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ORIGINAL ARTICLE

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Molecular Mechanism of the Additive Effects of Leukotriene Modifier in Asthmatic Patients Receiving Steroid Therapy

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ABSTRACT

Background: The addition of leukotriene modifier (LM) may be a useful approach for uncontrollable asthma despite treatment with inhaled corticosteroid (ICS), especially in asthmatics comorbid with allergic rhinitis (AR), although little is known about its molecular mechanism. We evaluated the additive effects of LM with ICS on pulmonary function and airway inflammation in asthmatics with or without AR.

Methods: Eighteen uncontrolled steroid-treated asthmatics, nine with and nine without AR, were enrolled. Spirometry, peak expiratory flow (PEF) measurements, and exhaled breath condensate sampling were performed before and 8 weeks after LM administration. The lowest PEF over the course of one week, expressed as a percentage of the highest PEF (Min%Max PEF), was used as an index of fluctuation of the airway caliber. Airway cytokine expression was analyzed with a protein array.

Results: A significant improvement in forced expiratory volume in one second as a percentage of the predicted value (%FEV_1) and Min%Max PEF was seen in the subgroup of asthma with AR. Although there was no significant difference in the baseline cytokine values between the groups, the exhaled RANTES level was significantly reduced by LM in the asthma with AR group. The changes in the RANTES level were significantly related to the changes in the %FEV_1 and Min%Max PEF values.

Conclusions: LM caused a greater improvement in pulmonary function and airway inflammation in asthmatics with AR. The RANTES-mediated pathway may be involved in the improvement of the airflow limitation and airway lability by LM additive therapy in asthmatics receiving steroid therapy.

KEY WORDS

airflow limitation, airway hyperresponsiveness, airway lability, exhaled breath condensate, RANTES

INTRODUCTION

A basic pathological feature of asthma and allergic rhinitis (AR) is airway inflammation, in which various inflammatory cells and molecules produced from them are involved.[1] The cysteinyl leukotrienes (CysLTs), common mediators of asthma and AR, induce bronchoconstriction and mucus hypersecretion, enhance airway responsiveness, and act as chemoattractants for eosinophils in the airway.[2] Leukotriene modifier (LM) has proven to be effective in the treatment of both asthma and AR, and is the only drug approved to treat both diseases in a single formulation.[3-5]

Despite treatment with inhaled corticosteroids (ICS), the suppression of inflammation in asthmatic airways is often incomplete,[6] and their effect on CysLTs biosynthesis is limited.[7,8] It has been demonstrated that LM added to ICS was as efficacious as double the dose of ICS in improving peak expiratory

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flow (PEF) in asthmatics.[9] However, when patients with comorbid AR were evaluated, the addition of LM was significantly better at improving airflow limitation than doubling the dose of ICS.[10] These results suggest that the addition of LM to ICS could be useful in treating asthmatics whose asthma is not well controlled with steroid therapy, especially in patients comorbid with AR. Although the additive anti-inflammatory properties of LM in asthmatics receiving steroid therapy have been examined using sputum eosinophil counting and exhaled nitric oxide measurements,[11-13] little is known about its molecular mechanism of action.

In the present study, we evaluated the additive effects of LM with ICS on pulmonary function and airway cytokine expression in asthmatics with or without AR. Furthermore, the relationship between the changes in the molecule expression and the physiological properties of asthma, such as airflow limitation and airway lability, was examined.

METHODS

STUDY SUBJECTS

Eighteen uncontrolled steroid-treated asthmatics, nine with AR and nine without AR, were enrolled in a randomized fashion after giving informed consent. To avoid the influence of the pollen season, the enrollment was performed from May to September 2006. The study was approved by the local ethics committee. All patients satisfied the American Thoracic Society criteria for asthma.[14] Patients with rhinitis were identified by specialists. All patients were receiving inhaled steroid therapy (equivalent dose of 400 μg fluticasone · day^{-1}) and used inhaled short acting β2 agonists as needed for symptom relief. Subjects were not included if they had had an exacerbation of asthma or a respiratory tract infection in the 2 weeks preceding the examination.

STUDY DESIGN

On the first day, spirometry and exhaled breath condensate (EBC) collections were performed. PEF monitoring had been started at least 4 weeks before this examination. After assessment of the baseline values, open, uncontrolled LM therapy (asthma with AR group, pranlukast in 5 cases and montelukast in 4 cases; asthma without AR group, pranlukast in 4 cases and montelukast in 5 cases) was administered for 8 weeks, and then the same examination was repeated.

EBC COLLECTION

EBC collection was performed with a standardized method according to the recommended procedure.[15] The EBC was collected by using a condenser, which permitted noninvasive collection of condensed exhaled air by freezing it to −20℃ (Ecoscreen; Jaeger, Hoechberg, Germany). The subjects breathed through a mouthpiece and a two-way non-rebreathing valve, which also served as a saliva trap. Subjects were asked to breath at a normal frequency and tidal volume while wearing a nose-clip. The collected EBC was stored at −70℃ and cytokine measurements were performed within 4 weeks.

Table 1 Subject demographics

	Asthma/AR +	Asthma/AR −
Number	9 (F/M = 6/3)	9 (F/M = 4/5)
Age (years)	42.3 ± 6.5	43.0 ± 4.6
FVC (L)	3.27 ± 0.26	3.72 ± 0.24
FEV_1 (L)	2.47 ± 0.24	2.75 ± 0.21
FEV_1% (%)	74.8 ± 3.8	73.8 ± 2.9
%FEV_1 (%)	84.6 ± 4.6	86.2 ± 3.6
Min%MaxPEF (%)	82.6 ± 2.1	84.4 ± 2.2

Definition of abbreviations: AR, allergic rhinitis; F, female; M, male; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; PEF, peak expiratory flow; Min%Max PEF, the lowest PEF over a week expressed as % highest PEF. Values are means ± SE.

CYTOKINE MEASUREMENTS

Human Inflammation Antibody III (Ray Biotech Inc., Norcross, GA, USA), consisting of 40 different cytokine and chemokine antibodies spotted in duplicate onto a membrane, was utilized as previously described.[16] The intensity of the signals was detected directly from the membranes using a chemiluminescene imaging system (Luminocapture AE6955; Atto Co., Tokyo, Japan). HRP-conjugated antibody served as a positive control at six spots and was also used to identify the membrane orientation. For each spot, the net intensity gray level was determined by subtracting the background gray levels from the total raw intensity gray levels. The relative intensity levels of the cytokine amounts were normalized with reference to the amount present on the positive control in each membrane on the following basis: average of the cytokine spot intensities/average of the positive control spot intensities, indicated as a percentage. Using this technique, we have previously shown that the expressions of IL-4, IL-17, RANTES, MIP-1α, MIP-1β, IP-10, IL-8, TNF-α, and TGF-β were increased in asthmatic airways.[16] Thus, these nine cytokines were selected as target molecules.

PEAK EXPIRATORY FLOW (PEF) MEASUREMENTS

PEF was measured using an Assess® peak flow meter (Respironics HealthScan Co., NJ, USA). Among PEF indices, the lowest PEF over a week, expressed as a percentage of the highest PEF (Min%Max PEF), has been suggested to be the best index of airway lability.[17] We have confirmed that Min%Max PEF showed a good correlation with the degree of airway

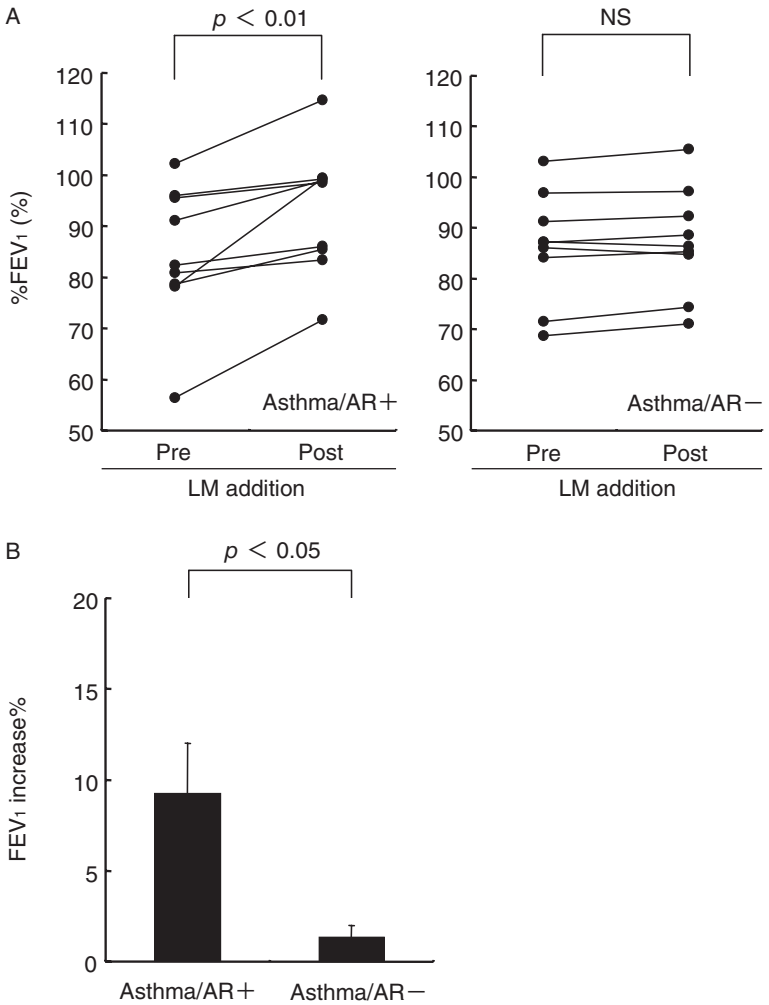

Fig. 1 Graphs of individual forced expiratory volume in one second (FEV_1) at baseline and at the end of additive leukotriene modifier (LM) therapy in asthma patients with or without allergic rhinitis (AR) (**A**), and mean change from baseline in FEV_1 for each subgroup (**B**).

hyperresponsiveness (AHR) measured by the inhalation challenge test,[18] and thus Min%Max PEF was used as an index of fluctuation of the airway caliber in this study.

PULMONARY FUNCTION TEST

Forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were measured with a Vitalograph Pneumotrac 6800™ (Vitarograph Co., Ennis, Ireland).

STATISTICAL ANALYSES

Comparisons of before and after LM therapy were performed by Mann-Whitney *U* tests and comparisons between groups were performed by Fisher's exact tests. Pearson's correlation coefficients were calculated to determine the correlation between the changes in the levels of cytokine expression and pulmonary physiological parameters by LM therapy. All data were expressed as means ± SE, and significance was defined as a *P* value of less than 0.05.

RESULTS

SUBJECT DEMOGRAPHICS

The clinical characteristics of the study subjects are shown in Table 1. There were no significant differences in baseline characteristics between the groups. The asthma control levels of all subjects were classified as partly controlled at baseline.[19] After LM additive therapy, asthma symptoms in seven of nine asthmatics with AR and five of nine asthmatics without AR were improved to a controlled level. The rates of improvement were higher in the asthma with AR group, but the differences were not significant. There were no subjects whose asthma control levels worsened. All of the asthma with AR subjects had nasal

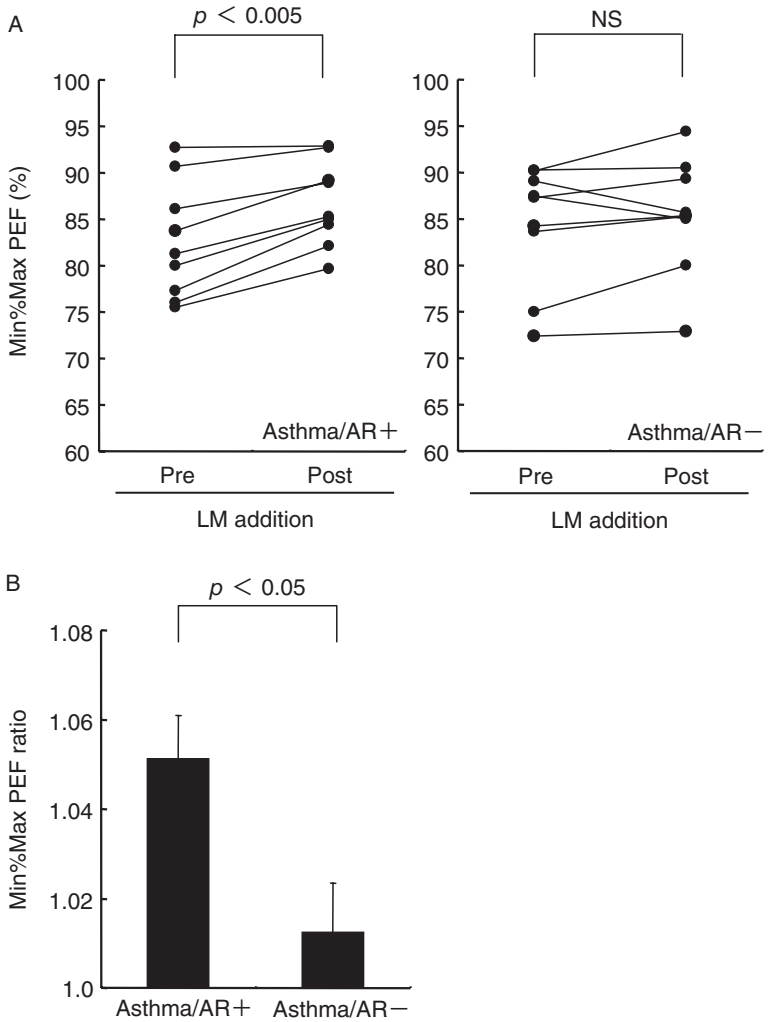

Fig. 2 Graphs of individual peak expiratory flow (PEF) variability (Min%Max PEF) at baseline and at the end of additive leukotriene modifier (LM) therapy in asthma patients with or without allergic rhinitis (AR) (**A**), and mean changes from baseline in Min%Max PEF for each subgroup (**B**).

symptoms at baseline; there was nasal discharge in seven subjects and nasal blockage in four subjects. Additive LM improved the nasal discharge in four subjects and nasal blockage in three subjects.

PULMONARY FUNCTION

A significant improvement in the parameter that represents airway caliber, FEV_1 as a percentage of the predicted value (%FEV_1), was seen in the subgroup of asthma with AR by additive LM therapy (Fig. 1A, B). LM therapy also improved the parameters that represent airway lability, Min%Max PEF, in the asthma with AR group but not in the asthma without AR group (Fig. 2A, B). The kind of LM used was not related to the additive effects on pulmonary function. The LM-mediated improvement in airflow limitation, namely the increase in %FEV_1, was significantly correlated with the changes of Min%Max PEF ($r = 0.754$, $p < 0.01$, [Fig. 3]).

AIRWAY CYTOKINE EXPRESSION

There was no significant difference in the baseline cytokine values between the two groups (Fig. 4). Among the nine examined molecules, the RANTES level in the asthma with AR group was significantly reduced by LM therapy ($p < 0.05$), whereas there were no significant changes in all examined cytokine levels in the asthma without AR group (Fig. 5A, B). The kind of LM used was not related to the changes in the cytokine expressions by LM additive therapy.

RELATIONSHIP BETWEEN CHANGES IN RANTES LEVELS AND PULMONARY PHYSIOLOGICAL PARAMETERS BY ADDITIVE LM THERAPY

The changes in the RANTES levels by additive LM therapy were significantly correlated with the im-

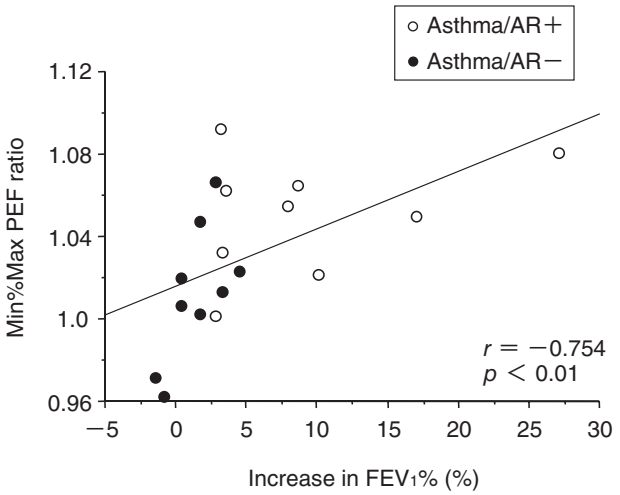

Fig. 3 Relationship between leukotriene modifier-mediated improvement in forced expiratory in one second and peak expiratory flow variability in asthma patients with (open circles) or without (closed circles) allergic rhinitis (AR). The lines correspond to the fitted regression equation.

provement in the FEV_1 increase% and the ratio of Min %Max PEF ($r = -0.736$, $p < 0.01$, Fig. 6A and $r = -0.622$, $p < 0.05$, Fig. 6B, respectively). Correlations between LM-mediated changes in the levels of other molecules and the physiologic properties were not seen.

DISCUSSION

In the present study, adding LM therapy to ICS improved the airflow limitation and airway lability, and improvement was significant in the subgroup of asthma with AR but not in the asthma without AR group. There was no significant difference in the baseline cytokine values between the groups. However, the exhaled RANTES levels were significantly reduced by LM in the asthma with AR group. The changes in the RANTES level were related to the changes in the physiologic properties, such as $\%FEV_1$ and Min%Max PEF values.

To our knowledge, the current report is the first direct comparison study to evaluate the additive effect of LM on pulmonary function and airway cytokine expression between steroid-treated asthmatics with AR and those without AR. Asthma and AR often co-exist and upper airway diseases can influence lower airway inflammation and function in some patients with asthma.[1] Allergen challenge to the lung leads to inflammation in the nose.[20] Similarly, allergen challenge to the nose leads to AHR in the lower airway.[21] CysLTs are key mediators and modulators of systemic allergic responses as well as a component of the inflammatory responses that lead to the typical symptoms of asthma and rhinitis.[2] CysLTs facilitate eosinophil recruitment into susceptible tissues and prolong their survival, contributing to the maintenance of the inflammatory reaction.[22] In addition, CysLTs have modulating effects on the cytokine activity and production from cells.[2] A previous study has shown that CysLTs stimulate lung mononuclear cells to release inflammatory mediators, such as RANTES.[23] Allergen challenge induces RANTES positive cells in accordance with increased eosinophils in the airway, and LM suppresses airway eosinophils and RANTES production.[24,25] These studies show that LM has the potential to suppress airway RANTES expression by the blockage of CysLTs.

In this study, LM provided significant improvements in airflow limitation in asthmatics with AR, in agreement with a previous study.[10] In addition, we are the first to demonstrate that LM causes a greater improvement in pulmonary function and exhaled RANTES expression in asthmatics with AR than in those without AR. Although airway inflammation seems likely to play a similar role in the pathogenesis of AR as in asthma, it may be difficult to explain our results by the differences in the degree of airway inflammation between the two groups. Even in the absence of rhinitis, asthma patients have increased eosinophil levels in nasal mucosa, and these levels are related to the bronchial eosinophil values.[26] The present study also showed that the cytokine values at baseline were similar in the two groups.

Previous studies have shown that there is increased excretion of urinary leukotriene E4 in asthmatics with AR.[27] Nasal allergen challenge causes a dose-dependent increase in CysLTs that correlates with nasal symptoms.[28] In addition, the sputum CysLTs levels obtained from asthmatics remain elevated despite ICS treatment.[29] Consequently, despite receiving steroid therapy, CysLTs are over-expressed in the asthmatic airway and possibly more so in patients comorbid with AR. This speculation may explain the present result that LM significantly reduced the RANTES levels only in the asthma with AR group. However, the expression of CysLTs in the lower airways has not been directly compared between groups, although increased CysLT levels have been shown in the BAL fluid and sputum of patients with asthma.[29,30] In addition, in other previously proposed theories of the interaction between asthma and AR, the irritant effects of nasal secretions directly entering the lower airways and systemic propagation of nasal inflammation to the lower airways,[31] may be involved in the mechanism of the present result. However, the current study was not able to prove these possibilities.

The RANTES-mediated pathway may be involved in the improvements of the airflow limitation and airway lability by the addition of LM in asthmatics receiving steroid therapy. A possible explanation for this association may be as follows. In asthmatic airways, RANTES have a potent role in eosinophil re-

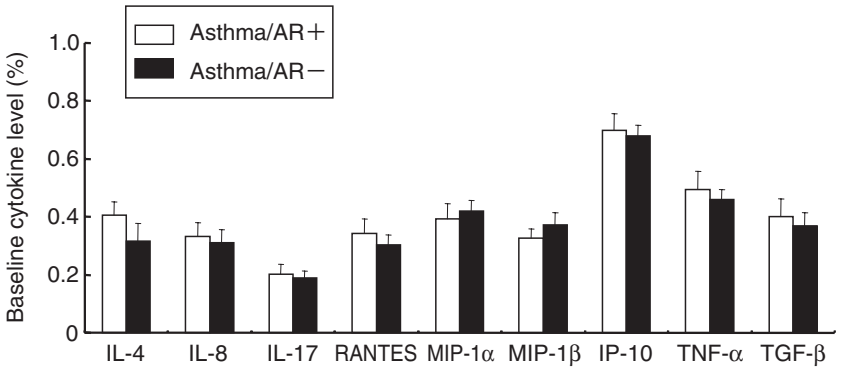

Fig. 4 Baseline expression levels of IL-4, IL-8, IL-17, RANTES, MIP-1α, MIP-1β, IP-10, TNF-α, and TGF-β in exhaled breath condensate obtained from asthma patients with (open bars) or without (filled bars) allergic rhinitis (AR).

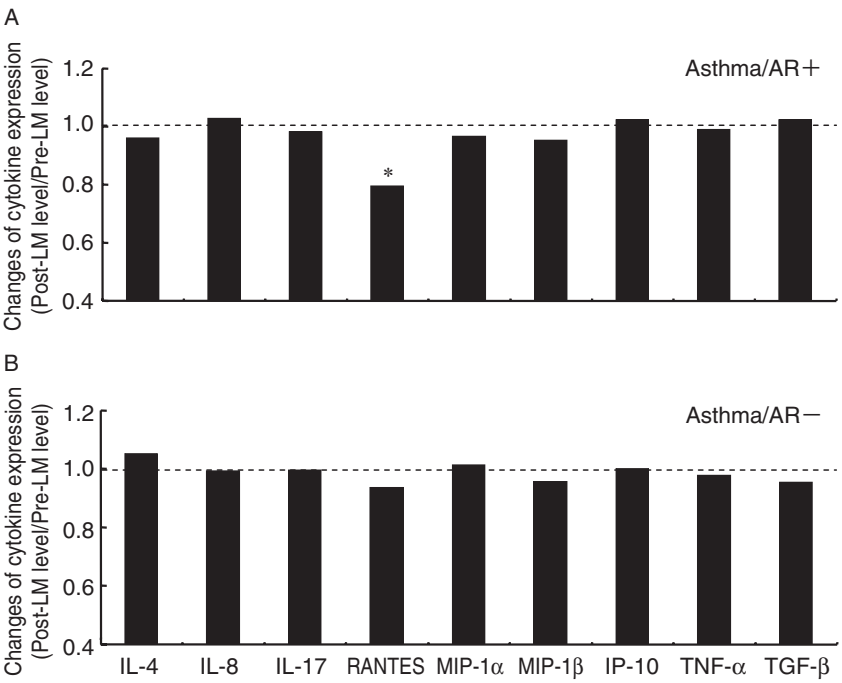

Fig. 5 Changes of expression levels of IL-4, IL-8, IL-17, RANTES, MIP-1α, MIP-1β, IP-10, TNF-α, and TGF-β in exhaled breath condensate by additive leukotriene modifier (LM) therapy in asthma patients with (**A**) or without (**B**) allergic rhinitis (AR). $*p < 0.05$ compared with baseline cytokine levels.

cruitment in the airway,[32,33] and RANTES-positive sputum eosinophils are correlated with the degree of %FEV_1 after allergen challenge.[33] LM therapy may modulate the cytokine expression, such as RANTES, with a consequent inhibition of the airway inflammation resulting in improvement of the pulmonary function. It has been shown that improvements in airflow limitation and AHR in asthmatics are accompanied by a decrease of airway inflammation and reduction in the RANTES expression,[33,34] which is compatible with our results.

Furthermore, RANTES activate immune cells and induce the exocytosis of bronchoconstrictive mediators resulting in airflow limitation.[32,33] Using a murine asthma model, a previous study has shown that the blockage of RANTES reduces AHR.[35] In the present study, the reduction in the exhaled RANTES levels was associated with improvements in both the airflow limitation and airway lability. The LM-mediated improvements in the airflow limitation were related to the changes in airway lability. These results suggest that LM can inhibit the airflow limitation induced by

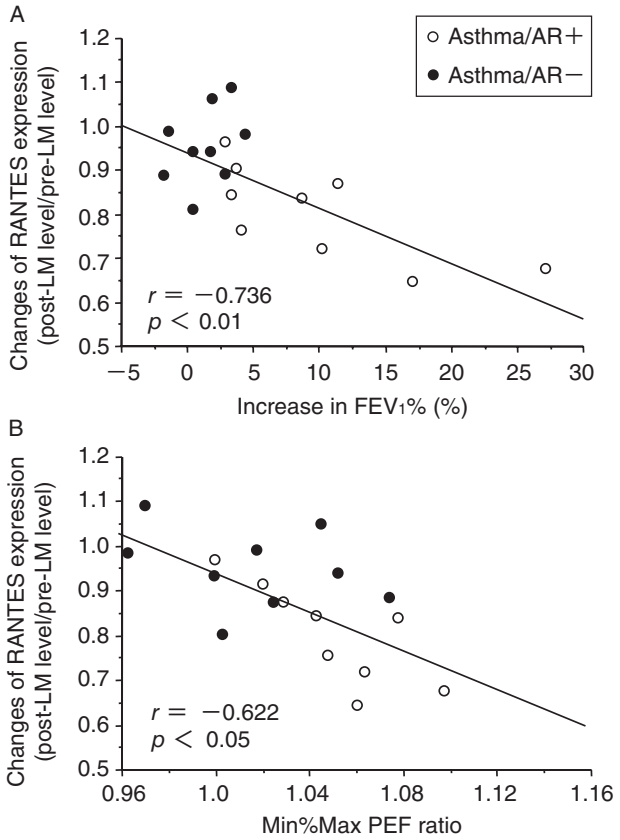

Fig. 6 Relationship between leukotriene modifier (LM)-mediated changes of RANTES expression (the ratio of post-LM level/pre-LM level) and improvement in physiological parameters: forced expiratory volume in one second (**A**) and peak expiratory flow variability (**B**) (open circles, asthma patients with allergic rhinitis [AR]; closed circles, asthma patients without AR). The lines correspond to the fitted regression equation.

RANTES and thereby improve the fluctuation of the airway caliber.

The limitations of the current study are as follows. The enrollment of subjects was carefully performed to avoid the influence of the pollen season. However, the possibility that the changes in the parameters could be attributed to a seasonal effect remained. Furthermore, the small number of study subjects may affect the result that LM did not significantly improve the examined parameters in the asthma without AR group. This report does not claim that LM should not be used for asthma without AR patients. Finally, this small-scaled study did not have enough power to examine the association between the LM-mediated changes in symptoms and RANTES levels in EBC.

In conclusion, LM caused a greater improvement in pulmonary function and airway inflammation in asthmatics with AR. The RANTES-mediated pathway may be involved in the improvement of the airflow limitation and airway lability by LM additive therapy in asthmatics receiving steroid therapy.

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