

Protective Effects of *Mentha haplocalyx* Ethanol Extract (MH) in a Mouse Model of Allergic Asthma

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Mentha haplocalyx Briq., a commonly used herb in traditional Oriental medicine, has a variety of known pharmacological properties. However, neither the protective effects of *Mentha haplocalyx* ethanol extract (MH) against inflammation of the airway in an asthmatic model nor the mechanisms involved, have previously been reported. In the present study, an ovalbumin (OVA)-induced mouse model of allergic asthma was used to investigate whether MH was effective against the disease through regulation of airway inflammation. The MH treatment significantly inhibited increases in immunoglobulin (Ig) E and T-helper 2 (Th2)-type cytokines such as IL-4 and IL-5 in bronchoalveolar lavage fluid (BALF) and lung tissue. Inflammatory cell infiltration of the airway in mice treated with MH was effectively alleviated when compared with infiltration seen in the OVA-induced group. These data indicated that decreased cytokine levels are the result of the decreased number of invaded leukocytes. Also, the generation of reactive oxygen species (ROS) in BALF was diminished by MH treatment. Taken together, these findings indicate that the administration of MH may have potential therapeutic value in the treatment of inflammatory disease. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: asthma; *Mentha haplocalyx*; eosinophilia; cytokines; IgE.

INTRODUCTION

Asthma is associated with a wide range of symptoms and effects that include tightness of the chest, wheezing and coughing, increased airway obstruction, and airway hyperresponsiveness (AHR). The body's response to inflammatory conditions such as asthma involves many cell types and cellular mediators (Wenzel, 1994). In 2003, Evans and colleagues reported that murine models of asthma exhibited T-helper 2 (Th2) lymphocyte-driven eosinophilic pulmonary inflammation as well as AHR (Evans *et al.*, 2003). Both symptoms are also very important features of the inflammatory process in human asthma patients. As an immunological disease caused by Th2-driven inflammation, asthma is characterized by increased mucus production in the lung, and by an inflammatory process involving various cell types including eosinophils, basophils, mast cells and Th2 lymphocytes (Umetsu *et al.*, 2002). Airway remodeling can begin at the initial stages of bronchial asthma and may facilitate the continuation of tissue damage and the inflammatory response. This is caused by alterations in the composition of the extracellular matrix (ECM) primarily through association of the ECM with eosinophils and fibroblast-released transforming growth factor-beta (TGF- β), along with other growth

factors (Macklem, 1996; Solway and Fredberg, 1997). The immunopathological hallmark of allergic asthma is the infiltration of eosinophils and Th2 cells into affected tissue. Immune deviation in favor of Th2 cells is prominent in allergic asthma (Kay, 2001), but the immunologic mechanism remains unclear. Th1 cells produce interleukin (IL)-2, which promotes cellular immune responses, whereas Th2 cells produce IL-4, IL-5 and IL-13, which are associated with allergic diseases. IL-4 drives naïve Th cells toward the Th2 phenotype and induces B cells to switch the isotype to IgE. Meanwhile, IL-5 is essential for eosinophil differentiation, accumulation of such cells at the sites of inflammation, and their continued survival (Mosmann *et al.*, 1986).

Oxidative stress plays an important role in inflammation and tissue damage in asthma patients. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), generated by macrophages and inflamed tissue, deplete antioxidants (Dunstan *et al.*, 2006; Dinakar, 2004; Murata *et al.*, 2002). ROS generation is enhanced in bronchoalveolar lavage (BAL) cells of stable asthmatics (Kelly *et al.*, 1988), and increases after antigen challenge (Sanders *et al.*, 1995). Many antioxidants are altered in level in asthma and several are markers of oxidative stress (Brasch-Andersen *et al.*, 2004; Corradi *et al.*, 2003; Shanmugasundaram *et al.*, 2001). Despite the wide variety of steroid and non-steroid medications employed, all currently available antiinflammatory or antiasthma drugs cause undesired, and possibly serious, side effects. Thus, the development of new and more powerful drugs is required. Compared with conventional agents, many medicinal plants provide equal

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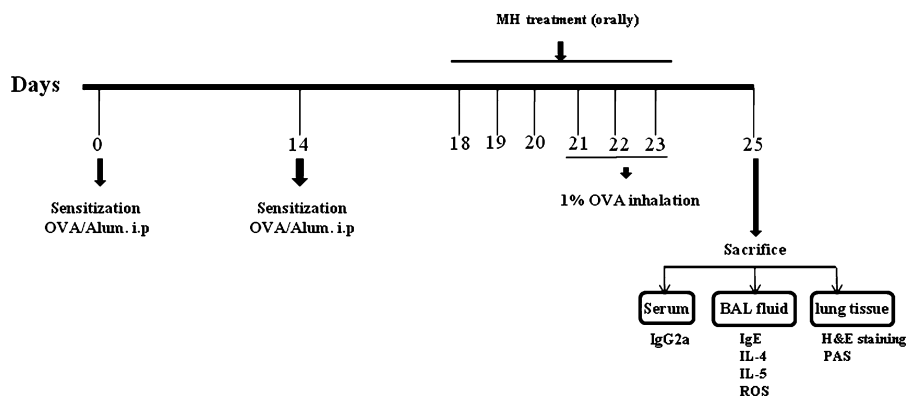


Figure 1. A mouse model of airway inflammation and treatment with MH.

symptom relief (Verpoorte, 1999). The reported pharmacological effects of *Mentha haplocalyx* Briq. include the lowering of body temperature and relaxation of the muscles of the digestive tract. The herb is traditionally used for the treatment of high fever, mild chills, cough, thirst and sore throat, and to combat nausea, vomiting and flatulence (Zheng *et al.*, 1998). The present study evaluates the scientific basis for the traditional use of a *Mentha haplocalyx* ethanol extract (MH).

METHODS

Preparation of *Mentha haplocalyx* Briq. ethanol extract (MH). Dried aerial parts of the *Mentha haplocalyx* plant were purchased in October 2008 from HMAX (Chungbuk, Korea). A voucher specimen has been deposited at the Korea Institute of Oriental Medicine (KIOM) (Daejeon, Korea). The dried aerial parts of *Mentha haplocalyx* (200 g) were suspended in 70% (v/v) ethanol (2 L) and extracted with sonication for 1 h. This process was repeated three times. The extract was then filtered through filter paper and allowed to evaporate to dryness, resulting in 30.5 g of product. The yield of dried extract from starting crude material was 15.2%.

Animals. Female BALB/c mice (7 weeks of age), weighing 20–24 g and routinely screened serologically for relevant respiratory pathogens, were purchased from the Daehan Biolink Co. Ltd (Seoul, Korea). Mice were maintained in an animal facility under standard laboratory conditions for 1 week before use, and were provided with food and water *ad libitum*. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and all handling of animals followed the dictates of the National Animal Welfare Law of Korea.

Sensitization and airway challenge. Mice were used in groups of 6 or 7, and received one of the following treatments: (1) sham-sensitization plus challenge with phosphate-buffered saline (PBS) (i.p./Neb); (2) sensitization plus challenge with OVA (Sigma A5503; Sigma, St Louis, MO) (i.p./Neb); or (3) sensitization with OVA (i.p.) plus challenge with OVA (Neb) and MH (100 mg/kg) or montelukast (30 mg/kg), (p.o.). Briefly, the mice were sensitized by intraperitoneal injection of either

PBS or 20 µg OVA emulsified in 2 mg aluminum hydroxide in 200 µL PBS buffer (pH 7.4), on days 0 and 14. Each mouse was subsequently challenged through the airway with either PBS or OVA (1%, w/v, in PBS) for 30 min using an ultrasonic nebulizer (Neb) (NE-U12 instrument; Omron Corp., Tokyo, Japan) on days 21, 22 and 23 after initial sensitization. Mice were orally administered 100 mg/kg of MH and 30 mg/kg of montelukast each day from day 18 to 23. To characterize the suppressive effects of MH, the mice were killed 48 h after the last challenge (i.e. day 25). A schematic diagram of this treatment schedule is shown in Fig. 1.

Bronchoalveolar lavage (BAL) and accumulation of inflammatory cells. Mice were killed with an overdose of 50 mg/kg pentobarbital 48 h after the last challenge, and tracheotomies were performed. After ice-cold PBS (0.6 mL) was instilled into the lungs, bronchoalveolar lavage fluid (BALF) was obtained by three aspirations (total volume 1.8 mL) via tracheal cannulation. Each suspension of BALF was centrifuged and the supernatant collected and stored at -70°C . The cell pellets were resuspended in 1.0 mL PBS and total inflammatory cell numbers were determined by counting the cells in at least five squares of a hemocytometer. Dead cells, identified by trypan blue staining, were excluded from the total (Daigle and Simon, 2001). BALF (100 µL) was placed on a slide and centrifuged ($200 \times g$, 4°C , 10 min) in a cytospin machine (Hanil Science Industrial, Seoul, Korea). After the slides were dried, the cells were fixed and stained using Diff-Quik® Stain reagents (B4132-1A; Dade Behring Inc., Deerfield, IL) according to the manufacturer's instructions. According to their microscopic morphology, differential cell counts were performed.

2',7'-Dichlorofluorescein (DCF) fluorescence assay. Induction of oxidative stress was monitored using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR), which is converted into the highly fluorescent DCF by cellular peroxides including hydrogen peroxide. In brief, BALF was treated with 25 µM DCF-DA for 10 min at 37°C . To measure intracellular ROS activity, fluorescence was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Perkin-Elmer, Waltham, MA).

Measurement of total IgE and IgG2a in BALF. Total IgE and IgG2a were measured by enzyme-linked

immunosorbent assay (ELISA). Microtiter plates were coated with 100 μ L/well anti-IgE (2 μ g/mL; Serotec, Oxford, UK) and IgG2a (1 μ g/mL; Bethyl Laboratories, TX) in PBS-Tween 20. Antibodies were detected in BALF using isotype-specific secondary antibodies. After washing four times, 200 μ L of *o*-phenylenediamine dihydrochloride (Sigma) was added to each well. The plate was incubated for 10 min in the dark before absorbance was measured at 450 nm. Total and OVA-specific IgE concentrations were calculated from a standard curve generated using recombinant IgE purchased at 250 ng/mL (Serotec, Oxford, UK).

Measurement of Th2 cytokines in BALF. IL-4 and IL-5 contained in BALF were measured using mouse-specific IL-4 and IL-5 ELISA kits (BioSource International, Camarillo, CA). ELISAs were performed according to the manufacturer's instructions.

Real-time quantitative RT-PCR. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. Briefly, lung tissue samples were weighed and homogenized using 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) per 50 mg of tissue. Total RNA was extracted according to the manufacturer's protocol with an additional chloroform extraction and phase separation as well as an extra wash of the isolated RNA in 70% ethanol. RNA was resuspended in nuclease-free water, and its concentration was quantified at OD₂₆₀. The RNA purity was assessed by determining the OD₂₆₀/OD₂₈₀ ratio. Then, 1 μ g of RNA was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI) in a total volume of 20 μ L with random hexamer primers. The following conditions were employed for reverse transcription: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The PCR amplification was then performed using 2 μ L of RT product, Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and predeveloped primer pairs and Taqman probes (Applied Biosystems) in a total volume of 25 μ L. The following thermocycling conditions were employed: 95°C for 10 min, followed by 95°C for 15 s and 60°C for 60 s (for up to 40 cycles). The concentration of each sample was calculated from the threshold cycle (Ct) and normalized according to the expression of GAPDH.

Lung tissue histopathology. After BALF was obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin, trimmed and embedded in paraffin. For observation, lung sections of 4 μ m thickness were stained with H & E solution (hematoxylin, Sigma MHS-16, and eosin, Sigma HT110-1-32) and periodic acid-Schiff (PAS) stain (IMEB Inc., San Marcos, CA). Tissues were subsequently mounted under coverslips using Dako-mounting medium (Dakocytomation, Denmark, CA) and the mucus production determined. The degree of cell infiltration in the airway was scored in a double-blind screen by two independent investigators (Myou *et al.*, 2003). The peri-bronchiole and perivascular inflammation was evaluated.

Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum. ALT and AST levels were determined to assess liver function using commercial kits (Beckman Coulter, Inc.,

Fullerton, CA). The levels of AST and ALT were determined using a Beckman CX4 auto-analyser (Beckman Coulter, Inc.).

Image capture and photomicrography. Photomicrographs were captured using a Photometric Quantix digital camera and montages were assembled in Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast but not otherwise manipulated.

Statistical analysis. Data are expressed as mean $\pm$ SD (standard deviations of the mean). Statistical significance was determined using Student's two-tailed *t*-test or a two-way ANOVA test to compare independent means. The critical level for significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

To evaluate the suppression of inflammatory cells by MH in OVA-challenged mice, cells recruited to the BALF were counted. Numbers of eosinophils, neutrophils, lymphocytes, macrophages and total cells in BALF were increased significantly at 48 h after the OV-challenged mice compared with the numbers of PBS-challenged mice (Fig. 2A). The oral administration of MH, at a dose of 100 mg/kg, 1 h before OVA challenge, significantly inhibited eosinophils, neutrophils, lymphocytes, macrophages and total cells when compared with OVA-challenged mice without MH exposure. Also, the decrease of infiltrated leukocytes was due mainly to the decrease of infiltrated macrophages and eosinophils. These results indicate that MH had an inhibitory effect on lung inflammation in our asthma model. A positive control, montelukast, which has been widely used as an antiasthmatic drug, showed a similar decrease of BALF cell with MH.

Airway inflammation and hyperreactivity in asthma are likely to involve oxidative stress to the lung, and excess production of ROS by immune cells may play an important role in airway injury (Talati *et al.*, 2006; Dworski, 2000). An increase in the generation of ROS in the airway (Nishida *et al.*, 2002; Zhang *et al.*, 2002) and BALF (Lee *et al.*, 2004) has been noted in OVA-induced asthma models. As illustrated in Fig. 2B, OVA-challenge of mice resulted in a markedly increased oxidative stress level as measured in BALF. Administration of MH (100 mg/kg), however, significantly decreased the level of DCF fluorescence in the BALF of OVA-challenged mice. These results demonstrate that MH reduced the ROS in the BALF of the asthmatic model as did montelukast.

One of the most prominent features of asthma, and one of the characteristics of airway inflammation (Bousquet *et al.*, 1990; Holgate *et al.*, 1991; Kay, 1991), is eosinophil infiltration of the peribronchial regions. Increased eosinophil numbers correlated with the severity of the disease (Corrigan and Kay, 1992). In the present study, lung tissue was examined and lung inflammation evaluated by histological examination and score. In OVA-challenged mice, leukocytes (mainly eosinophils) infiltrated the peribronchiole and perivascular connective tissue (Fig. 2C, D), whereas inflamma-

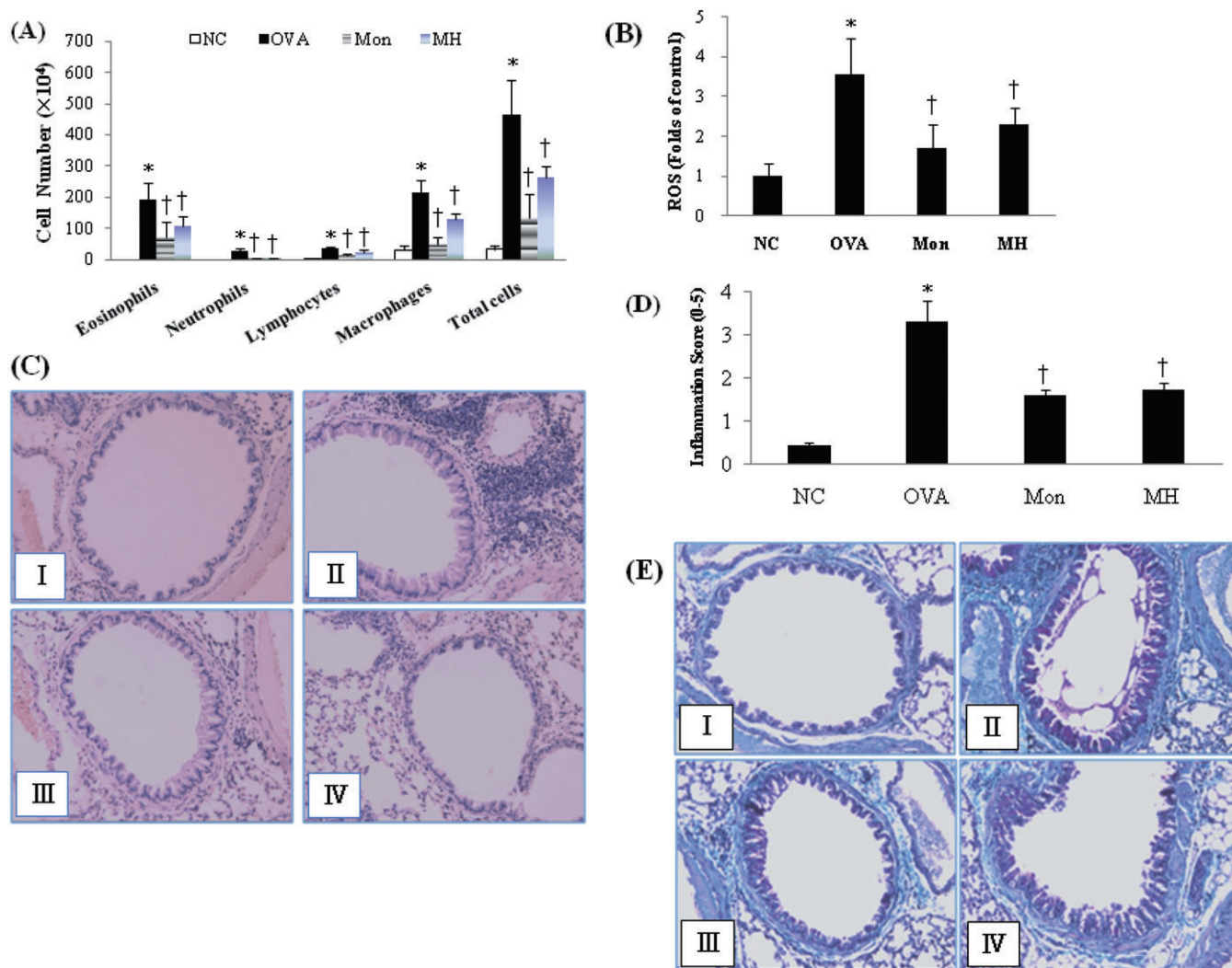


Figure 2. Effect of MH on the recruitment of inflammatory cells, reactive oxygen species (ROS) and mucus production in bronchoalveolar lavage fluid (BALF) and lung tissue of mice 48 h after the last OVA challenge. (A) The number of total cells, eosinophils, macrophages, and lymphocytes in BALF. Cells were isolated by centrifugation and stained with Diff-Quick® Stain reagents. Cell numbers were determined using a light microscope to count cells in at least five squares of a hemocytometer. Dead cells, stained with trypan blue, were excluded from the total. (B) Oxidative stress was quantified by DCF fluorescence in BALF. Values represent relative fluorescence units per milligram protein. NC, normal control mice (PBS only); OVA, OVA-sensitized/challenged mice; MH, MH (100 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. Histological examination of lung tissue was performed 48 h after the last OVA challenge. (C) Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with H&E solution (magnification $\times 200$). (D) Scoring the extent of inflammation by quantitative analysis of inflammatory-cell infiltration in lung sections was performed based on the methods of Myou *et al.* (2003). (E) Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with periodic acid–Schiff (PAS) for mucus production. I, normal control mice (PBS only); II, OVA-sensitized/challenged mice; III, MH (100 mg/kg) + OVA-sensitized/challenged mice; IV, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as mean $\pm$ SD. *Significant difference from NC, $p < 0.05$, and †significant difference from OVA, $p < 0.05$. This figure is available in colour online at <http://wileyonlinelibrary.com/journal/ptr>

tory cells were not detected in the same tissues of PBS-challenged mice. However, in the MH (100 mg/kg) + OVA-challenged mice, infiltration by eosinophils and macrophages were significantly attenuated compared with that seen in untreated OVA-challenged group's BALF and lung tissue (Fig. 2A, C, D).

To evaluate airway epithelial cell injury, hypersecretion of mucus and goblet cell hyperplasia, lung sections were stained with PAS. A significant induction of goblet cell hyperplasia was observed in the airway of OVA-challenged mice (mucus overproduction was clearly observed as a violet color) compared with PBS-challenged mice. Moreover, MH treatment of OVA-challenged mice appeared to suppress the hypersecretion of mucus in lung tissue (Fig. 2E). This is in agreement with the results shown in Fig. 2A, which

suggest that MH treatment inhibited airway inflammation in the asthma model. To investigate the possible inhibitory mechanisms of MH in airway inflammation, the levels of asthma-associated cytokines in BALF and lung tissue were next examined. The Th2 cells, together with other inflammatory cells such as eosinophils, B cells and mast cells, have been proposed to play a critical role in initiation, development and chronicity of the disease (Busse and Lemanske, 2001). IL-4 has three primary effects. First, IL-4 promotes B cell differentiation into plasma cells that secrete antigen-specific IgE antibodies; second, IL-4 promotes mast cell proliferation; and, third, increased IL-4 up-regulates endothelial cell expression of adhesion molecules specific for eosinophils (Tucker and Fanta, 2005). IL-5 mobilizes and activates eosinophils, leading to the release of a major

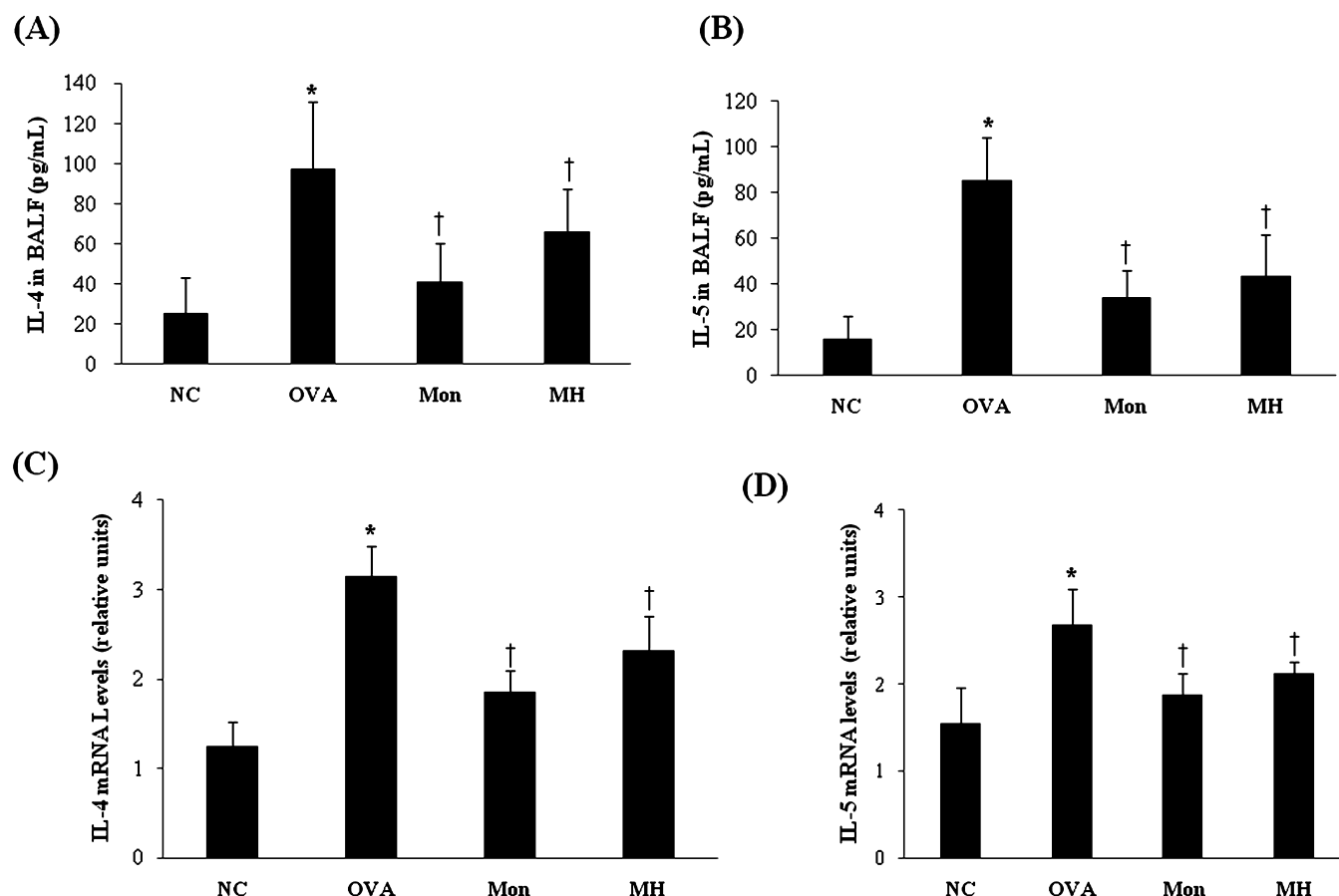


Figure 3. Effect of MH on cytokine levels in BALF and lung tissue. BALF and lung tissue were collected 48 h after the last OVA challenge of mice. Each sample was analysed by ELISA or real time RT-PCR. (A) IL-4 level in BALF; (B) IL-5 level in BALF; (C) mRNA level of IL-4 and (D) IL-5 in lung tissue. NC, normal control mice (PBS only); OVA, OVA-sensitized/challenged mice; MH, MH (100 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as mean $\pm$ SD. *Significant difference from NC, $p < 0.05$, and †significant difference from OVA, $p < 0.05$.

basic protein, cysteinyl-leukotrienes and eosinophil peroxidase, all of which contribute to tissue damage and AHR (Vargaftig and Singer, 2003, Foster *et al.*, 1996). Whereas IL-4 and IL-13 are important in directing B cell growth, differentiation, and secretion of IgE in mice (Emson *et al.*, 1998). The present study compared with the PBS-challenged mice, the levels of cytokines such as IL-4 and IL-5 in BALF of OVA-sensitized/challenged mice were significantly increased. MH treatment, at a dose of 100 mg/kg for 6 days, significantly suppressed any increase in the expression of IL-4 and IL-5 cytokines in OVA-sensitized/challenged mice (Fig. 3A, B). As shown Fig. 2A, decreased cytokine levels are the result of the decreased number of invaded leukocytes. Since MH reduced the number of inflammatory cells in the BALF, next both the mRNA levels of the Th2 type cytokine were investigated. As shown in Fig. 3, MH significantly inhibited the elevated mRNA levels of IL-4 (Fig. 3C) and IL-5 (Fig. 3D) following asthma induction. The data suggest that Th2 cells promote allergic diseases such as asthma and that Th2 cytokines play a leading role in generating the asthmatic phenotype (Kips, 2001; Schuh *et al.*, 2003; Cohn *et al.*, 2004). These effects may be related to inhibition of Th2-related IL-4 and IL-5 synthesis, and be responsible for eosinophil recruitment into the lung in asthma. This confirmed the allergic response in the asthmatic mouse model.

One of the characteristics of asthma is tissue alteration in the epithelium, basement membrane, smooth muscles

and nerves. The changes that occur in the epithelium of asthmatic patients include an abnormal epithelium with increased levels of mucus-secreting cells; a thickened sub-epithelial basement membrane, attributed to the action of eosinophils; and increased collagen deposition in connective tissue (Wenzel, 1994). Based on these findings, further IgE and IgG2a production in BALF and serum were examined by ELISA. Total IgE and IgG2a levels for each experimental group were determined by ELISA 48 h after the final OVA challenge. OVA-treated mice demonstrated dramatically elevated levels of IgE and IgG2a in BALF and serum compared with concentrations seen in control mice. When compared with the OVA-challenged group, mice that received MH exhibited reduced total IgE and IgG2a levels in BALF and serum (Fig. 4A, B). The control of allergic inflammation and asthma is complex, involving several different mechanisms and several different cell types. IL-4, which is pivotal in the pathogenesis of allergic disorders, acts on B cells to facilitate IgE production (Haas *et al.*, 1999; Ramshaw *et al.*, 2001). IgE, together with IgG1, has been closely associated with Th2-type responses, whereas IgG2a has been allied to Th1-type responses. The MH inhibited the serum total IgE level, probably as a result of the inhibition of IL-4 mRNA transcription.

Serum levels of AST and ALT, both serum toxicity marker enzymes, were assessed using AST and ALT assay kits, respectively, according to the manufacturer's instructions. The MH administration did not result in

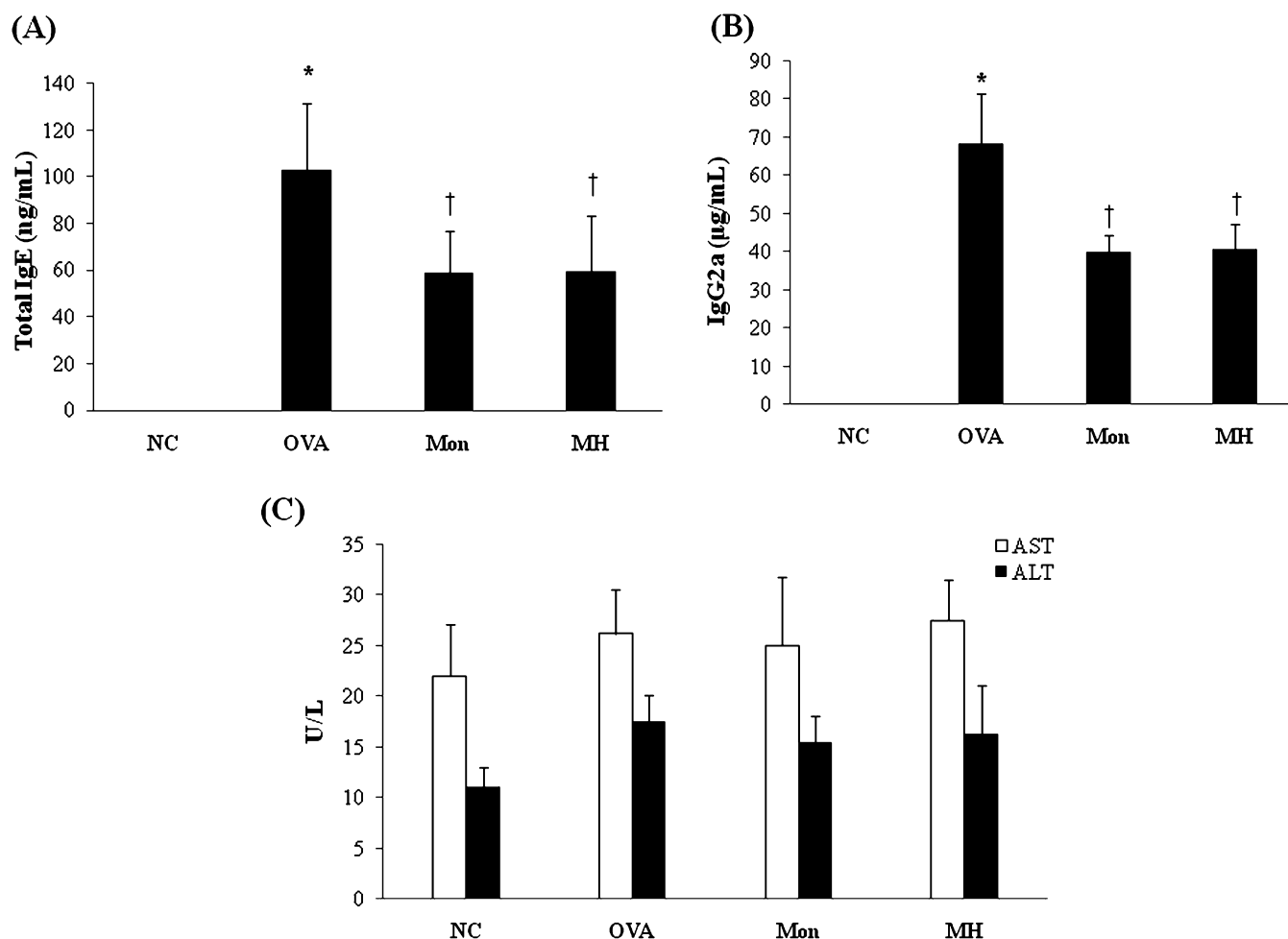


Figure 4. Effect of MH on total IgE, IgG2a, AST and ALT level in serum and BALF. BALF and serum were collected 48 h after the last OVA challenge of mice. Each sample was analysed by ELISA. (A) Total IgE level in BALF; (B) IgG2a level in serum; (C) AST and ALT levels in serum. Each sample was analysed by using commercial kits (Beckman Coulter, Inc., Fullerton, CA). NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; MH (100 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as mean $\pm$ SD. *Significant difference from NC, $p < 0.05$, and †significant difference from OVA, $p < 0.05$.

increased activity of ALT or AST, indicating that liver function was normal (Fig. 4C).

Here it is demonstrated, for the first time, that MH is effective in reducing airway inflammation in the asthmatic mouse model. In the present study, it was demonstrated that MH administration reduced mucus hypersecretion, recruitment of inflammatory cells, and levels of mRNA encoding Th2 type cytokines, in an OVA-sensitized/challenged mouse model. The results clearly indicated that MH treatment reduced the accumulation of eosinophils and other inflammatory cells (neutrophils, lymphocytes, macrophages) in the BALF and lung tissue of OVA-induced asthmatic mice; that IgE, IL-4, and IL-5 mRNA levels were up-regulated;

and that MH significantly reduced the severity of airway inflammation and the accompanying oxidative stress, which is essential if MH is to be used as a therapeutic agent.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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