



Astragalus Membranaceus prevents airway hyperreactivity in mice related to Th2 response inhibition

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Abstract

Aim of the Study: Asthma is recognized as a common pulmonary disease throughout the world. To date, there has been a growing interest in herbal products in Traditional Chinese Medicine, which is considered to be effective to treat asthma. A Chinese herb *Astragalus Membranaceus* (AM) was found useful in treating allergic diseases. The purpose of this study is to determine whether this herbal injection could suppress allergic-induced AHR and mucus hypersecretion in allergic mice.

Materials and Methods: A mouse model of chronic asthma was used to investigate AM injection on the airway lesions in compared with glucocorticoids. The study was conducted on mice sensitized and challenged with ovalbumin and the whole body plethysmography was performed to assess AHR. The bronchoalveolar lavage (BAL), histopathology were examined.

Results: We found 28-day AM administration significantly decreased inflammatory infiltration and mucus secretion in the lung tissues of allergic mice. 28-day AM administration enhanced Ova-induced decreased IFN- γ , and the Ova-induced elevations of IL-5 and IL-13 in BALF were prevented by 28-day injection. We also showed 28-day AM injection markedly suppressed increased AHR in allergic mice.

Conclusions: Our results indicate *Astragalus Membranaceus* has a potential role in treating allergic asthma.

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Keywords: *Astragalus Membranaceus*; Asthma; IL-5; IFN- γ

1. Introduction

Asthma is recognized as a common pulmonary disease and a serious public problem throughout the world. In the last two decades, asthma morbidity and mortality have been increased (Masoli, 2004). Airway hyperreactivity (AHR), chronic pulmonary inflammation with infiltration of eosinophils in lung, and mucus hypersecretion in the airways are hallmarks of allergic asthma (Ray, 1999; Wills-Karp, 1999; Adamko, 2003). The onset and progression of allergic asthma is accompanied by a complex series of overlapping and concurrent inflammatory responses in the lung orchestrated by CD4+ Th2 lymphocytes (Gavett, 1994). These responses include T cell mediated help of antigen-specific immunoglobulin production particularly

IgG1 and IgE by B cells, expression of Th2 proinflammatory cytokines (e.g., IL-4, -5, -9, and -13) (Jenmalm, 1999; Kon, 1999). Among Th2 cytokines, IL-5 promotes differentiation and migration of eosinophils (Foster, 1996). IL-4 and IL-13 have been implicated in goblet cell hyperplasia, the development of AHR, and lung remodelling (Wills-Karp, 1998; Larche, 2003).

The treatment of asthma has been improved by the implementation of management guideline in recent years, with further development in the study for the mechanism of asthma. Inhaled corticosteroids and β 2-agonists are used as the first line of treatment of asthma, reducing airway inflammation and bronchial constriction effectively. Many other medicines such as theophylline, anticholinergics, leukotriene modifiers etc. have also been used to control asthma. However, the effects of those drugs are not satisfied in the clinical practice, because of local or systemic side effects (Sally, 2005). Therefore, there is a need for new or alternative approaches to the control of asthma. To

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date, there has been a growing interest in alternative medicine, particularly herbal products in Traditional Chinese Medicine (TCM), which is much more appropriate to the prevention and treatment of disease in chronic condition such as asthma (Yu, 2006).

Astragalus Membranaceus (AM) is a traditional Chinese herb which has been used for more than 2000 years in China. Physician in TCM system used AM that has been manufactured into injection to boost the body's general vitality and strengthening resistance to exogenous pathogens, which was regarded as a potent tonic for increasing energy levels and stimulating the immune system (Block, 2003). Scientific investigation in the last two decades has revealed much insight into the pharmacological functions of AM (Bedir, 2000; Mckenna, 2002). Several studies focused on its immunomodulatory properties, and AM was shown to increase human lymphocyte proliferation, cytotoxic T cell responses and IgG production (Wang, 2002). It stimulated murine splenocytes to proliferate and macrophages to produce proinflammatory cytokines (Lau, 1994; Yoshida, 1997). Recently, AM in combination with other herbs, was used in treating allergic rhinitis, asthma and atopic dermatitis (Suzuki, 1999; Ishimitsu, 2001; Cui, 2003). AM has recently become popular with Western herbalist and alternative health care providers. However, the role of AM in asthma therapy in Western medicine is still uncertain. More study is needed to investigate the action and mechanism of this herbal medicine.

The purpose of this study is to determine whether AM could suppress allergic-induced AHR and mucus hypersecretion in allergic mice. We employed a mouse model of chronic asthma, which replicates many features of the human disease, to investigate inhibitory effect of AM injection on the airway lesions compared with glucocorticoids. We found AM injection significantly inhibited the enhanced AHR and mucus hypersecretion, with blockade of eosinophil recruitment in airway. Elevated IL-5, IL-13 in BALF in asthmatic mice were markedly decreased while increased IFN- γ was observed. Our results indicate that AM have a potential role in treating exacerbations of asthma triggered by allergen.

2. Materials and methods

2.1. Preparation of AM extract

The aqueous extract of AM was prepared as follows. Fifty grams of powdered *Astragalus radix* were placed in 600 ml water, and boiled for 1 h. Then filtered, and the decoction was diluted in distill water to concentration of 1 g/ml. (each ml of solution contained 1 g crude drug of *Astragalus Membranaceus*). The extract and the voucher specimens of the herbs were kept at Chitai Qingchunbao Pharmaceutical Co. Ltd. (Hangzhou China).

2.2. Mice

Eight to ten weeks old male *C57BL/6* mice weighing 20–25 g were purchased from the Laboratory Animal Center of the Medical School of Zhejiang University (certificate no. 220010342,

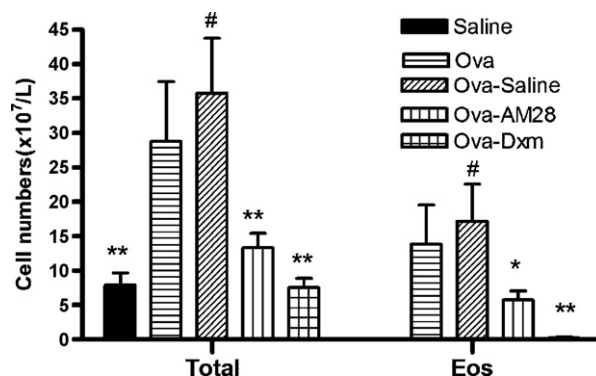


Fig. 1. Effect of AM injection on leukocytes in BALF of allergic mice. BALF was harvested on 48 h after last Ova challenge. Data are expressed as means $\pm$ S.E.M., $n=6$. ** $P<0.01$ vs. Ova; * $P<0.05$ vs. Ova; # $P>0.05$ vs. Ova. Saline: mice were sensitized and challenged with saline. Ova: mice were sensitized and challenged with Ova. Ova-AM28: Ova sensitized and challenged mice were treated with AM injection for 28 days at 10 g/kg per day. Ova-Saline: Ova sensitized and challenged mice were treated with Saline for 28 days. Ova-Dxm: Ova sensitized and challenged mice were treated with dexamethasone at 0.5 mg/kg 1 h before challenge.

conferred by the Zhejiang Medical Laboratory Animal Administration Committee). The present study was performed according to the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985). Fifty mice were divided into five groups, i.e., Ova group: mice were sensitized on the day 0 and day 14 by ip injection of ovalbumin (Ova) (Sigma-Aldrich, St. Louis, MO, USA) mixed with aluminum. At day 24, 25, and 26, mice were challenged by aerosolized 1% Ova in saline for 40 min. Saline group: mice were sensitized and challenged with saline. Ova-Saline group: mice sensitized and challenged as Ova group were administered with saline intraperitoneally for 28 days after first sensitization. Ova-AM28 group: mice sensitized and challenged as Ova group were administered with AM injection (Chitai Qingchunbao Pharmaceutical Co. Ltd., Hangzhou China) intraperitoneally for 28 days after first sensitization at dose of 10 g/kg per day. Ova-Dxm group: mice sensitized and challenged as Ova group were administered with dexamethasone at day 24, 25, and 26 before challenge at dose of 0.5 mg/kg.

2.3. Bronchoalveolar lavage fluid (BALF) of the lungs

At day 28, BALF was performed using 1 ml of PBS containing 2% FBS. Total BAL cellularity was determined with the use of a hemocytometer. Cytospin slides were fixed and stained by Wright-Giemsa staining. Differential cell counts by unbiased observers were based on counts of 200–300 cells using standard morphological criteria to classify individual leukocyte populations.

2.4. Airway hyperresponsiveness in response to methacholine

Airway responsiveness to methacholine (MeCh), was assessed by whole body plethysmography (Buxco Electronics,

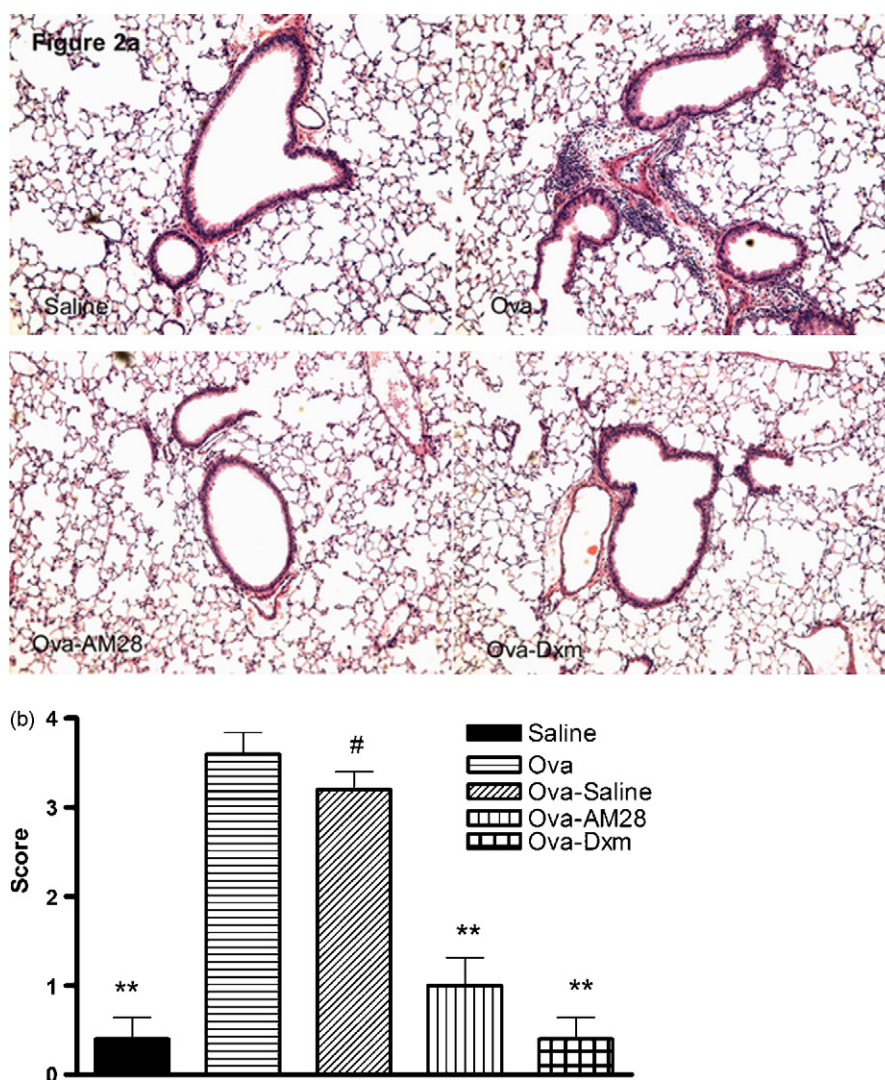


Fig. 2. Effect of AM injection on pulmonary inflammation in allergic mice. Lung tissue sections obtained from mice on 48 h after last Ova challenge were stained with H&E (100 $\times$, Fig. 2a). Semi-quantitative analysis of inflammatory cell infiltration in lung sections were performed as described in Section 2 (Fig. 2b). Data are expressed as means $\pm$ S.E.M., $n=6$. ** $P < 0.01$ vs. Ova, # $P > 0.05$ vs. Ova. Saline: mice were sensitized and challenged with saline. Ova: mice were sensitized and challenged with Ova. Ova-AM28: Ova sensitized and challenged mice were treated with AM injection for 28 days at 10 g/kg per day. Ova-Saline: Ova sensitized and challenged mice were treated with Saline for 28 days. Ova-Dxm: Ova sensitized and challenged mice were treated with dexamethasone at 0.5 mg/kg 1 h before challenge.

Troy, NY). Mice were exposed for 3 min to aerosolized saline and subsequently to increasing concentrations of aerosolized MeCh (0, 3.125, 6.25, 12.5, 25, and 50 in isotonic saline) dissolved in isotonic saline. Following each nebulization, enhanced paused (Penh) were recorded for 3 min. The Penh values measured during each 3-min sequence were averaged and expressed for each dose of MeCh. Penh data were plotted as change from baseline per dose.

2.5. Pulmonary histology

After BAL was performed, lungs were inflated with 1 ml of 10% neutral-buffered formalin via a tracheostomy tube. After instillation of fixative, the trachea was ligated, and the lung was excised and fixed in formalin for 24 h at 4 $^{\circ}$ C. These tissues were then embedded in paraffin, cut in 4 μ m frontal

sections, mounted onto slides, and stained with either hematoxylin and eosin (H&E), periodic acid-Schiff (PAS). Image-Pro Plus software (version 5.0.1, Media Cybernetics, Inc., Silver Spring, MD, USA) was used to analyze histopathology. The severity of inflammatory cell infiltration in lung was evaluated by a 5 point scoring system. Briefly, the scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells >4 cells deep. Scoring of inflammatory cells was performed in at least three different fields for each lung section. The goblet cell hyperplasia ratio (HR) and the epithelial cell mucus occupying ratio (MOR) were used to determine airway mucus: $HR = (\text{the number of the goblet cell}/\text{the length of the airway epithelial}) \times 100$, $MOR = (\text{the area of airway epithelium staining positive for mucus}/\text{the total area of airway epithelium}) \times 100$.

2.6. Cytokine assay

IL-4, IL-5, IL-13 and IFN- γ levels in BALF were determined using the Mouse ELISA kits (R&D, Minneapolis, MN, USA), as the manufacturer's instructions. The sensitivity for each cytokine is 2.0 pg/ml for IL-4, IL-5, IL-13 and IFN- γ .

2.7. Statistical analysis

Data are expressed as mean $\pm$ S.E. SPSS 13.5 software was used for statistical analysis. Differences between groups were tested using a one-way analysis of variance (ANOVA).

3. Results

3.1. Effect of AM injection on total leukocyte and eosinophils in the BALF of allergic mice

To determine whether AM injection influence the inflammatory cells accumulation in airway responding to allergen challenge, BALF was recovered. Total cells of BALF were counted and differentiated using morphological criteria. As shown in Fig. 1, the total number of leukocytes and eosinophils in the Ova group was significantly greater than that in the Saline group (28.7 ± 8.7 vs. 7.8 ± 1.08 $P < 0.01$, $n = 6$). Compared to allergic mice, the mice in AM28 group showed a decreased total number of leukocytes in BALF (13.3 ± 2.0 vs. 28.7 ± 8.7 $P < 0.01$, $n = 6$). The increase of eosinophils was also remarkably reduced in AM28 group (5.8 ± 1.3 vs. 13.8 ± 5.7 $P < 0.05$, $n = 6$), which accounted for the elevation of total number of leukocytes. Dexamethasone exhibited significant suppressive effect on the recruitment of total number of leukocytes (7.5 ± 1.4 vs. 28.7 ± 8.7 $P < 0.01$, $n = 6$) and eosinophils (0.22 ± 0.14 vs. 13.8 ± 5.7 $P < 0.01$, $n = 6$). No significant difference in total number and eosinophils was observed between the Ova group and Ova-Saline group.

3.2. Effect of AM injection on Ova-induced inflammatory infiltration in lung tissue of mice

To examine inhibition of inflammation in lung tissue by AM injection, we observed the pulmonary pathology stained with H&E in mice of all groups (Fig. 2a). Compared with mice treated with saline, the mice exposure to Ova resulted in the development of a characteristic peribronchial and perivascular airway tissue infiltration (Fig. 2a). In comparison with mice in Ova group, the mice administered with 28-day AM exhibited reduced inflammation in the lung tissue obviously (Fig. 2a). The inflammatory infiltration was also decreased in the mice administered with dexamethasone (Fig. 2a). As shown in Fig. 2b, the inflammation scores of peribronchial and perivascular regions were significantly decreased in AM28 and Dxm group, compared to Ova group.

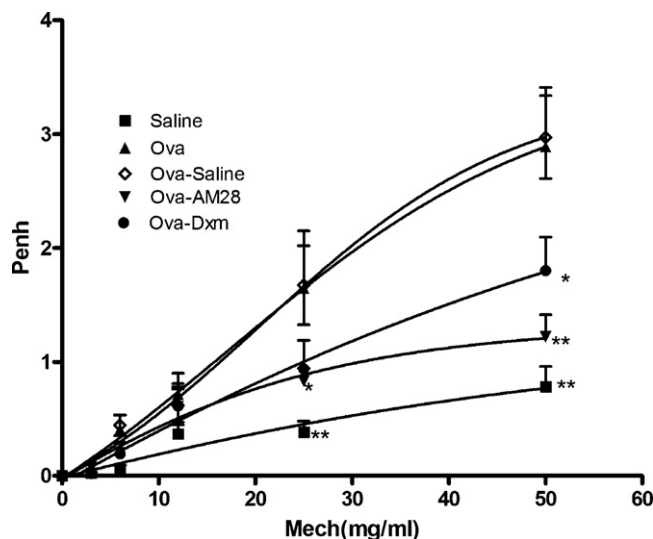


Fig. 3. Effect of AM injection on AHR in allergic mice in response to Mech. Whole body plethysmography was performed on 24 h after last Ova challenge to examine AHR in response to Mech. Data are expressed as means $\pm$ S.E.M., $n = 8$. *** $P < 0.01$ vs. Ova; * $P < 0.05$ vs. Ova. Saline: mice were sensitized and challenged with saline. Ova: mice were sensitized and challenged with Ova. Ova-AM28: Ova sensitized and challenged mice were treated with AM injection for 28 days at 10 g/kg per day. Ova-Saline: Ova sensitized and challenged mice were treated with Saline for 28 days. Ova-Dxm: Ova sensitized and challenged mice were treated with dexamethasone at 0.5 mg/kg 1 h before challenge.

3.3. Effect of AM injection on enhanced AHR in allergic mice in response to methocholine

To determine whether AM injection improves pulmonary function of allergic mice after challenge with Ova, whole body plethysmography was performed to examine the AHR in response to Mech (Fig. 3). Compared with control animals in Saline group, mice in Ova group developed higher Penh in response to Mech at the dose of 25 and 50 mg/ml (1.65 ± 0.5 vs. 0.38 ± 0.10 $P < 0.01$; 2.9 ± 0.52 vs. 0.78 ± 0.18 $P < 0.01$, $n = 8$). Administration of dexamethasone (1.8 ± 0.3 vs. 2.9 ± 0.52 $P < 0.05$) and 28-day AM administration (1.22 ± 0.19 vs. 2.9 ± 0.52 $P < 0.01$, $n = 8$) both significantly prevented the enhancement of Penh in response to Mech at 50 mg/ml. We were surprised to observe that 28-day AM administration resulted in significant decrease of enhanced Penh in allergic mice in response to Mech not only at the dose of 50, but also at 25 mg/ml (0.83 ± 0.11 vs. 1.65 ± 0.5 $P < 0.05$, $n = 8$). However, dexamethasone didn't affect Penh responding to Mech at the dose of 25 mg/ml (0.94 ± 0.25 vs. 1.65 ± 0.5 $P > 0.05$, $n = 8$). There is no difference of Penh between Ova and Ova-Saline group.

3.4. Effect of AM injection on Th1/Th2 cytokines in BALF of mice

To evaluate the effect of AM on the pulmonary immune response in allergic mice, the concentration of IL-4, IL-5, IL-13 and IFN- γ in BALF were measured (Fig. 4). The IL-4, IL-5 and IL-13 level in the Ova group were significantly elevated in comparison to Saline group. Though there was no effect on

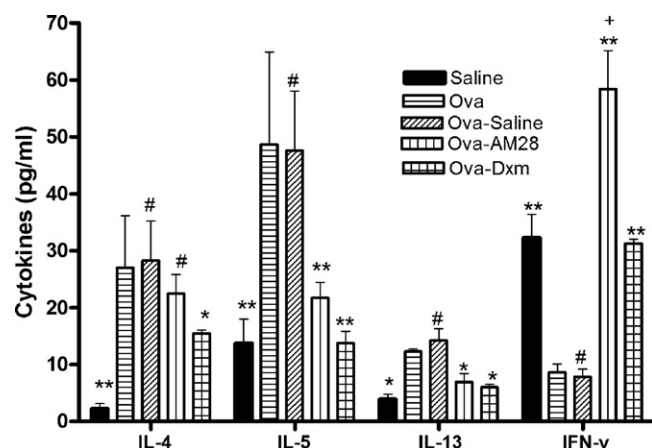


Fig. 4. Effect of AM injection on Th1/Th2 cytokines in allergic mice. BALF was harvested on 48 h after last Ova challenge. IL-4, IL-5, IL-13 and IFN- γ level were analyzed by ELISA. Data are expressed as means $\pm$ S.E.M., $n = 6$. ** $P < 0.01$ vs. Ova; * $P < 0.05$ vs. Ova; # $P > 0.05$ vs. Ova; + $P < 0.05$ vs. Dxm. Saline: mice were sensitized and challenged with saline. Ova: mice were sensitized and challenged with Ova. Ova-AM28: Ova sensitized and challenged mice were treated with AM injection for 28 days at 10 g/kg per day. Ova-Saline: Ova sensitized and challenged mice were treated with Saline for 28 days. Ova-Dxm: Ova sensitized and challenged mice were treated with dexamethasone at 0.5 mg/kg 1 h before challenge.

elevated levels of IL-4, 28-day AM administration reduced the increased IL-5 of BALF in the allergic mice. More inhibitory effect on elevated IL-4 and IL-5 levels was shown in mice of Dxm group. Elevated IL-13 in allergic mice was significantly decreased by 28-day AM administration, which was similar to the effect of dexamethasone. IFN- γ level in Ova group was less than that in the Saline group. Dexamethasone and 28-day AM administration significantly promoted IFN- γ level in the BALF of allergic mice, compared to Ova group. To our surprise, IFN- γ level enhanced by 28-day AM administration was greater than enhanced by dexamethasone. No significant difference of any cytokine was observed between the Ova group and Ova-Saline group.

3.5. Effect of AM injection on mucus overproduction of allergic mice

To demonstrate the inhibitory effect of AM injection on hypersecretion of mucus in airway, PAS stained lung sections were observed to analyze alteration of histopathology in various groups (Fig. 5a). Semi-quantitative analysis of mucus secretion was also performed that HR refers to the percentage of mucus cells hypertrophy, and MOR refers to the percentage of airway mucus. It was revealed that mucus secretion was markedly increased in mice of Ova group with enhanced HR and MOR. Compared to Ova group, no reduced mucus overproduction was observed in Ova-Saline group. As shown in Fig. 5a, AM injection and Dxm displayed suppressive effect on Ova-induced overproduction of mucus. Compared to mice in Ova group the hypersecretion of mucus was remarkably disappeared in the mice of AM28 group with HR and MOR decreased to zero, which is similar to the mice in Dxm group (Fig. 5b).

4. Discussion

In the present study, a mice model of asthma was employed to assess the effect of AM injection on airway inflammatory disease. We found 28-day AM administration significantly decreased the total number of leukocytes and eosinophil in the BALF of allergic mice. Pathology analysis documented 28-day AM administration resulted in the reduction of allergic inflammatory infiltration and mucus secretion in the lung tissues. Twenty-eight-day AM administration enhanced Ova-induced decreased IFN- γ . The Ova-induced elevations of IL-5 and IL-13 in BALF were prevented by 28-day injection. We also showed 28-day AM injection markedly suppressed increased AHR in allergic mice. Collectively, all of these results demonstrated that AM injection is a potent agent in pulmonary inflammatory disease.

Eosinophilia is a prominent pathological feature of allergic diseases, which is thought to contribute to the airways damage through release of a myriad of mediators and cytokines (Gavett, 1994). The infiltration of eosinophils into the airways has been linked to the production of IL-5, which is important for eosinophils proliferation, activation, and migration (Foster, 1996). In the present study, we showed that AM injection inhibited the pulmonary accumulation of eosinophils in mice, which is parallel to the decrease of IL-5 in BALF. Our results suggest that AM injection play a key role in blocking the recruitment of eosinophils in lung through IL-5 dependent pathway as dexamethasone did.

AHR is one of the hallmark features of asthma, which is defined as the abnormal increase in airflow limitation in response to a provoking stimulus. Although the studies to date have not elucidated the precise mechanisms by which Th2 cytokines mediate AHR, they have suggested that either eosinophils, mast cells, or both are likely to play a role in this process (Wills-Karp, 1999). In the present study, we found both dexamethasone and AM injection significantly decreased AHR in response to Mech at 50 mg/ml. However, what surprised us was that AM also inhibited allergen-induced AHR in response to Mech at 25 mg/ml, but dexamethasone did not exhibit inhibitory effect on Pehn at the same point. Moreover, decrease of AHR responding to Mech at 50 mg/ml in AM28 group mice was greater than that in dexamethasone treated mice. To our knowledge, it is the first investigation that AM inhibited AHR in an asthma model of mice was reported.

Mucus is a viscoelastic gel that coats and lubricates the epithelium of mucosal surfaces. Low levels of mucus are produced in the normal lung, and mucus metaplasia with mucus hypersecretion are characteristic features of airways disorders such as asthma. Many evidences support that inflammatory cells infiltration and mucus hypersecretion are associated with Th2 cytokines that regulate immune functions in mice. IL-4 was shown to play a key role in mucus production through the recruitment of Th2 cells to the lungs and induction of inflammation (Steinke, 2004). Blockage of the IL-4 receptor in mice sensitized and challenged with antigen markedly decreased airway goblet cells metaplasia. In the absence of IL-4R alpha in recipient mice, there was no goblet cell metaplasia or mucus hypersecretion in

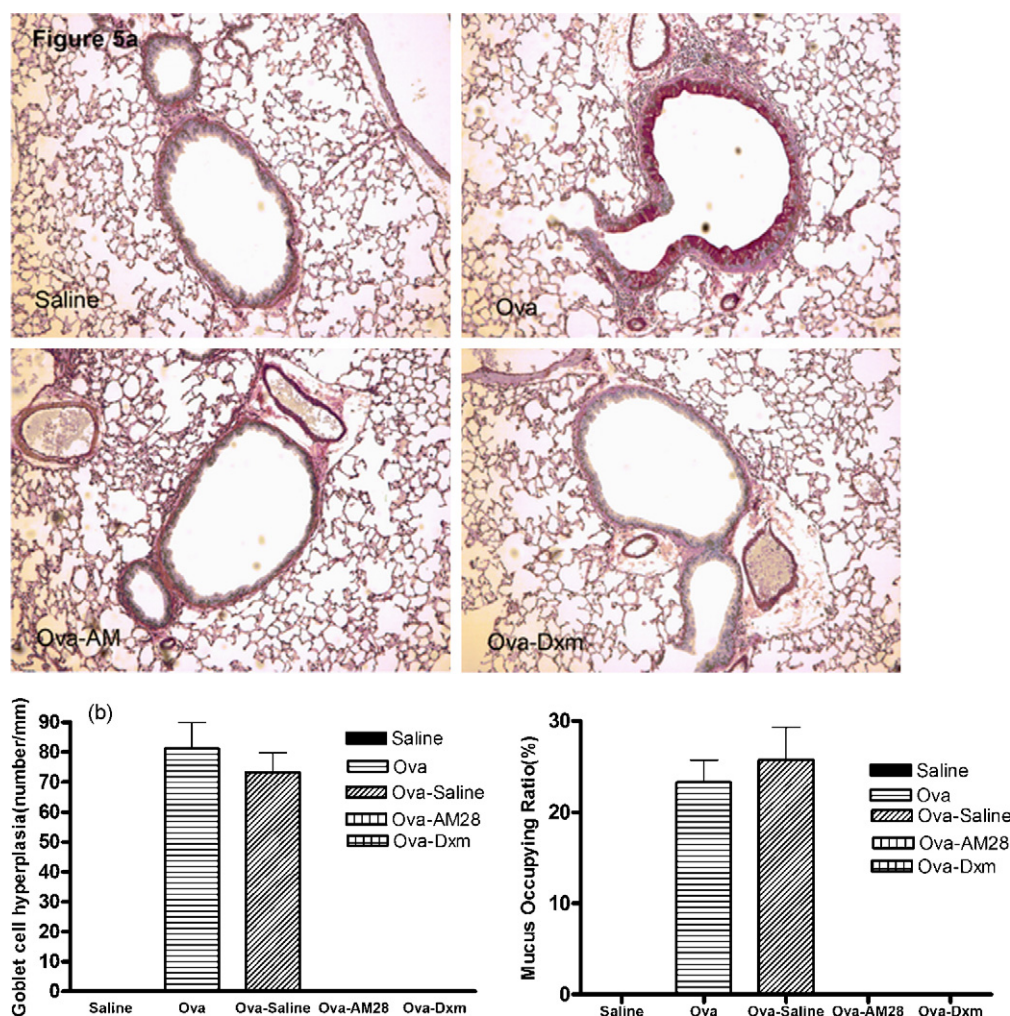


Fig. 5. Effect of AM injection on mucus overproduction in allergic mice. Lung tissue sections obtained from mice on 48 h after last Ova challenge were stained with PAS (100 $\times$, Fig. 5a). The cytoplasmic of cell stained with blue or purplish or purplish red was goblet cell. The goblet cell hyperplasia ratio (HR) and the epithelial cell mucus occupying ratio (MOR) were used to determine airway mucus (Fig. 5b): HR = (the number of the goblet cell/the length of the airway epithelial) $\times$ 100, MOR = (the area of airway epithelium staining positive for mucus/the total area of airway epithelium) $\times$ 100. Saline: mice were sensitized and challenged with saline. Ova: mice were sensitized and challenged with Ova. Ova-AM28: Ova sensitized and challenged mice were treated with AM injection for 28 days at 10 g/kg per day. Ova-Saline: Ova sensitized and challenged mice were treated with Saline for 28 days. Ova-Dxm: Ova sensitized and challenged mice were treated with dexamethasone at 0.5 mg/kg 1 h before challenge.

response to Ova, even in the presence of Th2 cells and substantial eosinophilic infiltration (Kelly-Welch, 2004). But IL-4 was shown to play an indirect role in mucus production through the recruitment of Th2 cells to the lungs and induction of inflammation. Another Th2 cytokine IL-13, was demonstrated to be a central mediator in the mucus obstruction phenotype evidenced in the mouse model of allergic asthma (Wills-Karp, 1998). Pulmonary aspiration of IL-13 alone could cause airway epithelial thickening and goblet cell hyperplasia, as reported earlier with intratracheal challenge (Cohn, 1997; Grunig, 1998). In a transgenic mice model that chronically overexpress IL-13 in their airways, obvious airway inflammation and mucus hyperplasia were observed (Zhu, 1999). Present study showed 28-day AM administration significantly prevented mucus overproduction in allergic mice concomitant with reducing allergen-induced IL-13. Our results suggested AM inhibited mucus overproduction in asthmatic mice dependent on IL-13 reduction.

Allergen-induced allergic inflammation is regulated by the balance between Th1 and Th2 cells, in which Th2 cells up-regulate but Th1 cells down-regulate the allergic inflammation by producing cytokines. There are a few publications on the immunomodulatory effect of AM, and its mechanism is still far from clear. The preferential inhibition on Th1 or Th2 response in allergy by AM also remains controversial. Kang (2004) reported that *in vitro* and *in vivo* treatment of AR increased IL-4 production but decreased IFN- γ , thus favoring the Th2 development pathway. However, in present study we observed decreased IL-5, IL-13, and increased IFN- γ in allergic mice treated with AM. Moreover, IFN- γ level in the AM28 mice was twofold higher than that of saline mice. The elevation of IFN- γ was also twofold higher than that of mice in Dxm group. One of the reason should be noted that the mouse strain used in this study is C57BL/6, which favors Th1 response, while it was BALB/c in Kang's report which tends to develop over-

whelming Th2 responses (Kang, 2004). Our results indicated that AM inhibited Th2 cells preferentially in allergic diseases, which is supported by the following study. Wei (2003) reported that cultured peripheral blood mononuclear cells collected from patients treated with astragalus root produced higher amounts of IFN- γ and IL-2, which are recognized as Th1 cytokines. It was also demonstrated that AR specifically increased the production of IL-2 and IFN- γ as well as the overall cytotoxic activity of lymphocytes from mice treated with a carcinogen called BBN (Kurashige, 1999). Gyokuheifusan of which AM is main component, significantly increased the production of IFN- γ released by Ova-treated splenocytes to a much higher level (Fang, 2005).

In summary, present study identified that a Chinese herb AM could suppress airway inflammation, hyperresponsiveness, and mucus overproduction, which were dependent on inhibition of Th2 cytokine. Our results suggested AM is a potential anti-inflammation agent in asthma treatment.

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