
Effects of anti-inflammatory therapies on recurrent and low-grade respiratory syncytial virus infections in a murine model of asthma

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Background: Recurrent and subclinical viral respiratory tract infections could immunologically exacerbate allergic airway inflammation. However, the most appropriate treatment for virus-induced asthma exacerbation is yet to be established. The effects of glucocorticoids in virus-induced acute asthma are controversial.

Objective: To determine the effects of representative anti-inflammatory therapies for asthma—glucocorticoids and leukotriene receptor antagonists (LTRAs)—in mite allergen-sensitized and repeatedly low-grade respiratory syncytial virus (RSV)–infected mice.

Methods: *Dermatophagoides farinae*–sensitized mice were inoculated twice with low-grade RSV and subcutaneously injected with either a glucocorticoid or an LTRA for 4 consecutive days. Lung inflammation, cytokine profiles, LT production, and viral RNA in lung tissues were compared in 5 groups of 8 mice each: controls, *D farinae* allergen sensitized, *D farinae* sensitized and RSV infected, *D farinae* sensitized and RSV infected with dexamethasone, and *D farinae* sensitized and RSV infected with pranlukast, an LTRA.

Results: Allergic airway inflammation in *D farinae* mice was significantly enhanced by recurrent and low-grade RSV infections (RLRIs). The glucocorticoid attenuated allergic airway inflammation, which was associated with interleukin 5 (IL-5) and interferon- γ (IFN- γ) suppression in lung-draining lymph nodes without affecting viral quantity. The LTRA also attenuated allergic airway inflammation in *D farinae*–RSV mice with concomitant inhibition of IL-5 but not IFN- γ . *Dermatophagoides farinae* allergen sensitization significantly increased LTs in the airway, whereas RLRIs did not further enhance LT production.

Conclusions: Glucocorticoids and LTRAs significantly inhibit RLRI-induced exacerbation of allergic airway inflammation by distinct pathways. Dexamethasone suppressed nonspecific cytokines, whereas viral RNA did not increase via suppression of immunity. In contrast, pranlukast specifically inhibited IL-5 but not IFN- γ .

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INTRODUCTION

Viral respiratory tract infections represent the most common trigger of acute exacerbation of asthma in children and adults.^{1–3} Although the underlying mechanism in virus-induced exacerbation of allergic airway inflammation remains unknown, a variety of inflammatory molecules (eg, cytokines, chemokines, and adhesion molecules) and inflammatory cells (eg, lymphocytes, eosinophils, and dendritic cells) are critically involved.^{4–6} Among the respiratory viruses, respiratory syncytial virus (RSV) has attracted attention because it could potentially exacerbate and induce asthma.^{1,7,8} The mouse provides an excellent model to investigate the possible synergy between allergen sensitization and RSV infection at the immunologic level.^{8–10} In natural infection, viral infection occurs in much lower grade in adults than in children, and it is unlikely that marked pulmonary infection

occurs in infected adults. Thus, in the present study, *Dermatophagoides farinae* allergen-sensitized BALB/c mice were repeatedly infected with low-grade RSV, which per se did not cause pulmonary inflammation but induced an immunologic response in the host. This model could mimic subclinical and recurrent RSV infection in adults but not severe RSV bronchiolitis in infants.¹¹

The international guideline for asthma management recommends the early introduction of oral glucocorticoids or increased inhaled glucocorticoids for the treatment of virus-induced exacerbation of asthma.¹² There is concern that glucocorticoids might worsen viral infections because these agents suppress cellular immunity. In fact, systemic administration of glucocorticoids delays viral clearance in infants with severe RSV bronchiolitis who have received mechanical ventilation.^{12,13} Thus, in the treatment of virus-induced exacerbation of asthma, it remains uncertain whether glucocorticoids are good players as an anti-inflammatory agent or bad players as an immunosuppressive agent. In addition, although the anti-inflammatory effects of perflubron, a perfluorocarbon liquid, on airway abnormalities in RSV-infected mice

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were demonstrated,¹⁴ its role in virus-induced asthma exacerbation is yet to be determined.

Cysteinyl leukotrienes (cys-LTs) are not only involved in the early asthmatic response through their actions of airway smooth muscle constriction and increase in vascular permeability but also show proinflammatory effects.^{15,16} Montelukast has been demonstrated to inhibit IL-5 messenger RNA and cys-LTs in mononuclear cells from human asthmatic patients.¹⁷ It had been reported that viral respiratory tract infections increase cys-LTs in the airway,¹⁸ which could exacerbate preexisting allergic airway inflammation in asthma.¹⁶ Several human and animal studies^{18,19} showed the usefulness of anti-LT therapy for RSV infection. Nonetheless, only a few studies examined the effects of anti-LT therapy in RSV-induced exacerbation of asthma.

Based on this background, the present study sheds light on representative anti-inflammatory therapies for asthma, ie, glucocorticoids and LT receptor antagonists (LTRAs), as drugs for RSV-induced acute exacerbation of asthma. Our working hypotheses are that (1) systemic administration of glucocorticoids might increase viral quantity and (2) anti-LT therapy might be effective in recurrent and low-grade RSV infection (RLRI) in a murine model of allergic asthma.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 4 to 5 weeks of age, were obtained from Charles River (Yokohama, Japan). They were housed under pathogen-free conditions at the Laboratory Animal Center for Biochemical Research, Nagasaki University School of Medicine. All experimental procedures were reviewed and approved by the Nagasaki University School of Medicine Committee on Animal Research.

RSV Preparation and Inoculation

The A2 strain of human RSV (American Type Culture Collection [ATCC], Rockville, MD) was propagated in HEp-2 cells (ATCC) in a monolayer culture. For preparation of a large stock of the virus, HEp-2 cells were infected with the ATCC stock virus, and cell supernatant samples were collected 5 or 6 days after infection. The supernatant was centrifuged at 2,000 rpm for 10 minutes at 4°C to remove cellular debris. The concentration of virus was adjusted as evaluated by quantitative plaque-forming assay. The clear supernatant was collected and stored at -70°C. Mice were infected under light ether anesthesia (Wako, Osaka, Japan) with low-grade RSV (2×10^3 plaque-forming units in 50 μ L) by means of intranasal inoculation as previously described.¹¹ Controls were sham-infected with HEp-2 cell culture medium in a similar manner. Each experiment was repeated at least 3 times.

Experimental Protocols

Five groups of 8 mice each were prepared. Mice were immunized twice intraperitoneally on days 1 and 14 with 0.5 mg of *D farinae* (LG-5339; CosmoBio, Tokyo, Japan) precipi-

tated in aluminum hydroxide (the *D farinae*, *D farinae*-RSV, *D farinae*-RSV-pranlukast, and *D farinae*-RSV-dexamethasone groups) or with 0.5 mL of phosphate-buffered saline (PBS) (control group). These mice were then challenged intranasally with 50 μ L of PBS (control group) or 50 μ g/50 μ L of *D farinae* allergen (all 4 *D farinae* groups) for 3 consecutive days (days 14 to 16 and days 35 to 37) and were infected twice with HEp-2 cell culture medium (control and *D farinae* groups) or with live RSV (all 4 *D farinae* groups) on days 17 and 38. After secondary infection, 0.5 mg of dexamethasone (Sigma-Aldrich Corp, St Louis, MO) or 0.5 mg of pranlukast, an LTRA (Ono Pharmaceutical Co, Osaka, Japan), was subcutaneously administered per mouse for 4 whole days on days 38 to 41 (the *D farinae*-RSV-dexamethasone and *D farinae*-RSV-pranlukast groups, respectively). Both drugs were dissolved in 99% ethanol. Preliminary experiments showed that the solvent did not have significant effects in *D farinae*-sensitized and RSV-infected mice. Lungs were harvested and bronchoalveolar lavage (BAL) was performed 4 days after secondary infections (day 42). Doses and routes of administration of *D farinae* allergen, dexamethasone, and pranlukast were based on previous studies.²⁰

Pulmonary Inflammation and BAL

The lungs were inflated via tracheostomy tube with 10% phosphate-buffered formalin and then stored in 10% phosphate-buffered formalin, paraffin embedded, cut into 5- μ m-thick sections, and stained with hematoxylin-eosin. The mice were humanely killed and the trachea was exposed and cannulated using a needle connected to a tuberculin syringe. Then the lung underwent BAL with 0.5 mL of PBS, and the recovered BAL fluid (BALF) was pooled. Recovered BALF volumes ranged from 75% to 85% of instilled PBS. There was no statistically significant difference in recovered BALF volumes between control and experimental groups. The supernatant was collected after centrifugation of BALF and was stored at -80°C until it was assayed for cys-LTs. The cell suspension was centrifuged onto a glass slide using a cytospin centrifuge at 850 rpm for 2 minutes at room temperature. Cytocentrifuged cell smears were air-dried and stained by Giemsa stain. At least 300 cells were examined microscopically in a blinded manner for a differential blood cell count. Concentrations of cys-LTs in BALF were assayed using enzyme immunoassays (Cayman Chemical Co, Ann Arbor, MI).

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Lung tissue samples were homogenized using a tissue homogenizer. All the reagents for RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) were purchased from Gibco BRL (Rockville, MD). Total RNA was isolated using TRIzol reagent, and 5 μ g of total RNA was reverse transcribed in 20 μ L of 50-mmol/L Tris-hydrochloride (pH 8.3), 10-mmol/L dithiothreitol, 75-mmol/L potas-

sium chloride, 3-mmol/L magnesium chloride, 0.5-mmol/L deoxyribonucleoside triphosphate, 2 U/ μ L of Moloney murine leukemia virus RT, and 25 ng/ μ L of random hexamer primers. The PCR was performed in 20 μ L of reaction buffer (50-mmol/L potassium chloride, 10-mmol/L Tris-hydrochloride [pH 9.0], 0.1% Triton X100, and 1.25-mmol/L deoxyribonucleoside triphosphate), 1 μ M each of sense-strand and antisense-strand primers, and 0.01 U/ μ L of Taq polymerase. For PCR, mouse β -actin and RSV N protein-specific primers were designed from previous studies.²¹ Twenty-five to 35 amplification cycles (denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 1 minute) were used. The PCR products were separated by means of agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

Analysis of Cytokines

Thoracic lymph nodes were prepared from the 5 groups of mice, and mononuclear cells were prepared as previously described.⁶ Mononuclear cells (2×10^5 /200 μ L per well) were cultured in the absence or presence of 100 μ g/mL of *D farinae* in 96-well plates for 48 hours as previously described.²⁰ The concentrations of interferon- γ (IFN- γ) and interleukin 5 (IL-5) in the supernatants, representing T_H1 and T_H2 cytokines, respectively, were determined by means of enzyme-linked immunosorbent assay (Endogen, Woburn, MA), using the procedure described in the instruction manual.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Data were evaluated by using repeated-measures analysis of variance with a Bonferroni multiple comparison test. $P < .05$ was considered statistically significant.

RESULTS

Dexamethasone and Pranlukast Attenuate *D farinae* Allergen-Sensitized and RLRI-Induced Allergic Airway Inflammation

Consistent with the previous study by our group,¹¹ RLRI enhanced *D farinae* allergen-induced allergic airway inflammation (Fig 1). Lung abnormalities in the *D farinae*-RSV-dexamethasone group were comparable with those in control mice, and pranlukast attenuated allergic airway inflammation in *D farinae*-RSV mice to a lesser degree than dexamethasone (Fig 1). The BALF analysis revealed that RLRI significantly increased airway eosinophilia in *D farinae* mice (Fig 2). This significance disappeared after either dexamethasone or pranlukast use (Fig 2). In this murine model, allergic airway inflammation was immunologically enhanced after RLRI, and, thus, viral RNA could be detected by RT-PCR in primary RSV infection, whereas it became undetectable in secondary RSV infection.¹¹ In agreement with this, viral RNA could not be detected in *D farinae*-RSV mice in the present study (data not shown). After dexamethasone or pranlukast treatment, viral RNA remained undetectable (data not

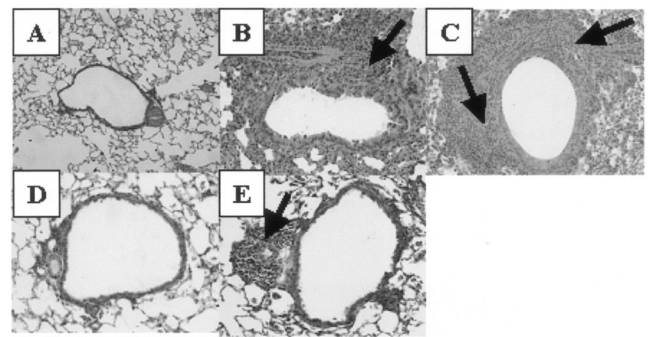


Figure 1. Lung tissue samples obtained from 5 groups of mice after secondary low-grade respiratory syncytial virus (RSV) infection: the control (A), *Dermatophagoides farinae* (B), *D farinae*-RSV (C), *D farinae*-RSV-dexamethasone (D), and *D farinae*-RSV-pranlukast (E) groups (hematoxylin-eosin, magnification $\times 100$). Arrows indicate pulmonary inflammation.

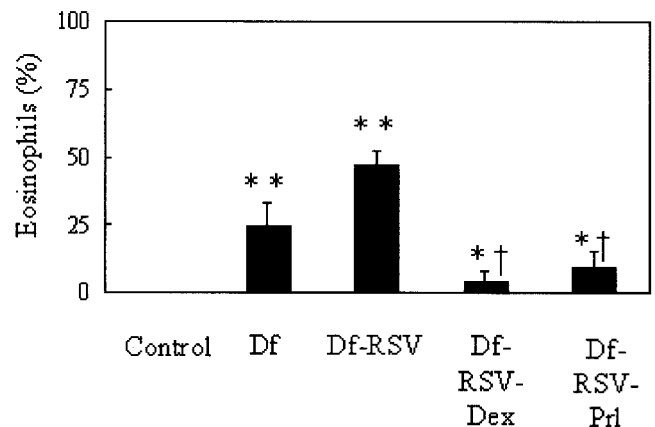


Figure 2. Mean eosinophil percentages in bronchoalveolar lavage fluid in the 5 study groups. Differential blood cell counts are based on May-Grünwald-Giemsa. Df indicates *Dermatophagoides farinae*; RSV, respiratory syncytial virus; Dex, dexamethasone; Pr, pranlukast; double asterisk, $P < .01$; asterisk, $P < .05$ vs control; and dagger, $P < .05$ vs Df. Error bars represent SEM.

shown). Collectively, the glucocorticoid and the LTRA attenuated virus-induced exacerbation of allergic airway inflammation without a significant effect on viral quantity.

Dexamethasone Attenuates T_H1 and T_H2 Responses, Whereas Pranlukast Attenuates Only T_H2 Response in RLRI and *D farinae*-Sensitized Mice

To determine the immunologic mechanisms of action of glucocorticoids and LTRAs, allergen-specific cytokine production in lung-draining lymph nodes was compared in the 5 groups of mice (Table 1). Compared with *D farinae* mice, *D farinae*-RSV mice showed significant increases in IL-5 and IFN- γ . Dexamethasone significantly inhibited IL-5 and IFN- γ production. Pranlukast also significantly inhibited IL-5 production but not IFN- γ production. These results suggest that the effects of glucocorticoids in cytokine inhibition are nonspecific, whereas those of LTRAs are T_H2 specific.

Table 1. Cytokine Production by Thoracic Lymph Node Mononuclear Cells After Secondary Low-Grade RSV Infection*

Group and stimulation	IFN- γ , pg/mL	IL-5, pg/mL
Control		
None	188.2 $\pm$ 11.4	0 $\pm$ 0
Allergen	177.9 $\pm$ 23.3	0 $\pm$ 0
<i>Dermatophagoides farinae</i>		
None	77.4 $\pm$ 35.6†	66.2 $\pm$ 15.4†
Allergen	106.2 $\pm$ 44.7†‡	134.6 $\pm$ 21.9†‡
<i>D farinae</i> -RSV		
None	1,593.4 $\pm$ 31.1†§	831.3 $\pm$ 53.2†§
Allergen	2,203.4 $\pm$ 11.8†‡§	1,100.0 $\pm$ 34.4†‡§
<i>D farinae</i> -RSV-dexamethasone		
None	112.2 $\pm$ 19.7†¶	32.5 $\pm$ 5.8†¶
Allergen	154.3 $\pm$ 22.6†‡¶	98.7 $\pm$ 12.6†‡¶
<i>D farinae</i> -RSV-pranlukast		
None	1,232.6 $\pm$ 22.9†§	241.5 $\pm$ 31.1†§¶
Allergen	1,786.5 $\pm$ 28.5†‡§	422.2 $\pm$ 22.5†‡§¶

Abbreviations: IFN- γ , interferon- γ ; IL-5, interleukin 5; RSV, respiratory syncytial virus.

* Data are given as mean $\pm$ SEM (n = 8 for each group).

† $P < .01$ vs control.

‡ $P < .05$ vs none.

§ $P < .01$ vs *D farinae*.

¶ $P < .01$ vs *D farinae*-RSV.

Dexamethasone and Pranlukast Attenuate Cys-LT Production in BALF

Concentrations of cys-LTs in BALF were compared in the 5 groups of mice (Fig 3). Compared with control mice, *D farinae* allergen sensitization resulted in a significant increase in cys-LTs in the airway. The RLRI did not increase airway cys-LTs in *D farinae* mice. Dexamethasone and pranlukast significantly inhibited cys-LT production in the airway. It is likely that cys-LTs did not play a critical role in RLRI-induced exacerbation of allergic airway inflammation and

that the effects of pranlukast are unlikely to represent cys-LT antagonism.

DISCUSSION

Using the RLRI murine model of allergic asthma, the present study found that (1) the systemic glucocorticoid inhibited allergic airway inflammation associated with suppression of T_H1 and T_H2 cytokines; (2) although the systemic glucocorticoid suppressed IFN- γ production, viral RNAs were not increased; (3) pranlukast, an LTRA, attenuated allergic airway inflammation associated with IL-5 suppression; and (4) the glucocorticoid and the LTRAs significantly reduced cys-LTs in the airway.

In contrast to previous studies,^{22,23} wherein mice were infected with high-grade RSV, which per se causes the development of pulmonary inflammation, mice in the present study were repeatedly infected with low-grade RSV, which per se does not cause the development of pulmonary inflammation but induces significant IFN- γ production in lung-draining lymph nodes.¹¹ Thus, the present study mimics recurrent and subclinical RSV infections in adults, which frequently occur in real life.^{24,25} A previous study¹¹ showed that RLRI enhanced preexisting allergic airway inflammation through immunologic mechanisms, wherein IL-5 is critically involved.

In addition to non-virus-induced exacerbation, glucocorticoids are used for the virus-induced acute exacerbation of asthma.¹² However, the effects of glucocorticoids in virus-induced asthma remain controversial in human and animal studies.^{26,27} In addition, although glucocorticoids are effective for virus-induced airway inflammation, they potentially delay viral clearance via the suppression of cellular immunity.²⁸ In

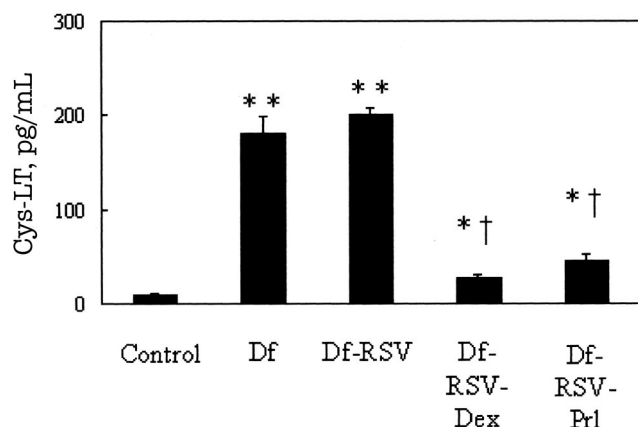


Figure 3. Mean concentrations of cysteinyl leukotrienes (cys-LTs) in bronchoalveolar lavage fluid were determined by means of enzyme immunoassays. Df indicates *Dermatophagoides farinae*; RSV, respiratory syncytial virus; Dex, dexamethasone; Pr1, pranlukast; double asterisk, $P < .01$; asterisk, $P < .05$ vs control; and dagger, $P < .05$ vs Df. Error bars represent SEM.

the present study, systemic administration of a glucocorticoid ameliorated allergic airway inflammation in mite allergen-sensitized and RLRI mice with concomitant suppression of T_H1 and T_H2 cytokines. Although IFN- γ was suppressed, viral RNA remained undetectable, supporting the current guideline, which recommends early induction of oral glucocorticoids for virus-induced acute asthma.¹²

Viral respiratory tract infections increase airway cys-LTs,^{18,29} which could worsen asthma.^{16,30,31} Antileukotriene therapies are effective for RSV infection in humans and animals.^{18,32} We had originally expected that RLRI might increase cys-LTs in the airway. Unexpectedly, cys-LTs were significantly increased in the BALF of *D farinae* allergen-sensitized mice but were not further increased in *D farinae*-RSV mice. As mentioned previously herein, the preexisting allergic airway inflammation was immunologically enhanced in the present study by low-grade RSV, which per se does not cause the development of pulmonary inflammation. Thus, cys-LTs, mediators of acute inflammation, do not seem to increase in contrast to primary high-dose RSV infection. Although the concentration of cys-LTs in BALF did not increase, LTRAs attenuated RLRI-induced allergic airway inflammation in *D farinae* mice, which was associated with the inhibition of IL-5 but not IFN- γ . Our group^{20,33} previously reported that pranlukast inhibited allergen-specific IL-5 production in a murine model of allergic asthma and in passively sensitized human lung tissue probably via a mechanism distinct from cys-LT receptor antagonism, which is currently under investigation in the Laboratory Animal Center for Biochemical Research. Because the LTRA did not inhibit IFN- γ , a T_H1 cytokine, it could be used more safely for the treatment of virus-induced asthma compared with glucocorticoids, although the anti-inflammatory effects of LTRAs were less potent than those of glucocorticoids.

The glucocorticoid and the LTRA significantly inhibited *D farinae*-induced cys-LT production in BALF. It had been reported that glucocorticoids do not inhibit cys-LT production³⁴ and that pranlukast is originally an LTRA but not a cys-LT synthase inhibitor. Although the exact mechanism is yet to be determined, it is possible that both drugs significantly reduced the number of eosinophils, cys-LT-producing cells in the airway, which subsequently resulted in cys-LT reduction. In addition to eosinophils, mast cells and dendritic cells also produce cys-LTs.^{35,36} The effects of number and activation of cys-LT-producing cells in the airway by these drugs is an interesting issue for future research.

In conclusion, the present study demonstrates that glucocorticoids and LTRAs significantly inhibit RLRI-induced exacerbation of allergic airway inflammation. The glucocorticoid suppressed nonspecific cytokines, whereas viral RNA did not increase via suppression of cellular immunity. Pranlukast, an LTRA, suppressed IL-5 but not IFN- γ , at least probably not through cys-LT receptor antagonism. The role of these anti-inflammatory therapies in virus-induced asthma seems promising.

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