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Suppressive effects of F-1322 on the antigen-induced late asthmatic response and pulmonary eosinophilia in guinea pigs

Akinori Mochizuki [a,*], Norihiko Tamura [a], Yoriko Yatabe [a], Sadayoshi Onodera [a], Toru Hiruma [a], Niro Inaba [a], Jun Kusunoki [a], Hisao Tomioka [b]

[a] *Pharmaceuticals Research Laboratories, FujiREBIO Inc., 51 Komiya-cho, Hachioji, Tokyo 192-0031, Japan*

[b] *Department of Internal Medicine, Toho University School of Medicine, Sakura Hospital, 564-1 Shimoshizu, Sakura, Chiba 285-0841, Japan*

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Abstract

We investigated the effects of F-1322 (*N*-[2-[4-(benzhydryloxy)piperidino]ethyl]-3-hydroxy-5-(3-pyridylmethoxy)-2-naphthamide), a new compound that inhibits both thromboxane A_2 synthetase and 5-lipoxygenase and that functions as a histamine antagonist, on the Ascaris antigen-induced late asthmatic response and pulmonary eosinophilia in guinea pigs. Oral administration of F-1322 (10–100 mg/kg) inhibited the antigen-induced late asthmatic response in a dose-dependent manner. Histological analysis revealed that F-1322 prevented the accumulation of eosinophils in the airways and this was paralleled by a decrease in the number of eosinophils and lymphocytes recovered in bronchoalveolar lavage fluid. F-1322 (0.1–10 μM) inhibited eotaxin-induced chemotaxis and actin polymerization of eosinophils in vitro in a concentration-dependent manner, while oral administration of F-1322 dose-dependently suppressed the migration of eosinophils into the airways in vivo in response to infusion of interleukin 5 and eotaxin in combination. F-1322 may, thus, improve the late asthmatic response in this model, in part, by preventing the accumulation of eosinophils in the airways. The pharmacological profile of F-1322 indicates that this drug is likely to be useful in the treatment of allergic diseases such as asthma.

Keywords: F-1322; Asthma; Anti-asthmatic agent; Asthmatic response, Late; Eosinophil migration; (Guinea-pig)

1. Introduction

In typical bronchial asthma, inhalation of antigen causes a biphasic bronchoconstriction: an immediate asthmatic response occurs within 3 h after antigen challenge followed by a late asthmatic response 4 to 8 h after antigen challenge (Hargreave et al., 1974; Robertson et al., 1974; O'Byrne et al., 1987). The late asthmatic response is reported to lead to more severe asthmatic symptoms such as hyperresponsiveness to a wide variety of stimuli (Cartier et al., 1982; Hargreave, 1989). Various chemical mediators, such as histamine (Schroeder and MacGlashan, 1997), cysteinyl leukotrienes (Piper, 1982; Drazen, 1988) and thromboxane A_2 (Iwamoto et al., 1988), are produced by inflammatory cells or epithelial cells after antigen challenge and are thought to be involved in both the immediate and the late asthmatic responses (Wenzel et al., 1991; Arakida et al., 2000). During the late asthmatic response, eosinophil infiltration into the airways is often seen in several animal species including human (De Monchy et al., 1985) and guinea pigs (Santing et al., 1994; Sugiyama et al., 1995). Eosinophils are able to release several proinflammatory mediators, such as lipids, specific granule proteins and active oxygen, and they are thought to be associated with not only bronchoconstriction in the late phase, but also hyperresponsiveness as a result of the damage to epithelial cells in the airways (Busse and Sedgwick, 1992). Eosinophilia is considered to be one of the dominant features of airway inflammation, and the accumulation of this cell type in the airways is likely to be associated with the late asthmatic response.

F-1322 (*N*-[2-[4-(benzhydryloxy)piperidino]ethyl]-3-hydroxy-5-(3-pyridylmethoxy)-2-naphthamide) is a new compound that inhibits both thromboxane A_2 synthetase and 5-lipoxygenase, and it can function also as a histamine receptor antagonist (Lee et al., 1995). We previously reported that F-1322 suppresses interleukin 5 production in

* Corresponding author. Tel.: +81-426-45-0071; fax: +81-426-46-8325.

E-mail address: aki.mochizuki@nifty.com (A. Mochizuki).

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bronchoalveolar lavage fluid of sensitized mice after specific antigen challenge, and that it also inhibits eosinophil infiltration in bronchial submucosal tissue and eosinophil migration in vitro (Mochizuki et al., 1998). However, it remains unknown whether F-1322 affects asthmatic response following antigen challenge.

In the present studies, we investigated the effect of F-1322 on the late asthmatic response in guinea pigs and on eosinophil accumulation in the airways following antigen challenge. We also determined whether F-1322 inhibits eosinophil migration in vivo induced by infusion of a combination of interleukin 5 and eotaxin.

2. Materials and methods

2.1. Reagents and drugs

Recombinant human eotaxin, recombinant human interleukin 5 and anti-human eotaxin monoclonal antibody (clone: 43911.11) were purchased from R&D Systems (Minneapolis, MN, USA). Polymyxin B sulfate and L-α-lysophosphatidylcholine were purchased from Wako (Osaka, Japan). *N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)phallacidin (NBD-phallacidin) was obtained from Molecular Probes (Oregon, USA). Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) was purchased from Nissui Pharmaceutical (Tokyo, Japan). RPMI1640 (Nissui Pharmaceutical) was supplemented with 2 mM L-glutamine, 27 mM HEPES, and 2 g/l sodium bicarbonate. Hanks' balanced salt solution (HBSS; Life Technologies, Maryland, USA) was supplemented with 25 mM HEPES and adjusted to pH 7.5. F-1322 (Lee et al., 1995) was synthesized by Pharmaceuticals Research Laboratories of FujiREBIO (Tokyo, Japan). F-1322 was administered orally as a suspension of 5% Arabic gum solution. For in vitro experiments, F-1322 was first dissolved in dimethylsulfoxide and polyoxyethylene hydrogenated castor oil-60 in ethanol (1:1) and then diluted with RPMI1640 or the appropriate buffer. The final concentration of dimethylsulfoxide was 0.01%, and the concentrations of polyoxyethylene hydrogenated castor oil-60 and ethanol were 0.005%.

2.2. Immunization and challenge

The late asthmatic response was induced in conscious guinea pigs by the inhalation of Ascaris antigen, according to the method of Pennock et al. (1979) with slight modification. Briefly, 3-week-old male Hartley guinea pigs (Japan SLC, Sizuoka, Japan) were immunized intraperitoneally twice, at a 2-week interval, with a mixture of 20 μg of antigen protein which was extracted from Ascaris suum according to Tada and Okumura (1971) and 20 mg of silica gel. One week after the second immunization, in order to sensitize the airways, guinea pigs were exposed to an aerosol of antigen solution (2.5 mg/ml protein in saline) for 15 s, generated by an ultrasonic nebulizer (NE-V; Omron, Tokyo, Japan). One week after this antigen inhalation, they were again challenged by inhalation of aerosolized antigen in the same way as described above. Specific airway resistance was measured before and 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h after the antigen challenge according to the method of Pennock et al. (1979) by means of a two-chambered, double-flow plethysmograph system (Pulmos-I; M.I.P.S., Osaka, Japan). Briefly, the animal was placed with its neck extending through the partition of a two-chambered box and the specific resistance of airways was measured by means of airflow sensors placed in the front and rear chambers. F-1322 (10–100 mg/kg) or vehicle (5% Arabic gum alone) was orally administered 2 h after the inhaled antigen challenge. Data are either summarized as the percent increase over basal airway resistance (ΔRaw) and are expressed as the mean for eight animals, or are summarized as maximal ΔRaw, which represents the maximal alteration of ΔRaw that occurred between 0.5 and 2 h after challenge for the immediate asthmatic response, and between 3 and 8 h after challenge for the late asthmatic response, and are expressed as the mean ± S.E.

2.3. Histological examination

The lungs were excised from normal (nonimmunized and those that had inhaled saline) and challenged guinea pigs (immunized and those that had inhaled antigen; with or without F-1322 treatment). Immunized guinea pigs, which had been challenged with saline, were not included because in preliminary studies, such animals did not differ significantly in histological findings from nonimmunized and saline challenged animals. Six hours after the antigen challenge, animals were anesthetized intraperitoneally with urethane (1.2 g/5 ml/kg) and killed by exsanguination from the abdominal aorta. The lung along with the trachea was excised, rinsed once with saline and fixed with 10% neutral formalin. Morphometry was performed on 4-μm-thick paraffin-embedded sections taken from the midsagittal regions of each lung and stained with Giemsa. The number of eosinophils in the submucosal membrane (taken as the region between the airway epithelium and the cartilage of the small bronchus) was counted in a blind fashion according to the method of Arima et al. (1995) and Iijima et al. (1987). This airway tissue was chosen for analysis on the basis the results of a previous study by Iijima et al. (1987) in which airway contraction was assessed. The infiltrated eosinophils in randomly selected eight microscopic fields (47 × 47 μm each) were counted using a 10× eye piece and 20× objective, and the number of eosinophils was calculated per area. Data are presented as the means ± S.E. number of the cells in tissue (cells per mm^2) for six animals. F-1322 (10–100 mg/kg) or vehicle (5% Arabic gum alone) was orally administered 2 h after the inhaled antigen challenge.

2.4. Determination of inflammatory cell numbers in bronchoalveolar lavage fluid

Antigen challenge and administration of F-1322 (30 mg/kg) or vehicle were performed as described above. Six hours after challenge, animals were anesthetized as above, and the tracheas were exposed and cannulated with a disposable catheter (8 Fr. size; Atom, Tokyo, Japan). The airway lumen was washed three times with 10 ml of PBS. Bronchoalveolar lavage fluid was pooled into a 50-ml plastic tube, and then centrifuged at $400 \times g$ for 10 min at 4 °C. The pellet was resuspended with 5 ml of saline, and the total cell number was counted in a hemocytometer. Differential cell counts were made from centrifuged preparations stained with Diff-Quick stain, counting 200 or more cells at a magnification of $\times 400$. Data are summarized as the percentage of inflammatory cells out of the total cell number counted and are expressed as the means $\pm$ S.E. for six animals.

2.5. Eosinophil isolation for in vitro experiments

Eosinophils were isolated from peritoneal cavity lavage fluid of polymyxin B-treated guinea pigs as described by Pincus (1978). One milligram of polymyxin B sulfate was injected intraperitoneally into 4-week-old male Hartley guinea pigs (Japan SLC) every week for 8 weeks. One week after the last injection, the peritoneal cavity was lavaged with PBS supplemented with 200 IU heparin sodium (Wako), and the lavage fluids were centrifuged at $400 \times g$ for 10 min at 4 °C. The pellet was resuspended in 1.070 g/ml Percoll (Pharmacia Biotech, Uppsala, Sweden) and layered onto discontinuous gradients of Percoll (1.085 and 1.100 g/ml). After centrifugation at $1200 \times g$ for 20 min at room temperature, the eosinophils were recovered from the 1.085/1.100 g/ml interface and washed twice with PBS by centrifugation. Eosinophil numbers were adjusted to 4.4×10^6 cells/ml in RPMI1640 containing 0.4% bovine serum albumin for the migration assay, or to 1.1×10^7 cells/ml in 20 mM HEPES-buffered HBSS for the actin polymerization analysis. Cell purity and viability were determined using Diff-Quick stain and the Trypan blue dye exclusion test, respectively. Eosinophils with a purity and viability of more than 95% were used for experiments.

2.6. Eosinophil chemotaxis assay

Eosinophil chemotaxis was assayed according to a modified Boyden chamber technique, using a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD, USA) as described previously (Mochizuki et al., 1998). Briefly, 100 ng/ml eotaxin in RPMI1640 containing 0.05% bovine serum albumin was dispensed into the lower wells. The eosinophils were treated with test compound or vehicle for 5 min at 37 °C (final cell concentration: 4×10^6 cells/ml) and were then added to the upper chamber, which was separated from the lower well by a nitrocellulose filter (3-μm pore size) (Sartorius, Gettingen, Germany). Migration was allowed to proceed at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 2 h. After this time, the filters were fixed with a solution containing 50% saturated $HgCl_2$ and 50% ethanol. They were then stained with Carrazi's hematoxylin solution followed by chromotrope 2R. The numbers of cells that had migrated through to the reverse side were counted in 10 high-power-fields with a light microscope at a magnification of $\times 400$. Data are expressed as the means $\pm$ S.E. for three experiments.

2.7. Fluorescent staining of filamentous actin

The filamentous actin content was analyzed by flow cytometry with NBD-phallacidin staining according to a slightly modified method of Howard and Meyer (1984). Briefly, eosinophil numbers were adjusted to 1.1×10^7 cells/ml in 20 mM HEPES-buffered HBSS. Then 90 μl of cell suspension (containing 1×10^6 eosinophils) in 1.5-ml microtubes was preconditioned for 30 min at 37 °C; 10 μl of drug or vehicle was then added to the cells, which were incubated for a further 2 h at 37 °C. The cells were stimulated by addition of 100 μl prewarmed eotaxin (final concentration 100 ng/ml) for 30 s, and the reaction was terminated by the addition of 200 μl ice-cold 7.4% formaldehyde and fixed for 10 min at 4 °C. After fixation, 100 μl of staining cocktail containing lysophosphatidylcholine and NBD-phallacidin in 20 mM HEPES-buffered HBSS (final concentrations 100 μg/ml and 0.17 μM, respectively) was added to the cells and incubated for 15 min at 37 °C in the dark. Cells were washed once with PBS, centrifuged and analyzed on a flow cytometer (Coulter) with a linear fluorescence channel. Data are summarized as the relative ratio of mean fluorescence intensity and expressed as the means $\pm$ S.E. for three individual animals. Percent inhibition of actin polymerization was calculated according to the following formula: $[1 - (A - B)/(C - B)] \times 100$, where A is the mean fluorescence intensity of drug-treated cells, B is the mean fluorescence intensity of nonstimulated cells, and C is the mean fluorescence intensity of vehicle-treated cells.

2.8. In vivo eosinophil migration

Eosinophil migration in vivo was induced by the combined infusion of interleukin 5 and eotaxin. First, a blood sample was collected and then 0.6 μg/kg of interleukin 5 or vehicle (PBS containing 0.1% bovine serum albumin) was intravenously infused into the upper limb of conscious guinea pigs in order to induce eosinophilia. In a preliminary test, the number of eosinophils in the blood increased

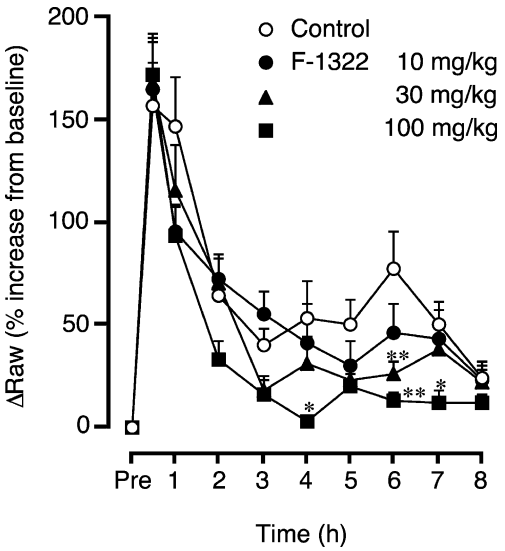

Fig. 1. Effect of F-1322 on the antigen-induced late asthmatic response in actively sensitized guinea pigs. F-1322 was orally administered 2 h after antigen inhalation. Airway resistance was measured with a two-chambered, double-flow plethysmograph system before and 0.5 to 8 h after antigen challenge. Results are summarized as percent increase from basal airway resistance (ΔRaw). Each point represents the mean + S.E. ΔRaw for eight animals. *, ** Significantly different from time-matched control group at $P < 0.05$ and $P < 0.01$, respectively (Williams' multiple test).

rapidly within the first hour after intravenous infusion of interleukin 5, and this eosinophilia remained for at least 2 h. Therefore, 1 h after the interleukin 5 injection, animals were anesthetized with 50% ketamine solution and diethyl ether, and 10 pmol/250 μl/body of eotaxin solution or vehicle (PBS containing 0.1% bovine serum albumin) was injected intratracheally. F-1322 (10–100 mg/kg) or vehicle (5% Arabic gum alone) was orally administered 2 h before and 10 h after the eotaxin injection. Twenty-four hours after the eotaxin injection, guinea pigs were anesthetized with an intraperitoneal injection of urethane (1.2 g/5 ml/kg), and exsanguination was carried out from the abdominal aorta. Bronchoalveolar lavage and differential cell counts were performed as above. The number of eosinophils in blood was also counted as above after the hemolysis of heparinized blood. Results are expressed as the means ± S.E. of the number of eosinophils in blood or bronchoalveolar lavage fluid from six animals.

2.9. Statistics

Statistical significance was assessed using Student's *t*-test for comparisons between normal animals and control groups. Thereafter, Williams' multiple test was used when the dose–response relationship was recognized by means of a simple regression method (SAS system ver. 6; SAS Institute Japan, Tokyo, Japan). *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. Effect of f-1322 on the late asthmatic response

In a time-course study, the airway resistance of antigen-challenged guinea pigs without drug treatment increased rapidly 1 h after challenge, but improved within 3

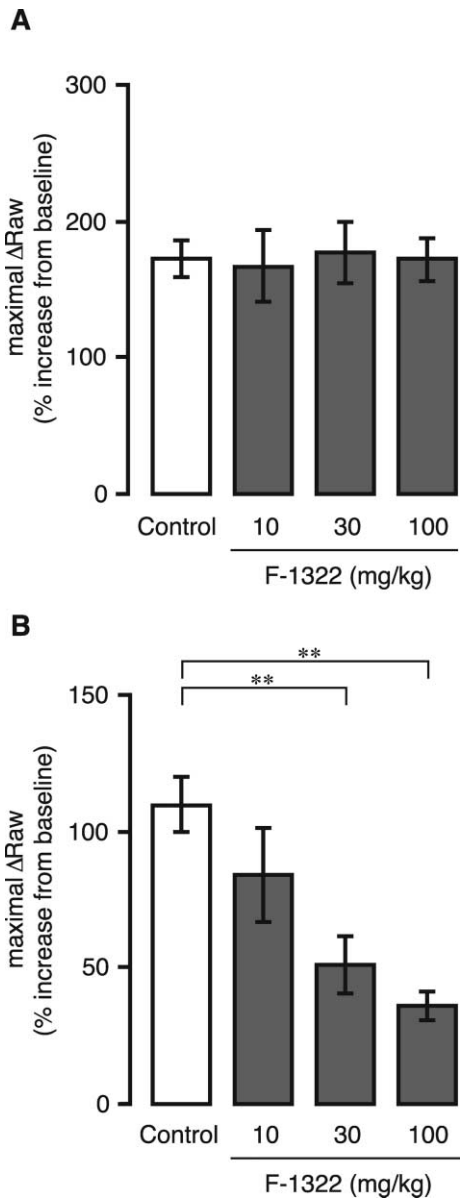

Fig. 2. Alteration of the maximal ΔRaw of antigen-induced immediate asthmatic response (A) and late asthmatic response (B) in actively sensitized guinea pigs. F-1322 was orally administered 2 h after antigen inhalation. Airway resistance was measured with a two-chambered, double-flow plethysmograph system. Results are summarized as maximal ΔRaw, which is the maximal alteration of ΔRaw that occurred between 0.5 and 2 h after challenge for the immediate asthmatic response (A), and between 3 and 8 h after challenge for the late asthmatic response (B). Each column represents the mean ± S.E. of results from eight animals. ** Significantly different from the control group at $P < 0.01$ (Williams' multiple test).

h. Between 4 and 7 h after challenge, the airway resistance increased again in a typical late asthmatic response (Fig. 1). By contrast, the airway resistance during the late response in F-1322-treated animals clearly improved compared with that of the control animals (Fig. 1). Although the maximal ΔRaw occurring in the immediate phase did not differ between the treated and nontreated animals (Fig. 2A), F-1322 significantly decreased the maximal ΔRaw occurring in the late phase in a dose-dependent manner ($P < 0.01$ vs. control group, $n = 8$) (Fig. 2B).

3.2. Histological changes of the submucosal tissue of antigen-challenged animals

Histological examination showed that 6 h after antigen challenge, eosinophils had clearly infiltrated the submucous membrane of the bronchus. Representative micrographs of the bronchus are shown in Fig. 3. The number of eosinophils that had infiltrated the airway was significantly increased following antigen challenge ($P < 0.01$ vs. normal group; $n = 6$). Single oral administration of F-1322 inhibited this accumulation of eosinophils in a dose-dependent manner, and significant reductions were observed at doses of 30 and 100 mg/kg ($P < 0.05$ and $P < 0.01$, respectively) (Table 1).

3.3. Inflammatory cell numbers in bronchoalveolar lavage fluid of antigen-challenged animals

Six hours after antigen challenge, the total number of cells in bronchoalveolar lavage fluid was significantly increased compared with that in normal animals ($P < 0.01$, Table 2). Oral administration of F-1322 at 30 mg/kg significantly decreased the total number of cells in

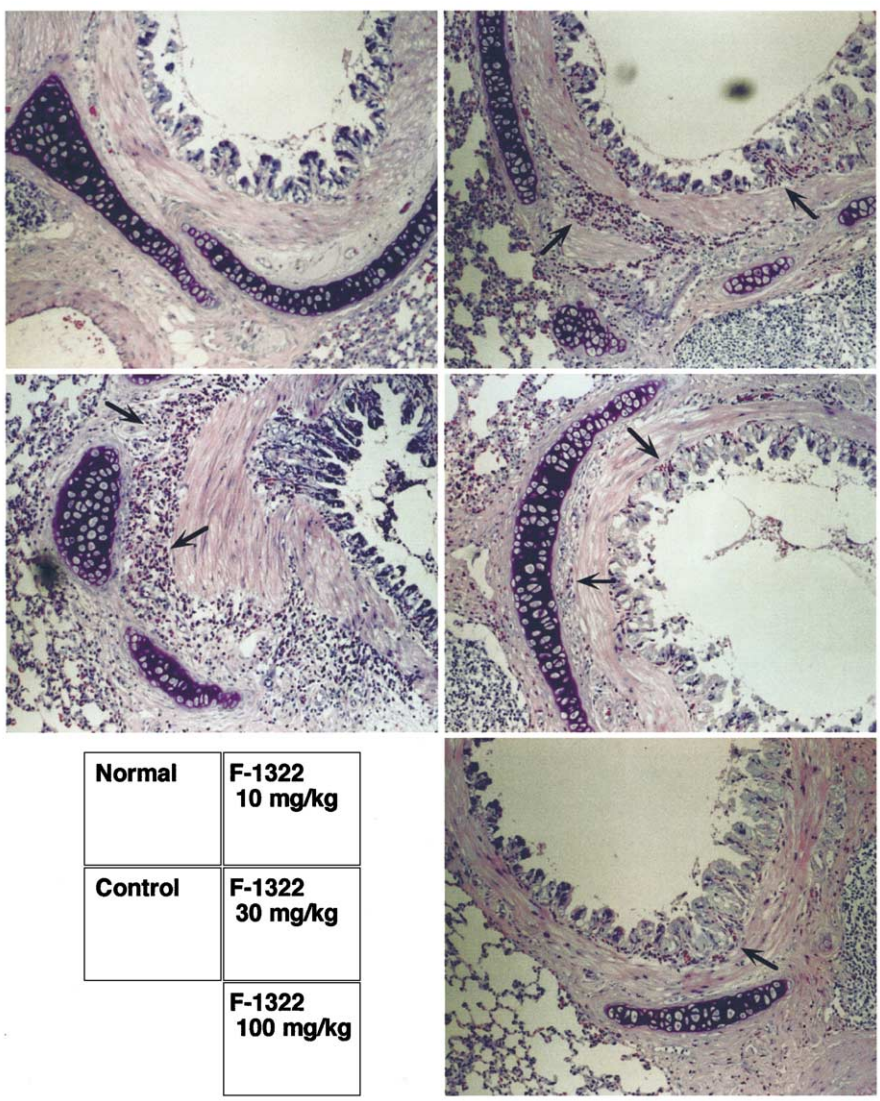

Fig. 3. Photomicrograph of the bronchus obtained either from sensitized guinea pigs 6 h after antigen challenge or from a normal, immunized control guinea pig. F-1322 or vehicle was orally administered 2 h after antigen inhalation. The bronchus was sectioned and stained with Giemsa as described in Materials and methods (magnification, ×100). Infiltrated eosinophils are indicated by arrows.

Table 1
Effect of F-1322 on eosinophil infiltration into the bronchus of actively sentisized guinea pigs 6 h after antigen challenge

Treatment	Dose (mg/kg)	Eosinophil number ($\times 10^2$ cells/mm^2)
Normal	–	1.7 ± 1.5
Control	–	56.4 ± 6.2[a]
F-1322	10	40.0 ± 7.4
F-1322	30	25.8 ± 11.5[b]
F-1322	100	14.6 ± 5.1[c]

The number of eosinophils was counted in eight randomly selected microscopic fields as described in Materials and methods, and the average number of cells that had infiltrated bronchus tissue was determined (cells/mm^2). Each value represent the mean ± S.E. for 6 animals.

[a] Significantly different from the normal group at $P < 0.01$ (Student's *t*-test).

[b] Significantly different from the control group at $P < 0.05$ (Williams' multiple test).

[c] Significantly different from the control group at $P < 0.01$ (Williams' multiple test).

bronchoalveolar lavage fluid ($P < 0.01$ vs. control group). In addition, F-1322 at a dose of 30 mg/kg significantly decreased the number of eosinophils and lymphocytes ($P < 0.01$ and $P < 0.05$, respectively, vs. the control group).

3.4. Effect on eosinophil chemotaxis in vitro

The chemotaxis of guinea pig eosinophils in vitro increased significantly in response to 100 ng/ml eotaxin (spontaneous migration: 17.0 ± 1.7, control: 289.0 ± 10.3 cells/10 high-power-fields; $n = 3$). As shown in Fig. 4, F-1322 significantly inhibited eotaxin-induced chemotaxis of eosinophils in a concentration-dependent manner. At a concentration of 10 μM F-1322, chemotaxis was inhibited by 84.7%. As a positive control, anti-eotaxin monoclonal antibody (100 μg/ml) completely inhibited eosinophil chemotaxis induced by eotaxin.

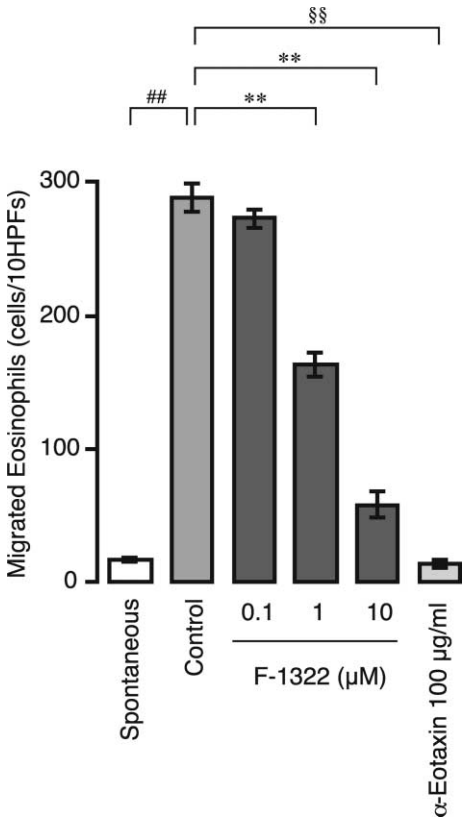

Fig. 4. Effect of F-1322 on guinea pig eosinophil migration in vitro induced by eotaxin. Guinea pig eosinophils were treated with F-1322 (0.1–10 μM), and eosinophil migration was induced by the addition of 100 ng/ml eotaxin. The number of cells that had migrated through to the reverse side of the membrane was counted in 10 high-power-fields (HPFs) with a light microscope at a magnification of ×400. Data are expressed as the means ± S.E. of results from three experiments. ##, §§ Significantly different from the spontaneous migration and the control at $P < 0.01$, respectively (Student's *t*-test). ** Significantly different from the control at $P < 0.01$ (Williams' multiple test).

3.5. Eotaxin-induced actin polymerization in eosinophils

Actin polymerization was observed to occur rapidly in guinea pig eosinophils following their stimulation with

Table 2
Effect of F-1322 on the infiltration of inflammatory cells into bronchoalveolar lavage fluid of actively sensitized guinea pigs 6 h after antigen challenge

Treatment	Number of cells ($\times 10^5$ cells in bronchoalveolar lavage fluid)				
	Total cells	Eosinophils	Neutrophils	Macrophages	Lymphocytes
Normal	180.2 ± 26.0	17.0 ± 3.9	2.7 ± 0.7	135.1 ± 30.1	29.4 ± 20.7
Control	667.4 ± 53.9[a]	394.3 ± 32.2[a]	41.5 ± 8.9[a]	151.1 ± 15.2	81.2 ± 10.9[b]
F-1322 30 mg/kg	282.0 ± 41.0[c]	102.7 ± 26.1[c]	21.4 ± 5.5	106.8 ± 21.0	51.4 ± 4.5[d]

F-1322 (30 mg/kg) was orally administered 2 h after antigen inhalation. Bronchoalveolar lavage was performed as described in Materials and methods. Differential cell counts were made from centrifuged preparations stained with Diff-Quick stain, counting 200 or more cells at a magnification of ×400. Data are expressed as the means ± S.E. for six animals.

[a] Significantly different from the normal group at $P < 0.05$ (Student's *t*-test).

[b] Significantly different from the normal group at $P < 0.01$ (Student's *t*-test).

[c] Significantly different from the control group at $P < 0.05$ (Student's *t*-test).

[d] Significantly different from the control group at $P < 0.01$ (Student's *t*-test).

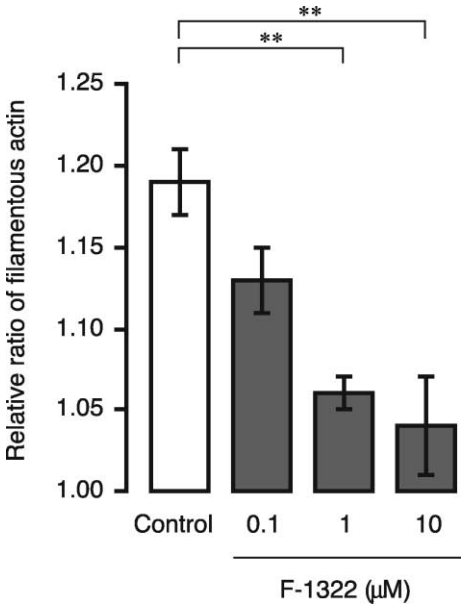

Fig. 5. Effect of F-1322 on actin polymerization induced by eotaxin in eosinophils. After treatment with F-1322 (0.1–10 μM), actin polymerization was induced by the addition of 100 ng/ml eotaxin for 30 s. Filamentous actin content was measured by flow cytometry. Data are summarized as the ratio of linear fluorescence intensity of stimulated to unstimulated cells. Each column represents the mean ± S.E. of results from three animals. ** Significantly different from the control at $P < 0.01$ (Williams' multiple test).

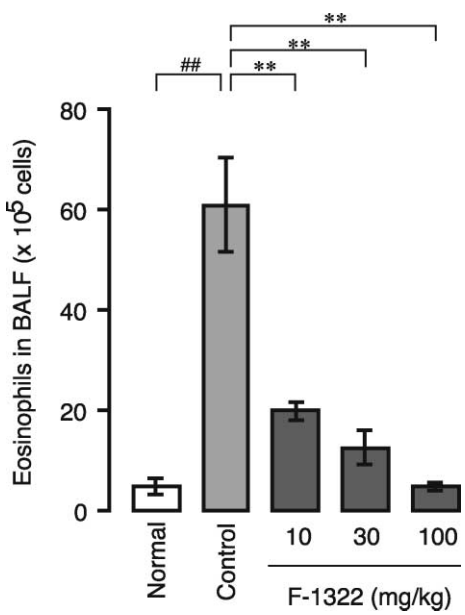

Fig. 6. Effect of F-1322 on eosinophil migration into the bronchoalveolar lavage fluid in vivo in guinea pigs. Migration was induced by the combined administration of intravenous interleukin 5 plus intratracheal eotaxin. Eosinophil accumulation was induced by intratracheal injection of eotaxin (10 pmol/body) 1 h after intravenous infusion of interleukin 5 (0.6 μg/kg) in guinea pigs. F-1322 was orally administered 2 h before and 10 h after the eotaxin injection. Bronchoalveolar lavage fluid was collected 24 h after the eotaxin injection. Results are expressed as the mean ± S.E. number of eosinophils in bronchoalveolar lavage fluid from six animals. ## Significantly different from the normal group at $P < 0.01$ (Student's *t*-test). ** Significantly different from the control group at $P < 0.01$ (Williams' multiple test).

eotaxin. As maximal actin polymerization was observed 30 s after stimulation in a preliminary examination, we evaluated the effect of F-1322 in eosinophils at this time point. As shown in Fig. 5, in the untreated eosinophils, the relative fluorescence intensity of filamentous actin was 1.19 ± 0.02 after stimulation by eotaxin ($n = 3$). Treatment with F-1322 significantly decreased the fluorescence intensity of filamentous actin and, hence, actin polymerization in a concentration-dependent manner: at 10 μM of F-1322, actin polymerization was suppressed by 79.3% (relative fluorescence 1.04 ± 0.03, $P < 0.01$ vs. untreated cells).

3.6. Effect on eosinophil migration in vivo

As shown in Fig. 6, administration of eotaxin together with interleukin 5 caused significant infiltration of eosinophils into bronchoalveolar lavage fluid: the number of eosinophils in bronchoalveolar lavage fluid from normal guinea pigs was $4.94 \pm 1.58 \times 10^5$ cells, whereas the number in bronchoalveolar lavage fluid from control animals treated with interleukin 5 plus eotaxin, but not F-1322, was significantly higher ($60.90 \pm 9.42 \times 10^5$ cells, $P < 0.01$ vs. normal group, $n = 6$). When the animals were treated with F-1322 in addition to interleukin 5 and eotaxin, the number of eosinophils in bronchoalveolar lavage fluid decreased significantly in a F-1322 dose-dependent man-

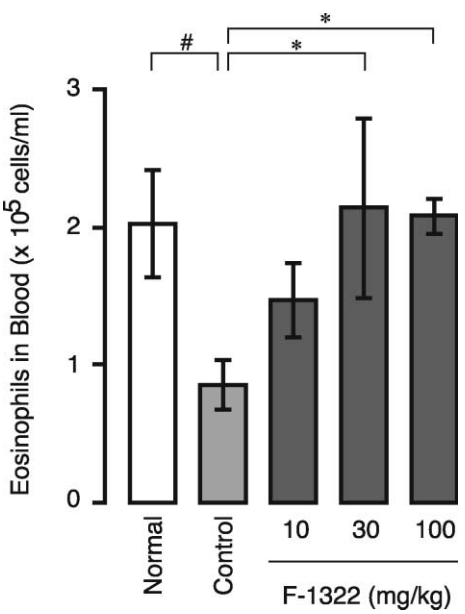

Fig. 7. Effect of F-1322 on eosinophil numbers in blood after the combined administration of intravenous interleukin 5 and intratracheal eotaxin. Administration of both interleukin 5 and eotaxin, and F-1322 treatment were carried out as described in the legend of Fig. 6. Blood samples were collected 24 h after the eotaxin injection. Data are expressed as the mean ± S.E. of results from six animals. # Significantly different from the normal group at $P < 0.05$ (Student's *t*-test). * Significantly different from the control group at $P < 0.05$ (Williams' multiple test).

ner. In contrast, in the interleukin 5 and eotaxin-treated animals, the number of eosinophils in blood was significantly higher when animals were treated with F-1322 at a dose of more than 30 mg/kg ($P < 0.05$ vs. control group), whereas the number in the control group was significantly lower than in the normal group ($P < 0.05$, $n = 6$) (Fig. 7).

4. Discussion

We have shown previously that F-1322 has an inhibitory effect on interleukin 5 production and eosinophil infiltration in sensitized mice and has an inhibitory effect on the migration of human and guinea pig eosinophils in vitro (Mochizuki et al., 1998). Here, to study the effect of F-1322 on the late asthmatic response, we used a guinea pig model in which the animals were actively sensitized with Ascaris suum extract. We also evaluated the effects of F-1322 on the accumulation of eosinophils in the airways of both antigen-sensitized and eotaxin-induced guinea pigs. In the present studies, the effect of F-1322 on the immediate asthmatic response was not examined specifically. However, this compound has an antagonistic effect on histamine and an inhibitory effect on both 5-lipoxygenase and thromboxane A_2 synthetase (Lee et al., 1995), and we have confirmed in a preliminary study (unpublished observation) that F-1322 can suppress antigen-induced immediate asthmatic responses when this compound is administered orally 2 h before antigen challenge.

Eosinophils have been implicated as key effector cells in allergic disease, and the number of eosinophils in the bronchial mucosa and in bronchoalveolar lavage fluid are correlated with the severity of asthma (Bousquet et al., 1990). The accumulation of eosinophils is observed specifically between 3 and 8 h after antigen challenge in both human (De Monchy et al., 1985) and other animal models (Santing et al., 1994; Sugiyama et al., 1995). These eosinophils produce several proinflammatory mediators, such as leukotriene B_4, platelet-activating factor, and granule proteins, such as eosinophil-derived neurotoxin, eosinophil cationic protein, eosinophil peroxidase, and major basic protein (Giembycz and Lindsay, 1999). Prevention of the infiltration of eosinophils into the airways has been postulated to alleviate airway hypersensitivity and to improve the symptoms of the late asthmatic response. Hence, the search for compounds that can downregulate eosinophil function has been a focus of drug development (Dent et al., 1998; Grutters et al., 1999; Aoki et al., 2000; Zhao et al., 2000).

Here, we first examined whether F-1322 improves airway resistance in a late asthmatic response guinea pig model. We found that F-1322 decreased the maximal resistance of the airways during the late phase in a dose-dependent manner (Figs. 1 and 2B). Johns et al. (1990) have reported that not only the lower, but also the upper, airways can contribute to the late phase airway obstruction following antigen challenge in sensitized guinea pigs. Therefore, although the whole-body plethysmograph system is quite often employed for analysis of the late asthmatic response, this system detects the resistance of both the upper and the lower airways. Histological analysis revealed that a single oral administration of F-1322 at a dose of 30 mg/kg potently prevented eosinophil accumulation in the mucosal tissue of the bronchus (Fig. 3, Table 1), an effect that we have previously shown to occur in mice (Mochizuki et al., 1998). These findings were in keeping with analysis of the cells recovered from the airways by bronchoalveolar lavage. F-1322 given orally at a dose of 30 mg/kg significantly decreased the number of eosinophils and lymphocytes in bronchoalveolar lavage fluid (Table 2). In our previous study, we observed that F-1322 administration prior to antigen challenge decreased interleukin 5 production in bronchoalveolar lavage fluid of immunized mice (Mochizuki et al., 1998). F-1322 may, thus, influence lymphocyte function and inhibit their infiltration into the airways, and this could result in a decrease interleukin 5 release. Karras et al. (2000) have reported that interleukin 5 antisense treatment can inhibit antigen-induced pulmonary eosinophilia and the late asthmatic response in a mouse model. Therefore, a suppressive effect on interleukin 5 production by F-1322 may also contribute to the prevention of eosinophil accumulation in the airways, though this compound can directly inhibit migration in guinea pig (Figs. 4 and 5) as well as human eosinophils (Mochizuki et al., 1998) in vitro. The reduction in airway resistance during the late asthmatic response in F-1322-treated animals may be associated, at least in part, with inhibition of inflammatory cell accumulation. However, a recent study has indicated that intravenous infusion of a humanized monoclonal antibody against interleukin 5 to asthmatic patients fails to protect against the late asthmatic response or airway hyperresponsiveness, despite a decrease in eosinophil numbers in blood and sputum being maintained following antigen challenge (Leckie et al., 2000). Interleukin 5 is likely to have a different role in the pathogenesis of airway disease in human and animal models.

Eosinophil accumulation in the airways and in the late asthmatic response has been reported to be inhibited not only by specific inhibitors of 5-lipoxygenase (Tohda et al., 1997) and thromboxane A_2 synthetase (Itoh et al., 1993, 1996), but also by leukotriene receptor antagonists (Matsumoto et al., 1994; Ihaku et al., 1999; Arakida et al., 2000) in animal models. Eosinophils produce several proinflammatory mediators, such as lipid mediators, cytotoxic granule protein, and cytokines (Giembycz and Lindsay, 1999), and these mediators are thought to be associated with both bronchoconstriction and hyperresponsiveness. Therefore, in the development of anti-asthmatic drugs, not only prevention of eosinophil accumulation, but also inhibition of the production of proinflammatory mediators should be considered. Further studies involving the

measurement of concentrations of leukotrienes or thromboxane A_2 in bronchoalveolar lavage fluid will be needed in order to determine whether F-1322 can inhibit the production of these chemical mediators in vivo. However, taking its pharmacological profile into account, F-1322 may suppress the production of proinflammatory mediators by eosinophils that have infiltrated the airways even in the presence of the drug. Roquet et al. (1997) have reported that the use of leukotriene receptor and histamine antagonists in combination can result in a greater alleviation of airway obstruction (during both the immediate and the late asthmatic response) than that achieved with these drugs administrated singly. A broad-spectrum pharmacological profile, such as that possessed by F-1322, could be an advantage in an anti-asthmatic drug.

We have reported previously that F-1322 can potently suppress rodent and human eosinophil migration in vitro (Mochizuki et al., 1998), but we did not examine whether this action could be seen in vivo. As eotaxin is a potent chemotactic factor, we developed an in vivo model in which we could observe eosinophil migration induced by this chemokine and evaluate the effect of F-1322. This model comprises two different phases: interleukin 5 is infused intravenously to induce blood eosinophilia and then a solution of eotaxin is injected intratracheally to induce infiltration of eosinophils into the airways. In a preliminary study, we found that F-1322, when administered 1 h before the infusion of interleukin 5, did not affect interleukin 5-induced blood eosinophilia (data not shown). As F-1322 is reported to be able to suppress the interleukin 5-induced migration of eosinophils in vitro (Mochizuki et al., 1998), this result suggest that F-1322 either had not been distributed into or had not reached an effective concentration (Mochizuki et al., 1998) in the bone marrow, which is thought to be the source of the 'rapidly mobilizable pool' of eosinophils (Collins et al., 1995; Mould et al., 1997). One hour after the intravenous infusion, further injection of eotaxin into the airways of these animals caused abundant infiltration of eosinophils into bronchoalveolar lavage fluid 24 h after the injection. Administration of F-1322, 2 h before and 10 h after the eotaxin injection, markedly suppressed eosinophil infiltration into the airways in a dose-dependent manner (Fig. 6). In contrast, although the number of eosinophils in blood significantly decreased in control animals, this decrease was suppressed by F-1322 treatment in a dose-dependent manner (Fig. 7). Taken together, these results show that F-1322 inhibits the distribution of eosinophils from blood to tissue and sustains eosinophilia. Thus, F-1322 potently suppresses eosinophil migration in vivo as well as in vitro and presumably prevents the accumulation of eosinophils in the airways of antigen-challenged animals.

The processes by which F-1322 suppresses eosinophil migration in vivo will require further clarification, but we investigated several possible mechanisms. First, we carried out tests using specific inhibitors against the enzymes that F-1322 is known to affect, 5-lipoxygenase (AA861) and thromboxane A_2 synthase (ozagrel) as well as a histamine antagonist (mepyramine). Although it has been reported previously that inhibitors of 5-lipoxygenase (Munoz and Leff, 1995) or thromboxane A_2 synthetase (Itoh et al., 1993) can suppress eosinophil migration in vitro, we did not observe a suppressive effect with such inhibitors either individually or in combination in our experimental system (data not shown). This discrepancy may be due to differences in the species or chemoattractant used. In addition, we assessed the effects of F-1322 on type IV phosphodiesterase activity, and on intracellular cAMP levels, which are known to modulate eosinophil function (Cohan et al., 1996; Momose et al., 1998), but they were not altered by the treatment of F-1322 (unpublished data). It is possible, therefore, that F-1322 may affect other pathways that have not yet been discovered. In order to confirm the inhibitory effect of F-1322 on eosinophil migration and to determine whether F-1322 modulates intracellular signal transduction, we analyzed eotaxin-induced filamentous actin formation in eosinophils. As shown in Fig. 5, F-1322 suppressed filamentous actin formation in a concentration-dependent manner. As F-1322 nonspecifically suppresses eosinophil migration induced by several stimuli, this compound may affect a common signal pathway between ligand-receptor recognition and the modulation of cytoskeletal changes. Further studies will be needed to clarify the inhibitory mechanisms of F-1322 on eosinophil function.

In conclusion, we have shown that administration of F-1322 in vivo improves airway resistance in the antigen-induced late asthmatic response as well as the accumulation of eosinophils in the airways during the late phase response. In addition to this direct effect of F-1322, the indirect effects of F-1322 on eosinophils, such as suppression of the production of proinflammatory mediators, may contribute to improvement of the late asthmatic response. Such a broad-spectrum pharmacological profile is likely to be a useful in the treatment of allergic diseases, in particular, in the treatment of asthma.

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