The Additional Effects of Clarithromycin And Pranlukast On The Cytokine Suppression By Corticosteroids Using Murine Allergic Bronchopulmonary Aspergillosis Model

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Abstract

Few medicines other than oral corticosteroids and anti-fungal medicines are currently known as reliable treatments for allergic bronchopulmonary aspergillosis (ABPA). The efficacies of macrolide or leukotriene receptor antagonist (LTRA) with or without corticosteroid on ABPA are unknown. Mice were sensitized to *Dermatophagoides farinae* (Df) allergen intranasally and infected with *Aspergillus fumigatus* (Af). After Af infection, corticosteroid (Dexamethasone; Dex) was administered for five days in DfAf/Dex group. The effects of macrolide (clarithromycin; CAM) or LTRA (pranlukast; PRN) with or without Dex were also evaluated. Pathologically, the combined treatment with Dex and CAM decreased the airway inflammation. The interleukin (IL)-5, IL-13 and macrophage inflammatory protein (MIP)-2 concentrations in homogenized lungs were significantly elevated in DfAf mice compared to control mice ($p < 0.05$, each). CAM significantly decreased the elevations of MIP-2 of DfAf mice ($p < 0.05$). The addition of CAM on Dex suppressed both of the MIP-2 and IL-5 elevation ($p < 0.05$, each, DfAf/Dex vs DfAf/Dex/CAM group), but the addition of PRN on Dex did not. It was suggested that combination of CAM and corticosteroid enhanced the suppressing effect of both eosinophilic and neutrophilic airway inflammations. This finding will give a new hope for the treatment of severe fungus-related asthma.

Background

Allergic bronchopulmonary aspergillosis (ABPA) caused by sensitization of the *Aspergillus* antigen, accounts for 1–4% of the asthma patients and involves in the asthma severity [1–3]. Exposure to fungal spores or mycelial fragments results in the formation of IgE and IgG antibodies, and T cells also play important roles in ABPA progress [4, 5]. The working group of the International Society for Human and Animal Mycology (ISHAM) had suggested the disease criteria with a preceding lung disease (Bronchial asthma or cystic fibrosis), and have both type I allergy (type I skin reaction or specific IgE antibody against *Aspergillus* antigen and/or increase of serum total IgE value) and type II allergy (image findings indicate the airway inflammation or bronchial injury [6]. Without adequate diagnosis and treatment, ABPA will progress to severe respiratory failure with bronchiectasis and fibrosis [7].

Systemic glucocorticoids are considered the mainstay of treatment of ABPA [8, 9]. Oral corticosteroid (OCS) and antifungal therapies appear to be partially successful in certain patients with ABPA [10–12], which like via suppression of cytokine production [4, 5, 13].

Other than anti-fungal medication, we hypothesized that cysteinyl leukotriene receptor antagonist (LTRA) or macrolide would be expectable for treatment of ABPA. LTRA, e.g. pranlukast (PRN) is effective for asthma control [14], and has been recommended as additional medicine on inhaled corticosteroid (ICS) / long acting beta agonist (LABA) treatment [1]. PRN suppressed the airway eosinophilic inflammation of asthmatic patients via interleukin (IL)-5 suppression [15, 16]. It is reported that macrolide improved peak expiratory flow, clinical symptoms, quality of life, and airway hyper-reactivity in asthmatics [17], and that macrolides improved the symptoms in the patients with severe neutrophilic asthma and in asthma exacerbations [18, 19].

To clarify the efficacies of LTRA and macrolide on ABPA especially in cytokine level for the direct or additional effect on OCS, in this study, we investigated the single or additional effect of LTRA (pranlukast; PRN) and macrolide (clarithromycin; CAM) with or without OCS (dexamethasone; Dex) on *Aspergillus fumigatus* (Af) and *Dermatophagoides farinae* (Df) co-exposed ABPA murine model.

Methods

Animals and Experimental protocol

Six groups of mice were prepared in the present study (Fig. 1). Four-week-old female BALB/c mice (Charles River Japan, Inc, Yokohama, Japan) were immunized twice intraperitoneally on days 1 and 14 with 0.5 mg per mouse of *Dermatophagoides farinae* (Df) (LG-5339; Cosmo Bio, Tokyo, Japan) precipitated in aluminum hydroxide. These mice were then challenged intranasally (i.n.) with 50 µg/50 µL Df allergen (crude extract of the mite) on days 14, 16 and 18. Following that, 5×10^6 of Af conidia were administered i.n. on days 19, 21 and 23. Various drugs were administered from on day 21 to 25 and ten groups of mice were prepared as follows. DfAf, no drug was administered; DfAf/Dex, 0.02mg of Dexamethasone (Sigma, St Louis, Mo) were injected subcutaneously (SC); DfAf/PRN, 0.5mg of pranlukast hydrate (ONO Pharmaceutical Co., Osaka, Japan) were injected subcutaneously (SC); DfAf/CAM, 4mg of clarithromycin (Taisho Toyama, Tokyo, Japan) were orally administered; DfAf/Dex/PRN, combination therapy with Dexamethasone and pranlukast hydrate; DfAf/Dex/CAM, combination therapy with Dexamethasone and clarithromycin. On day 26, all mice were sacrificed. Bronchoalveolar lavage fluid (BALF) and lung tissues were obtained from each group. The procedures were reviewed and approved by Nagasaki University School of Medicine Committee on Animal Research. All experiments were repeated at least 3 times.

Preparation of Af conidia

A. fumigatus MF-13, which was isolated from the sputum of a patient with pulmonary aspergilloma, was subcultured on Sabouraud dextrose agar (Becton Dickinson, Cockeysville, MD) at 30°C for 7 days. The conidia were then harvested with sterile saline containing 0.02% Tween 80 (Wako Pure Chemical Industries, Tokyo, Japan), counted in a hemocytometer, and diluted with sterile saline for intranasal infection.

Bronchoalveolar lavage and lung pathology

On day 26, mice were sacrificed and BAL was conducted utilizing 1 ml of PBS in the immediate postmortem period. The obtained BALF was centrifuged. Differential cell counts were performed using cytocentrifuged BALF stained with May-Grünwald-Giemsa. Formaldehyde fixative was gently infused through the lavage catheter set in the trachea. Resected lungs were fixed for an additional 24 hours and embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin (HE).

Analysis of cytokines concentrations in homogenized lung

Lung homogenates were prepared by homogenizing a freshly excised lung. Concentrations of IL-5, IL-13 and MIP-2 in homogenized lung samples were measured using enzyme-linked immunosorbent assay using the methods described by the manufacturer. Detection limits for IL-5, IL-13 and MIP-2 were 5 pg/ml, 1.5 pg/ml and 5 pg/ml respectively.

Statistical analysis

Results are expressed as mean (standard error of mean). Differences between groups were examined for statistical significance using repeated-measures ANOVA with a Bonferroni multiple comparison test. *p* values of < 0.05 were considered significant.

Results

The pulmonary pathology findings and the cell differentiation in BALF of ABPA Mice

Figure 2 shows some examples of the pulmonary pathology findings. DfAf mice had severe airway inflammation (Fig. 2A), and this inflammation was suppressed by combination of Dex and CAM (Fig. 2F).

Table 1 presents the BALF cell differentiations of each group. The eosinophil rate, neutrophil rate, and lymphocyte rate increased by *Der f* and *Af* exposure (DfAf group, $p < 0.05$ vs Control group). This eosinophil rate elevation was not suppressed by any medication. However, lymphocyte rate elevation was suppressed by Dex/PRN, CAM or Dex/CAM.

Table 1. Differentiation of cells of BALF

	Control	DfAf	DfAf/Dex	DfAf/PRN	DfAf/Dex/PRN	DfAf/CAM	DfAf/Dex/CAM
Macrophage rate, %	99 (2)	12 (2) †	30 (18) †	27 (15) †	26 (17) †	24 (10) †	26 (13) †
Neutrophil rate, %	1 (0)	13 (5) †	3 (1) ‡	5 (1) †‡	29 (12) †	1 (1) ‡	29 (10) †
Lymphocyte rate, %	0 (1)	18 (6) †	19 (7) †	18 (4) †	6 (2) †‡	3 (1) ‡	6 (1) †‡
Eosinophil rate, %	0 (0)	57 (22) †	48 (12) †	50 (19) †	39 (10) †	72 (21) †	39 (12) †

Data are shown as mean (standard error of mean). †: $p < 0.05$ vs Control group, ‡: $p < 0.05$ vs DfAf group

The cytokine concentrations in homogenized lungs

Figure 3 presents each cytokine concentration in homogenized lung of the groups. The concentrations of IL-5 and IL-13 (Th2 cytokines) and that of MIP-2 (murine cytokine corresponding to human IL-8) were significantly elevated in DfAf mice compared to control mice ($p < 0.05$, each). Dex or PRN did not suppress the measured cytokine levels. CAM suppressed the elevation of MIP-2 solely ($p < 0.05$, DfAf/CAM group vs DfAf group). Interestingly, the combination of Dex and CAM suppressed both of the MIP-2 elevation and the IL-5 elevation ($p < 0.05$, each DfAf/Dex/CAM group vs DfAf/Dex group). None of Dex, PRN, or CAM affected the IL-13 concentration.

Discussion

In this study, we found mainly two results. Firstly, our DfAf co-exposed murine ABPA model showed airway inflammation with increased eosinophils, neutrophils, and lymphocytes. Of these cells, neutrophil rate was suppressed by Dex, PRN, and CAM; lymphocyte rate was suppressed by Dex/PRN, CAM, and Dex/CAM. Secondly, in our ABPA model, IL-5, IL-13, and MIP-2 of the homogenized lung were significantly elevated. Of these cytokines, IL-5 elevation was suppressed by Dex/CAM, and MIP-2 elevation was suppressed by CAM and Dex/CAM. The combination of Dex and CAM evidently boosted the suppression of IL-5 and MIP-2 elevation than those by Dex solely.

In ABPA management, it is still a problem that there are only two creditable medications, OCS and anti-fungal medicines [1–5]. In this study, we expected the effect of LTRA and macrolide on airway eosinophilic or neutrophilic inflammation with or without corticosteroid. Several evidences suggested that LTRA reduces airway inflammation and remodeling [20–22]. It is known that cysteinyl LTs are not only critically involved in the pathogenesis of asthma but are also produced in the airway during respiratory infection and are involved in protection against respiratory pathogens. [23]. In this study, however, PRN and Dex/PRN did not decrease the elevated cytokines in this ABPA model. In a previous study, PRN did not significantly reduce cytokine production regardless of infection (*aspergillus fumigatus* or *respiratory syncytial*; *RS virus*) and increased IL-10 and IL-12 in Df sensitized mice after RS virus infection but not after aspergillus infection [24]. The role of PRN might not be so effective on ABPA therapy.

In previous studies, ABPA showed both of eosinophilic and neutrophilic inflammation in the airways [25, 26], and our ABPA model had both inflammations. Proteolytic enzymes and mycotoxins released by fungi, in concert with Th2-mediated eosinophilic inflammation and IL-8-mediated neutrophilic inflammation [26], may result in airway damage and central bronchiectasis. Findings from several clinical trials have shown that macrolides significantly reduce sputum eosinophil and neutrophil counts and pro-inflammatory cytokine concentrations [27, 28]. Simpson and co-workers showed that CAM significantly reduced sputum concentrations of IL-8 and neutrophil numbers which increased after withdrawal of the treatment in patients with severe asthma [29]. Macrolides have been known to have anti-inflammatory effect on bronchiectasis [30] or panbronchitis [31]. Macrolides have also been reported to reduce airway hyper-responsiveness and improve pulmonary function [32, 33]. Taking these ideas together, we hypothesized that CAM might be effective to ABPA which has both of neutrophilic and eosinophilic inflammation. As expected, CAM helped the suppression of the elevation of neutrophilic (MIP-2) and eosinophilic (IL-5) cytokine levels of the ABPA lung. Additionally, CAM fostered the Dex suppression of the cytokine levels.

There are some limitations in the study. Firstly, this model represented only some parts of the mechanism of the ABPA, which mimics the early onset state of the disease. The periodically type III allergy development bronchiectasis or fibrosis may be important for macrolide and corticosteroid combination treatment. Secondly, we have measured only three kinds of cytokines (IL-5, IL-13, and MIP-2). Some other important cytokines and proteins (IL-17, IL-33, Thymic stromal lymphopoietin, Metalloproteinases etc.) should have been measured for understanding the LTRA and macrolide pathological roles widely. We need clinical trials of the CAM effects on the ABPA or allergic bronchopulmonary mycosis in the future.

Conclusions

CAM may foster the Dex suppression of eosinophilic and neutrophilic cytokine levels of ABPA murine model. This may indicate the novel treatment strategy for ABPA using macrolide combination therapy with OCS in the future.

Abbreviations

ABPA: allergic bronchopulmonary aspergillosis

Af: *Aspergillus fumigatus*

BALF: Bronchoalveolar lavage fluid

CAM: clarithromycin

Dex: Dexamethasone

Df: Dermatophagoides farinae

HE: hematoxylin and eosin

ICS: inhaled corticosteroid

IL: interleukin

ISHAM: International Society for Human and Animal Mycology

LABA: long acting beta agonist

LTRA: leukotriene receptor antagonist

MIP: macrophage inflammatory protein

OCS: Oral corticosteroid

PRN: pranlukast

RS: respiratory syncytial

Declarations

Ethics approval

The procedures were reviewed and approved by Nagasaki University School of Medicine Committee on Animal Research.

Consent of publication: All authors have read and agreed to the manuscript publication

Availability of data and materials:

Conflicts of Interest:

The authors declare no conflict of interest.

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Figures

Figure 1

The experimental protocol All mice were immunized twice intraperitoneally on days 1 and 14 with *Dermatophagoides farinae* (Df) precipitated in aluminum hydroxide (black circle), then challenged with Df allergen (crude extract of the mite) intranasally on days 14, 16 and 18 (standard down arrow). Following that, 5×10^6 of Af conidia were administered intranasally on days 19, 21 and 23 (down arrow head). Various drugs were administered from on day 21 to 25 and six groups of mice were prepared as follows. DfAf, no drug was administered; DfAf/Dex, 0.02 mg of Dexamethasone (Sigma, St Louis, Mo.; shown as D) were injected subcutaneously; DfAf/PRN, 0.5 mg of pranlukast hydrate (Onon®, ONO Pharmaceutical Co., Osaka, Japan; shown as P) were injected subcutaneously; DfAf/CAM, 4mg of clarithromycin (Clarith®, Taisho Toyama, Tokyo, Japan; shown as C) were orally administered; DfAf/Df/PRN, combination therapy with Dexamethasone and pranlukast hydrate; DfAf/Dex/CAM, combination therapy with Dexamethasone and clarithromycin; On day 26, all mice were sacrificed (white star).

Figure 2

Some examples of pulmonary pathology observed in the study. DfAf mice had severe airway inflammation, and this inflammation was suppressed by combination of Dex and CAM.

Figure 3

The cytokine concentrations in homogenized lung of the groups. The concentrations of IL-5 and IL-13 (Th2 cytokines) and that of MIP-2 (murine cytokine corresponding to human IL-8) were significantly elevated in DfAf mice compared to control mice ($p < 0.05$, each, †: $p < 0.05$). Dex or PRN did not suppress the measured cytokine levels. CAM suppressed the elevation of MIP-2 solely ($p < 0.05$, DfAf/CAM group vs DfAf group, ‡: $p < 0.05$). Interestingly, the combination of Dex and CAM suppressed both of the MIP-2 elevation and the IL-5 elevation ($p < 0.05$, each DfAf/Dex/CAM group vs DfAf/Dex group, §: $p < 0.05$). None of Dex, PRN, or CAM affected the IL-13 concentration.