Effects of the Immunomodulatory Agent *Cordyceps militaris* on Airway Inflammation in a Mouse Asthma Model

Chia-Hsiu Hsu[1,4], Hai-Lun Sun[1,2], Ji-Nan Sheu[1,2], Min-Sho Ku[1], Chun-Ming Hu[1], You Chan[3], Ko-Huang Lue[1,2]*

[1]*Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, Chung Shan Medical University Hospital, Taichung, Taiwan*
[2]*School of Medicine, Chung Shan Medical University, Taichung, Taiwan*
[3]*Institute of Microbiology, Chung Shan Medical University, Taichung, Taiwan*
[4]*Department of Pediatrics, St. Martin De Porres Hospital, Chia-Yi, Taiwan*

Background: *Cordyceps militaris* is a well-known fungus with immunomodulatory activity. It is generally used in traditional Chinese medicine to treat hemoptysis, bronchial or lung inflammation, and urogenital disorders. The purpose of our study was to evaluate the effect of cultivated *C. militaris* on airway inflammation in a mouse asthma model.
Methods: BALB/c mice were sensitized with intraperitoneal ovalbumin (OVA) on Days 0 and 14, and were then given intranasal OVA on Day 14 and Days 25–27. Randomized treatment groups of sensitized mice were administered *C. militaris*, prednisolone, montelukast, or placebo by gavage from Days 15–27. Airway hyperreactivity to aerosolized methacholine was determined. Bronchoalveolar lavage fluid and serum were analyzed to assess airway inflammation.
Results: OVA-sensitized mice developed a significant airway inflammatory response that was inhibited by prednisolone and montelukast, whilst *C. militaris* reduced airway inflammation less effectively. Airway hyperresponsiveness to methacholine was observed in OVA-sensitized mice and was reversed by both prednisolone and montelukast. *C. militaris* initially reversed airway hyperreactivity, but this effect disappeared at higher methacholine doses.
Conclusion: *C. militaris* can modulate airway inflammation in asthma, but it is less effective than prednisolone or montelukast. These results demonstrate that *C. militaris* is unable to adequately block the potent mediators of asthmatic airway inflammation.

1. Introduction

Asthma is a complex disease that affects millions of people and its incidence has increased substantially over the last two decades. Asthma is initially characterized by airway hyperresponsiveness (AHR) to a variety of specific or nonspecific provocative stimuli,[1] by mucus hypersecretion, pulmonary

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*Corresponding author. Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, Chung Shan Medical University Hospital, 110 Section 1, Chien-Kuo North Road, Taichung 402, Taiwan.
E-mail: cshy095@csh.org.tw

eosinophilia, airway edema, and elevated serum immunoglobulin E (IgE) levels.[2] Persistent allergen stimulation induces chronic airway inflammatory changes, which are usually accompanied by airway remodeling.[3,4] Chronic asthma is thus difficult to control using inhaled β_2 agonists alone.[5–7] These airway changes, however, can be found in both newly diagnosed and chronic asthma patients.[4]

Cysteinyl leukotrienes (CysLTs) are now recognized as major mediators involved in the pathogenic changes of airway inflammation. They exert most of their bronchoconstrictive and proinflammatory effects through activation of a putative, 7-transmembrane domain, G-protein-coupled CysLT$_1$ receptor.[8] CysLT receptors are distributed diffusely in human lung smooth muscle cells, interstitial macrophages, precursor granulocytes, mast cells, basophils, eosinophils, and monocytes. Thus, CysLTs can augment inflammatory cell recruitment into the asthmatic airway through autocrine and paracrine mechanisms.[8,9] Corticosteroids exert well documented, anti-inflammatory effects by which they decrease the influx of eosinophils and other inflammatory cells into the blood, airways, and interstitial lung tissue.[10,11] The administration of corticosteroids during or after an allergen challenge blocks the airway inflammatory response.[10,12,13] To date, corticosteroids, the leukotriene receptor antagonist, montelukast, and β2 agonists are therefore the major medicines used in clinical practice for the treatment of asthma. Nevertheless, no satisfactory therapy is available for long-term asthma control.

Cordyceps militaris (also called north winter worm summer grass) is a major parasitic fungus that grows on the larvae of *Lepidoptera*.[14–16] It is one of the well known fungi used in traditional Chinese medicine for the treatment of asthma, and bronchial and lung inflammation.[17] It has multiple therapeutic functions including anti-tumor, anti-metastatic, immunomodulatory, and antioxidant effects.[18] Owing to the rarity of wild *C. sinensis* (also called winter worm summer grass), large amounts of cultivated *C. militaris* are manufactured by fermentation technology. Based on the similar compositions and bioactive effects of *C. militaris* and *C. sinensis*,[14,18–21] *C. sinensis* is gradually being replaced by *C. militaris* in the marketplace. However, the pharmacological activity and the effect of *C. militaris* on asthma remain unclear.

The purpose of the present study was to compare the effects of *C. militaris* with the corticosteroid, prednisolone, and the leukotriene receptor antagonist, montelukast, on systemic IgE production, airway inflammation and pulmonary function in a mouse asthma model. In the present *in vivo* study, the therapeutic agents were all administered by gavage, which is rarely used in such studies.[11] This method of administration may more closely approximate the physiological conditions encountered in daily practice.

2. Methods

2.1. Allergen immunization/challenge protocol

All animal procedures were approved by the Ethic and Animal Care Committee of Chung-Shan Medical University. Female BALB/c mice, 6–8 weeks of age, were obtained from the National Laboratory Animal Center in Taiwan (six mice/group). The mice were maintained on ovalbumin (OVA)-free diets.

The mice were immunized by intraperitoneal (i.p.) injections of 100 μg OVA (0.2 mL of 0.5 mg/mL) (grade V; Sigma Chem. Co., St Louis, USA) complexed with alum (Sigma Chem. Co.) on Days 0 and 14.[22,23] In the age and sex-matched control group, unsensitized mice received an i.p. injection of 0.2 mL saline complexed with alum. Intranasal (i.n.) OVA challenge was first given at a dose of 100 μg (0.05 mL of 2 mg/mL) on Day 14. The i.n. OVA challenges were then repeated on Days 25, 26, and 27 using a dose of 50 μg (0.05 mL of 1 mg/mL). In the control group, unsensitized mice received 50 μL of normal saline i.n. on Day 14 and Days 25, 26, and 27. Normal saline was used as a negative control.

2.2. Preparation and extraction of the fruiting bodies of *C. militaris*

The dry fruiting bodies of *C. militaris* were harvested from the larvae of *Bombyx mori L.* by a fermentation process.[20] One hundred grams of *C. militaris* fruiting body powder (obtained from the Microbiology and Immunology Department of Chung-Shan Medical University) were added to 1 L deionized water at 100°C for 30 minutes.[24] The solid residue was dissolved and filtered using Waterman No. 3 filter paper. A concentration of 100 mg/mL crude extract was then stored at 4°C until use.

2.3. Therapeutic intervention with a corticosteroid, a leukotriene receptor antagonist, and *C. militaris*

On Days 15–27, randomized treatment groups were fed different agents by gavage. An OVA-treated group was fed with the leukotriene receptor antagonist, montelukast (0.8 mg/kg/day, dissolved in apple juice; Merck and Co., Inc., Northumberland, UK).[9,12,25]

Another OVA-treated group was fed with cultivated *C. militaris* fluid (4 g/kg/day). This dosage was previously reported to inhibit airway inflammation.[17,26] The positive control group was fed with prednisolone sodium phosphate solution (3 mg/kg/day; Central Laboratories, Inc., Hsinchu County, Taiwan).[11] Another group of unsensitized mice and a group of OVA-treated mice were fed apple juice as a placebo.

2.4. Determination of airway responsiveness using noninvasive pulmonary function testing

On Day 28, 24 hours after the last i.n. challenge with either normal saline or OVA, pulmonary mechanics were determined noninvasively using whole-body plethysmography (Model PLY 3211; Buxco electronics, Inc., Sharon, CT, USA).[27] AHR to aerosolized methacholine was determined in conscious, freely moving, spontaneously breathing mice. Different concentrations of aerosolized methacholine (0, 5, 10, 20 mg/mL) were used. The degree of bronchoconstriction was expressed as the enhanced pause (Penh), a calculated dimensionless value that correlates with measurements of airway resistance, impedance, and intrapleural pressure.

2.5. Immunoassay for specific serum antibody levels

The mice were sacrificed after plethysmography and each mouse was exsanguinated by cardiac puncture. Blood was centrifuged at 6000 rpm for 5 minutes at 4°C to separate the serum. An indirect enzyme-linked immunosorbent assay (ELISA) (Alpha Diagnostics Intl., Inc., San Antonio, TX, USA) was used to determine serum IgE antibody titers. Optical density (OD) readings of the samples were obtained by measuring the absorbance at 450 nm using an ELISA reader. The antibody titers of the samples were related to pooled standards generated in the laboratory and expressed as ng/mL.

2.6. Acquisition of bronchoalveolar lavage fluid (BALF) and quantitation of BALF cells

Bronchoalveolar lavage (BAL) was performed after exsanguination. The right lung was separated by ligation at the right mainstem bronchus and BAL of the left lung was performed. The BALF was centrifuged at 1000 g for 5 minutes at 4°C. The supernatant was then removed and frozen at −80°C for subsequent testing. The pellet was resuspended and 10 μL of the resuspended BALF was stained with 0.06% methylene blue to determine the total leukocyte counts using a hemocytometer. Then, 200 μL of diluted BALF cell suspension was aliquoted into the cytocentrifuge's single chamber cytospin device (Rotofix 32; Andreas Hettich GmbH & Co. KG., Tuttlingen, Germany) and the samples were centrifuged at 500 rpm for 4 minutes at room temperature. Slides were stained with hematoxylin and eosin for eosinophil counts. Differential counts were performed in a blinded fashion by counting at least 300 cells under a light microscope.

2.7. Lung histology

After BAL, the trachea and right lung were fixed in formalin at room temperature for about 15 hours. The right lung tissue was then embedded in paraffin and cut into 5 μm sections. The lung sections were stained with hematoxylin and eosin to determine the degree of airway inflammatory cell infiltration and smooth muscle thickness. Between five and eight fields on each slide were randomly examined to evaluate morphometric variation.

2.8. Statistical analyses

The data are reported as the mean±SEMs of the combined experiments. The results of the different groups were compared using analysis of variance (ANOVA) with the protected least significant difference method. Differences between groups were considered statistically significant when p values were < 0.05.

3. Results

3.1. Serum IgE production

Serum IgE levels were significantly elevated in the OVA sensitized/challenged group (1833.52± 402.03 ng/mL) compared with the saline-treated control group (121.61±10.48 ng/mL, $p < 0.001$; Figure 1). In OVA-treated mice given prednisolone (574.03± 120.71 ng/mL, $p = 0.001$ *vs.* sensitized group) or montelukast (284.46±45.41 ng/mL, $p < 0.001$ *vs.* sensitized group) during repeated OVA challenges, serum IgE release was significantly decreased in both groups. In OVA-treated mice given *C. militaris* (1591.35±350.68 ng/mL, $p = 0.49$ *vs.* sensitized group), the serum IgE level was reduced by only 13.2%, which was not significantly different from the sensitized control group.

3.2. Airway inflammatory cell infiltration

Airway inflammatory cells markedly infiltrated into the BALF in the OVA-sensitized/challenged group (11.83±2.20×10⁵/mL) compared to the
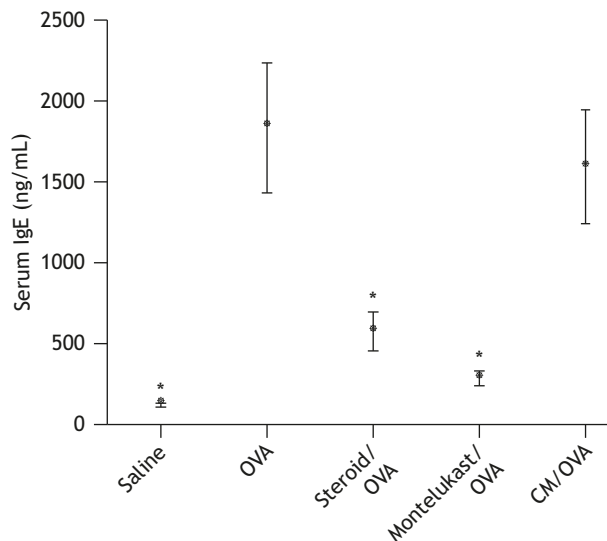
Figure 1 Effect of treatment with *C. militaris*, a CysLTs receptor antagonist and a corticosteroid on serum IgE levels in OVA-treated mice. Serum IgE levels were determined in the saline-treated group and the OVA-sensitized/challenged group with and without treatment with prednisolone (3 mg/kg), montelukast (0.8 mg/kg) or *C. militaris* (CM, 4 g/kg). The results are expressed as means± SEM. $n=6$ in each group. *$p<0.05$, compared with the OVA-treated group (ANOVA).
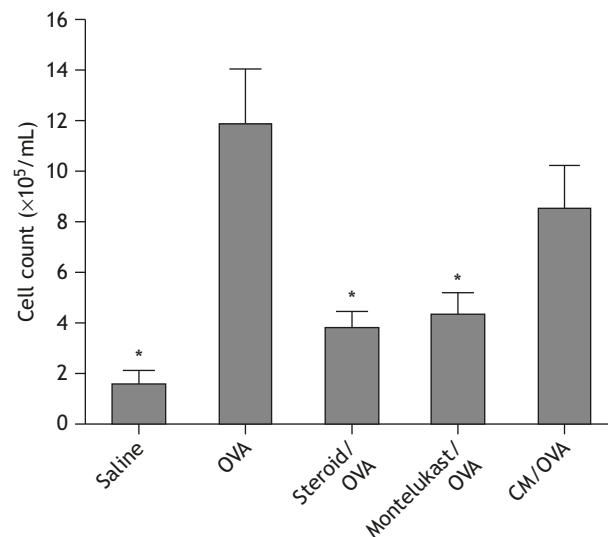

Figure 2 Effect of treatment with *C. militaris*, a CysLTs receptor antagonist and a corticosteroid on inflammatory cell infiltration in the BALF of OVA-treated mice. BALF was obtained from the saline-treated group and the OVA-sensitized/challenged group with and without treatment with prednisolone (3 mg/kg), montelukast (0.8 mg/kg) or *C. militaris* (CM, 4 g/kg). The total cell counts ($\times 10^5$) per mL of BALF were determined based on morphometric evaluation of cytospin preparations. *C. militaris* treatment had no significant effect on decreasing total BALF cell counts. The results are expressed as means±SEM. $n=6$ in each group. *$p<0.05$, compared with the OVA-treated group (ANOVA).

saline-treated control group ($1.58\pm0.55\times10^5$/mL, $p<0.001$; Figure 2). Both prednisolone ($3.83\pm0.60\times 10^5$/mL, $p<0.001$ *vs.* sensitized group) and montelukast ($4.33\pm0.85\times10^5$/mL, $p=0.001$ *vs.* sensitized group) given to OVA-treated mice during repeated OVA challenges significantly reduced the infiltration of airway inflammatory cells into the BALF. In contrast, the effect of *C. militaris* on the reduction of BALF inflammatory cells ($8.50\pm1.71\times10^5$/mL) was not significantly different from that seen in the control OVA-treated group ($p=0.094$). Airway inflammatory cells in BALF were only reduced by 28% by *C. militaris*.

3.3. Bronchoalveolar lavage fluid eosinophilia

The percentage of BALF eosinophils was significantly greater in the OVA-treated group ($50.83\%\pm 4.35\%$) than in the normal saline-treated group ($0.89\%\pm0.17\%$, $p<0.001$; Figure 3). Both prednisolone ($22.94\%\pm8.90\%$, $p=0.002$ *vs.* sensitized group) and montelukast ($16.28\%\pm6.52\%$, $p<0.001$ *vs.* sensitized group) significantly reduced eosinophil influx into BALF in OVA-treated mice. The administration of *C. militaris* ($44.0\%\pm4.89\%$, $p=0.41$ *vs.* sensitized group) during repeated OVA challenges caused no significant reduction in the percentage of eosinophils in the BALF.

3.4. Noninvasive *in vivo* plethysmography

Airway reactivity to aerosolized methacholine was evaluated using noninvasive plethysmography on Day 28, 24 hours after the last intranasal challenge with OVA or saline (Figure 4). Penh (% of air) was significantly increased in the OVA-treated group compared with saline controls after challenge with methacholine 5 mg/mL ($p<0.001$, OVA *vs.* saline), 10 mg/mL ($p=0.003$, OVA *vs.* saline), and 20 mg/mL ($p=0.004$, OVA *vs.* saline). Penh was significantly reduced in the prednisolone/OVA group after methacholine challenge at 5 mg/mL ($p=0.004$, OVA *vs.* prednisolone/OVA), 10 mg/mL ($p=0.009$, OVA *vs.* prednisolone/OVA), and 20 mg/mL ($p=0.014$, OVA *vs.* prednisolone/OVA). Similar results were observed in the montelukast/OVA group; Penh was significantly reduced after methacholine challenge at 5 mg/mL ($p=0.003$, OVA *vs.* montelukast/OVA), 10 mg/mL ($p=0.02$, OVA *vs.* montelukast/OVA), and 20 mg/mL ($p=0.012$, OVA *vs.* montelukast/OVA). In the *C. militaris*/OVA group, Penh was significantly reduced after methacholine challenge at 5 mg/mL ($p=0.025$, OVA *vs. C. militaris*/OVA) and 10 mg/mL ($p=0.031$, OVA *vs. C. militaris*/OVA). However, after higher doses (20 mg/mL) of methacholine, *C. militaris*
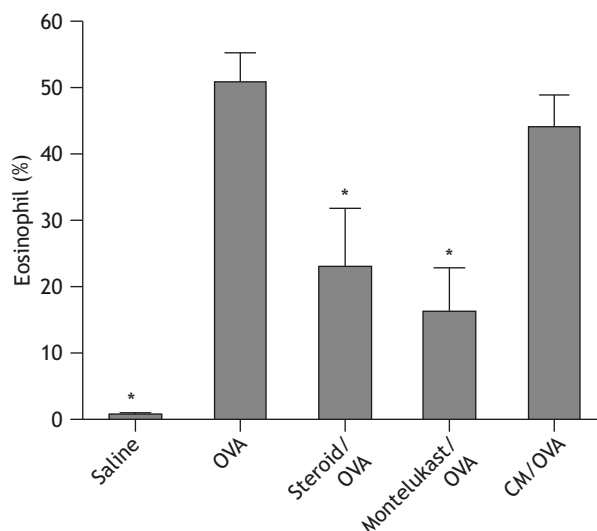
Figure 3 Effect of treatment with *C. militaris*, a CysLTs receptor antagonist and a corticosteroid on the percentage of eosinophil infiltration in the BALF of OVA-treated mice. The percentages of eosinophil (%) infiltration in the BALF were determined based on morphometric evaluation of cytospin preparations. *C. militaris* treatment had no significant effect on reducing the percentages of eosinophil (%) infiltration. The results are expressed as means±SEM. $n=6$ in each group. *$p<0.05$, compared with the OVA-treated group (ANOVA).

Figure 4 Effect of treatment with *C. militaris*, a CysLTs receptor antagonist and a corticosteroid on the pulmonary function response to aerosolized methacholine. The degree of bronchoconstriction (expressed as Penh [% of air]) to aerosolized methacholine (0, 5, 10 and 20 mg/mL) was determined on Day 28 in saline-treated mice and OVA-treated mice in the absence or presence of prednisolone, montelukast or *C. militaris* treatment. The results are expressed as means±SEM. $n=6$ in each group. *$p<0.05$, compared with all other groups; **$p<0.05$, compared with the OVA-treated group (ANOVA).

had no significant effect on the airway responses to methacholine in the OVA-sensitized/challenged group (Figure 4).

3.5. Airway inflammatory changes demonstrated by histology

Cross-sections of the right lung obtained from OVA-treated and saline-treated control groups were examined using light microscopy to assess inflammatory cell and smooth muscle cell hyperplasia in the airways (Figure 5). No evidence of inflammatory cell infiltration or airway smooth muscle thickening was observed in controls. In contrast, morphologic evidence of widespread inflammatory cell infiltration and airway smooth muscle thickening was seen in the OVA-treated group (Figure 5B). In the airways of OVA-sensitized/challenged mice, inflammatory cell infiltration was effectively blocked by both prednisolone and montelukast treatment (Figures 5C and 5D). Furthermore, both prednisolone and montelukast reversed the thickened airway smooth muscle in the OVA sensitized/challenged group (Figures 5C and 5D) shown by hematoxylin and eosin staining. However, *C. militaris* only partially blocked the development of airway inflammatory cell infiltration and smooth muscle layer thickening in the OVA-treated group (Figure 5E).
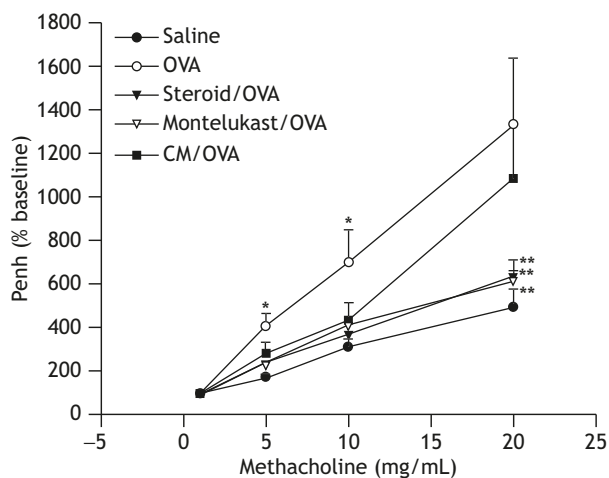
4. Discussion

Cultivated *C. militaris* is now widely used in Asia as a substitute for wild *C. sinensis* because of its similar bioactive effects and most importantly, the facilities for its mass production in a laboratory. However, the use of different species of *Cordyceps* with different proportions of components results in variable effects of this traditional Chinese medicine when used for asthma treatment.[16,17,26,28] An allergen-induced, mouse airway inflammatory model was used to study the effects of *C. militaris* on pulmonary inflammation. *C. militaris* was compared with the potent inflammatory inhibitor, prednisolone, and the leukotriene receptor antagonist, montelukast, as CysLTs are now recognized to be the most potent mediators involved in promoting airway inflammation, remodeling and hyperreactivity to methacholine exposure.

Mice can develop IgE-mediated allergic responses. In addition to elevated serum IgE levels in OVA-sensitized/challenged mice, AHR to methacholine and a late-phase influx of eosinophils were also noted in this mouse asthma model. Eosinophils modulate the function of mesenchymal cells, including fibroblasts and myofibroblasts, thus changing the composition of the airway wall matrix.[29] The development of allergic airway disease with consequent structural changes driven by IgE or
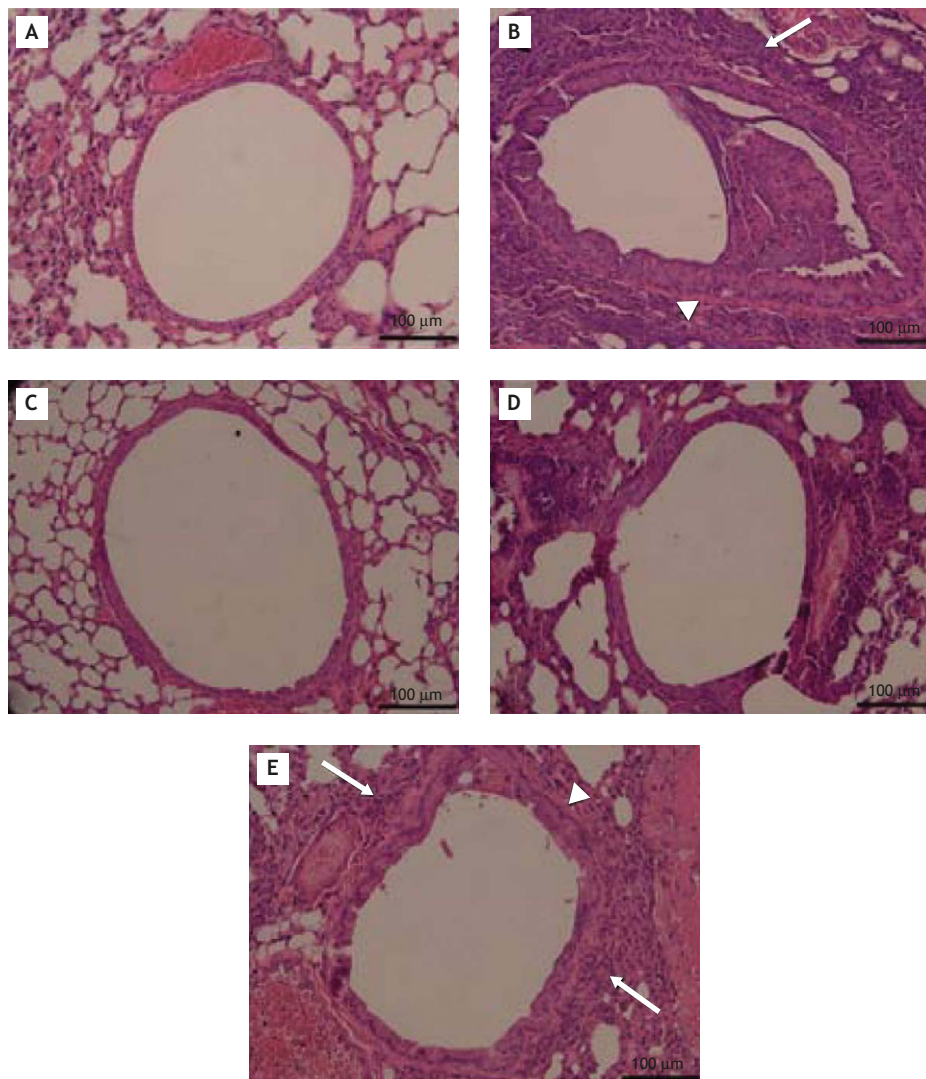
Figure 5 Effect of treatment with *C. militaris*, a CysLT receptor antagonist and a corticosteroid on allergen-induced airway inflammation. Lung tissue was obtained on Day 28 from saline controls (A) and OVA-treated mice in the absence (B) or presence of prednisolone (C), montelukast (D) or *C. militaris* (E); the tissues were stained with hematoxylin and eosin. (A) There is a scarcity of inflammatory cells around the airway in saline-treated controls; no airway smooth muscle thickening is observed. (B) Large numbers of inflammatory cells (arrow) are seen near the peribronchial tissue of OVA-treated mice; thickening of the airway smooth muscle layer (arrowhead) is also noted in this group. (C) Massive inflammatory cell infiltration and hyperplasia of the airway smooth muscle layer were markedly reduced when prednisolone was given during repeated OVA challenges. (D) Inflammatory cell infiltration and hyperplasia of the airway smooth muscle layer were also markedly reduced by montelukast during repeated OVA challenges. (E) Inflammatory cell infiltration (arrow) and hyperplasia of the airway smooth muscle layer (arrowhead) were partially reversed when *C. militaris* was given during repeated OVA challenges.

other Th2 cytokines can then follow. Levels of serum IgE, AHR and airway inflammatory cells, including eosinophils, can be suppressed by the use of corticosteroids and montelukast during allergen challenge. These results suggest that CysLTs are the major mediators leading the process of airway inflammation.

Here, *C. militaris* treatment was less effective than corticosteroids or montelukast in inhibiting serum IgE levels, eosinophil infiltration and AHR. Unlike the potent anti-inflammatory effects that

corticosteroids exert on most cells, *Cordyceps* mainly acts by inhibiting phagocytosis and chemotaxis of neutrophils and natural killer cells.[26,28] However, the characteristic of allergic airway inflammation is eosinophil recruitment. Eosinophil is not the main cell upon which *Cordyceps* can act. *Cordyceps* is also unable to inhibit the production of leukotrienes,[26] which are potent mediators of bronchoconstriction and airway inflammation.[30,31] This may explain the limited reversal of airway hyperresponsiveness in sensitized mice and the need

for higher *C. militaris* doses to suppress the hyperreactive airway.[17] Recent studies have suggested that the therapeutic activity of *Cordyceps* in asthma may be related to its content of immunomodulatory agents. These immunomodulatory agents may enhance a Th1 cell-mediated immune response, so reducing the Th2 cell-mediated immune response.[16,17] The prominent cytokine associated with the Th1 cell immune response is interferon-γ, which enhances IgG2a production and inhibits IgG1 and IgE production in B lymphocytes. Nevertheless, limited enhancement of IgG2a but no significant inhibition of IgG1 and IgE were noted in the present study (data not shown). Different species of *Cordyceps* and different culture conditions may contribute to such discrepancies. In previous studies, only specific fractions of *Cordyceps* resulting from the extraction process have been examined for immunomodulatory effects.[16,28,32,33] In contrast, our study used whole *C. militaris* extract, as do most people who use it as a health food product. This may be responsible for the lower efficacy of *C. militaris* in reducing airway inflammation.

In summary, our results indicate that a crude extract of *C. militaris* was unable to adequately inhibit asthmatic airway inflammation. *C. militaris* was not as effective as prednisolone or montelukast in reversing AHR; neither did *C. militaris* block the potent bronchoconstriction mediators–leukotrienes. *C. militaris* is therefore only suitable for use as an auxiliary agent in asthma control. The isolation of pure bioactive factors from *C. militaris* is required in order to help determine the mechanisms of *C. militaris* action on the asthmatic airway.

Acknowledgments

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