. There were numerous goblet cells containing large amounts of AB–PAS-positive mucosubstances in the nasal epithelium of the control antibody-treated sensitized, non-challenged animals (Fig. 3A). In contrast, a significant decrease in the AB–PAS-stained area in the nasal epithelium of the control antibody-treated sensitized–challenged animals (Fig. 3B and Table 4) was observed 5 h after the antigen challenge. This decrease in the AB–PAS-stained area in the nasal epithelium of the control antibody-treated sensitized–challenged animals was found to be the result of a decrease in the content of AB–PAS-stained mucosubstances in each goblet cell (Fig. 3B), indicating that degranulation of the goblet cells in the control antibody-treated sensitized–challenged animals, with the release of the mucosubstances contained in them, occurred after antigen challenge. Pretreatment of the sensitized–challenged animals with TRFK5 did not inhibit this antigen-induced goblet cell degranulation (Fig. 3C), and there was no significant difference in the mean score of the AB–PAS-stained area between the control antibody- and TRFK5-treated sensitized–challenged animals (Table 4).

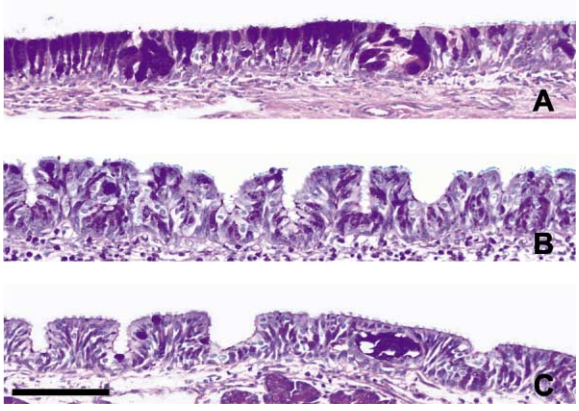

Fig. 3. Representative light photomicrographs of AB–PAS-stained sections of nasal mucosa from the control antibody- and TRFK5-treated guinea pigs. Numerous goblet cells containing AB–PAS-positive mucosubstances (dark purple) were observed in the nasal epithelium of the control antibody-treated sensitized, non-challenged guinea pigs (A). In contrast, a decrease in the content of AB–PAS-positive mucosubstances in the goblet cells was seen in the nasal epithelium of control antibody-treated sensitized–challenged animals 5 h after the 18th pollen inhalation challenge, indicating that degranulation of goblet cells with release of the mucosubstances had occurred after the antigen challenge (B). Pretreatment of the sensitized–challenged animals with TRFK5 scarcely influenced the antigen-induced goblet cell degranulation (C). Scale bar = 100 μm.

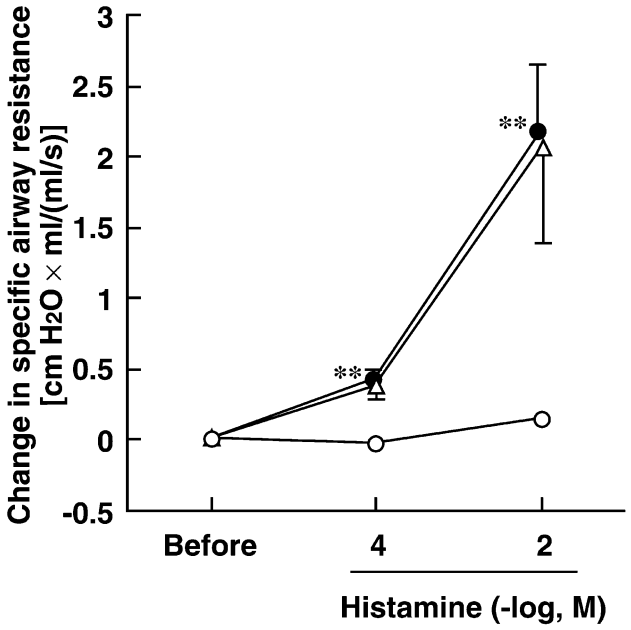

Fig. 4. Influence of pretreatment with TRFK5 on the nasal hyperresponsiveness to histamine 2 days after the 18th pollen inhalation challenge in sensitized guinea pigs. Significant and marked increase in the nasal responsiveness to histamine was observed in the control antibody-treated sensitized–challenged guinea pigs (closed circles) as compared with that in the non-sensitized, non-challenged guinea pigs (open circles). There were no significant differences in the nasal hyperresponsiveness to histamine between the control antibody-treated sensitized–challenged and TRFK5-treated sensitized–challenged guinea pigs (open triangles). The results represent the means ± S.E.M. for 10 animals/group. ** $P < 0.01$, compared with non-sensitized, non-challenged guinea pigs.

3.7. Nasal hyperresponsiveness to histamine

As shown in Fig. 4, a significant increase in the nasal responsiveness to histamine was seen in the control antibody-treated sensitized–challenged guinea pigs 2 days after the pollen inhalation challenge, as compared with that in the non-sensitized, non-challenged animals. The pretreatment with TRFK5 did not have a significant effect on the development of the hyperresponsiveness to histamine, and the TRFK5-treated sensitized–challenged animals showed the same responsiveness to histamine as the control antibody-treated sensitized–challenged animals.

Values for specific airway resistance before histamine (10^{-4} M) instillation did not differ between the non-sensitized, non-challenged animals, the control antibody-treated sensitized–challenged animals, and the TRFK5-treated sensitized—challenged animals [1.87 ± 0.14, 1.56 ± 0.14 and 1.64 ± 0.10 cm H_2O × ml/(ml/s), respectively].

3.8. Levels of thromboxane B_2 and cysteinyl leukotrienes in the nasal cavity lavage fluid

As shown in Fig. 5, the levels of thromboxane B_2 and cysteinyl leukotrienes in nasal cavity lavage fluid collected

5 h after the antigen challenge in the control antibody-treated sensitized–challenged animals were significantly higher than those measured in the control antibody-treated sensitized, non-challenged animals. The pretreatment with TRFK5 tended to inhibit the antigen-induced local production of thromboxane B_2 by 34%, but the inhibition was not significant ($P=0.42$). In addition, the pretreatment with TRFK5 did not have any effect on the production of cysteinyl leukotrienes.

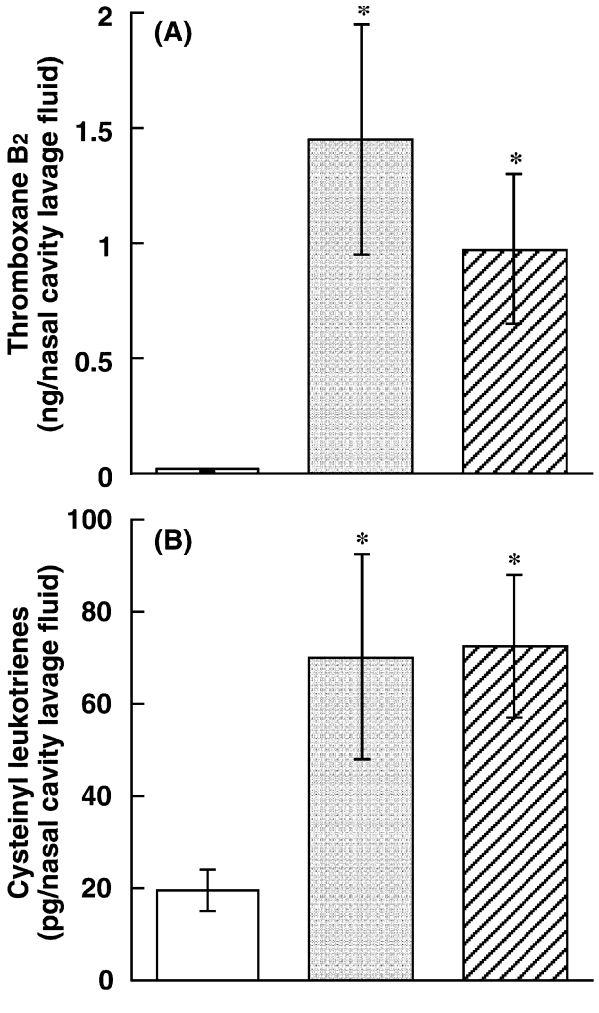

Fig. 5. Influence of pretreatment with TRFK5 on the late release of thromboxane B_2 and cysteinyl leukotrienes after the 18th pollen inhalation challenge in sensitized guinea pigs. Significant increases in the concentrations of thromboxane B_2 and cysteinyl leukotrienes were observed in the nasal cavity lavage fluid collected 5 h after the antigen challenge in the control antibody-treated (shaded bars) and the TRFK5-treated (hatched bars) sensitized–challenged guinea pigs compared with the control antibody-treated sensitized, non-challenged guinea pigs (open bars). There were no significant differences in the antigen-induced late release of these arachidonic acid metabolites into the nasal cavity between the control antibody-treated and the TRFK5-treated sensitized–challenged guinea pigs. The results represent the means ± S.E.M. for 10–13 animals/group. * $P<0.05$, compared with the control antibody-treated sensitized, non-challenged guinea pigs.

4. Discussion

There is no direct evidence that nasal airway eosinophilia is a true pathogenetic component of allergic rhinitis. In view of the lack of eosinophil-deficient animal species, we considered that the most direct way to assess the role of eosinophils in allergic rhinitis would be to selectively reduce the antigen-induced influx of eosinophils into the nasal airway. In this study, we successfully accomplished this using an anti-interleukin-5 antibody, TRFK5. Treatment of sensitized–challenged animals with TRFK5 selectively and markedly prevented the antigen-induced increase in the number of eosinophils in the nasal mucosa and nasal cavity lavage fluid. Thus, this antibody was extremely useful for evaluating the role of not only interleukin-5, but also of eosinophils in the pathogenesis of allergic rhinitis in this model. Treatment with TRFK5 also inhibited the induction of blood eosinophilia and the accompanying decrease in the number of eosinophils in the femoral bone marrow. These results suggest that interleukin-5 plays a pivotal role in the mobilization of eosinophils from the bone marrow pool into the circulation and in the subsequent migration of these cells to the nasal airway in allergic rhinitis, as reported for allergic asthma (Humbles et al., 1997). With respect to the contribution of interleukin-5 to this sequential process of eosinophil migration, Collins et al. (1995) reported that interleukin-5 has the ability to induce the rapid mobilization of eosinophils from bone marrow into blood, whereas it has limited direct activity to induce the migration of these cells from the circulation into tissue in normal guinea pigs. Therefore, we propose that the primary mechanism underlying the inhibitory effect of TRFK5 on antigen-induced nasal airway eosinophilia is blockage of the release of bone marrow eosinophils, which is the first step in eosinophil trafficking to the nasal airway.

Despite this clear effect on the eosinophilia, treatment with TRFK5 failed to inhibit the antigen-induced immediate sneezing, early and late nasal blockage responses, and goblet cell degranulation (which contributes at least partly to nasal hypersecretion) (Berger et al., 1999). To the best of our knowledge, this is the first report clearly demonstrating that neither interleukin-5 nor interleukin-5-induced nasal airway eosinophilia is necessary for the development of antigen-induced nasal symptoms in allergic rhinitis. It is well accepted that IgE-dependent activation of nasal mucosal mast cells is required for the onset of the early response in allergic rhinitis (Baraniuk, 1997). Consistent with this finding, we previously reported that histamine, which is a major chemical mediator released from mast cells, was closely involved in the development of antigen-induced sneezing in this model (Nabe et al., 2001; Yamasaki et al., 2001a). Furthermore, Takaishi et al. (1994) have reported that interleukin-5 has no effect on anti-IgE-induced mediator release by sensitized mast cells in vitro. Consequently, it is suggested that the immediate sneezing and early nasal blockage observed in the present model might also be mediated by activated nasal mucosal mast cells, without any involvement of interleukin-5 and eosinophils.

In contrast to the early response, the late response, in which the predominant symptom is nasal blockage, has been reported to be associated with eosinophil accumulation and activation in the human nasal airway (Pastorello et al., 1994). In the present guinea pig model, the development of the late nasal blockage response was also associated with nasal airway eosinophilia. In addition, we previously found two lines of evidence to suggest that thromboxane A_2 and cysteinyl leukotrienes, which are the major products of arachidonic acid metabolism in guinea pig and human eosinophils, are chemical mediators of the development of the late nasal blockage response in this model (Mizutani et al., 2001; Yamasaki et al., 2001a). (1) The thromboxane A_2 receptor antagonist seratrodast and the cysteinyl leukotriene receptor antagonist pranlukast alleviated this late nasal blockage response; (2) the levels of thromboxane B_2 and cysteinyl leukotrienes in nasal cavity lavage fluid increased during the late response. Thus, it was considered plausible that the accumulated eosinophils, as the source of thromboxane A_2 and cysteinyl leukotrienes, might contribute to the development of the late nasal blockage response in this model. Contrary to this hypothesis, however, the prevention of nasal airway eosinophilia by treatment with TRFK5 did not influence the development of the late nasal blockage response. Furthermore, the treatment resulted in very slight inhibition of the antigen-induced local production of thromboxane A_2, and had no effect whatsoever on that of cysteinyl leukotrienes, during the late response. These results indicate that almost all of these arachidonic acid metabolites might be produced by cell types other than eosinophils, although thromboxane A_2 might be produced, in some part, by accumulated eosinophils. Taking all these findings into consideration, we can conclude that nasal eosinophilia is not causative, but might rather be a parallel event in the development of the late nasal blockage response following antigen challenge, and that cell types other than eosinophils may be responsible for the development of this symptom in this experimental system.

At 2 days after the pollen inhalation challenge, the TRFK5-treated sensitized–challenged animals showed the same degree of nasal hyperresponsiveness to histamine as the control antibody-treated sensitized–challenged animals. This result suggests that antigen-induced nasal hyperresponsiveness also occurs independently of interleukin-5 and eosinophilia, because the hyperresponsiveness to histamine was reproducibly observed 10 h and 2 days, but disappeared at 7 days, after each pollen inhalation challenge in the sensitized guinea pigs (Mizutani et al., 1999). We previously reported that the histamine-induced increase in specific airway resistance in sensitized–challenged animals is mainly due to dilation of the nasal blood vessels followed by a reduction in the volume of the nasal airway cavity, whereas increases in nasal vascular permeability and nasal secretion are minor contributors to this response (Mizutani et al., 1999). Therefore, it is conceivable that the hyperresponsiveness is at least in part due to the increased dilative response of nasal blood vessels to stimuli, which may be induced by the actions of factors and cells other than interleukin-5 and eosinophils.

The antigen-induced accumulation of neutrophils and mononuclear cells in the nasal airway was not affected by treatment with TRFK5. These types of cells have the ability to produce lipid mediators (including thromboxane A_2 and cysteinyl leukotrienes), cytokines and other various biologically active substances (Garrelds et al., 1996). Interestingly, Agusti et al. (1998) demonstrated that the degranulation of tracheal goblet cells after antigen challenge resulted from neutrophil accumulation and the release of elastase from these cells in sensitized guinea pigs. Also, lymphocytes, in particular $CD4^+$ T-cells, have been shown to play a critical role in the development of the late response and airway hyperresponsiveness in some rodent models of allergic asthma (Gavett et al., 1994; Watanabe et al., 1995; Haczku et al., 1997). We therefore suspect that neutrophils and/or mononuclear cells, rather than eosinophils, may be the cells involved in the development of nasal disorders in this model. However, the roles of neutrophils and mononuclear cells in the pathogenesis of allergic rhinitis require further clarification in future studies.

We should consider the differences between the biological characteristics of guinea pig and human eosinophils before extrapolating the findings of this study to human allergic rhinitis. It has been reported that while guinea pig eosinophils do not have the ability to produce cysteinyl leukotrienes, they produce large amounts of thromboxane A_2 (Sun et al., 1991). Consistent with this finding, eosinophil depletion by TRFK5 in this model did not inhibit the local production of cysteinyl leukotrienes during the late response, while it suppressed, although very slightly, the release of thromboxane A_2, as described above. In contrast, human blood eosinophils have been shown to produce cysteinyl leukotrienes but not thromboxane A_2 (Sun et al., 1991). These arachidonic acid metabolites have been implicated in the pathogenesis of allergic rhinitis in humans as well as in our guinea pig model (Donnelly et al., 1995; Terada et al., 1998). Thus, we cannot completely exclude the possibility that in human allergic rhinitis, unlike in the guinea pig model, eosinophils as the source of cysteinyl leukotrienes may contribute to the development of the nasal responses. However, Godthelp et al. (1996) and Klementsson et al. (1991) reported that the development of nasal symptoms and hyperresponsiveness after antigen challenge in patients with allergic rhinitis were not correlated with either the number of eosinophils or the level of eosinophil cationic protein in nasal washings. In addition, it has been reported that epithelial destruction, which is thought to be a major cause of airway hyperresponsiveness and to be induced by activated eosinophils in asthmatics, was not found in nasal biopsy specimens obtained from patients with allergic rhinitis (Lim et al., 1995). These findings, as well as the results of the present study, question the contribution of eosinophils to these nasal disorders in human allergic rhinitis. Recently, an intriguing result was reported from a double-blind randomized placebo-controlled

trial in which the humanized monoclonal antibody against interleukin-5 significantly reduced the number of eosinophils in the blood and sputum of patients with asthma, while it had no effect on the late-asthmatic response following antigen challenge or on airway hyperresponsiveness to histamine, suggesting that eosinophils are not implicated in the pathogenesis of asthma (Leckie et al., 2000). More such clinical studies in patients with allergic rhinitis will be helpful in determining the role of eosinophils in human allergic rhinitis.

In conclusion, the findings of this study provide direct evidence that interleukin-5 is essential for the induction of nasal airway eosinophilia through the release of eosinophils from the bone marrow pool, and that neither interleukin-5 nor eosinophils are required for the development of the nasal symptoms and nasal hyperresponsiveness which are the most important features of allergic rhinitis.

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