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Effective Microorgainsm (EM) Fermentation Extract Attenuates Airway Hyperreactivity and Lung Inflammation In A Mouse Model of Asthma

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Effective microorganism (EM) fermentation extract has been widely used for agricultural and environmental application. It has been recently revealed that EM cocktail treatment may be effective for treatment of diseases including cancer. In the present study, effectiveness of EM cocktail to control asthma was investigated using a mouse model of allergic asthma. Asthmatic mice sensitized and intranasally challenged with OVA were orally given EM fermentate (EM-1®) during antigen challenge. Administration of EM-1® resulted in a significant reduction in airway hyper-reactivity (AHR) and airway recruitment of total leukocytes and eosinophils. Cytokine (IL-4, IL-5 and IFNγ) levels in bronchoalveolar lavage fluid (BALF) and lung tissues were not altered by EM-1® treatment. However, IL-13 level in BALF was considerably lower in EM-1® treated mice than in controls. Moreover, Ag-specific IL-4, IL-5 and IL-13 production of draining lymph node cells were markedly downregulated by EM-1® treatment when compared to controls, whereas their IFNγ production was not significantly different. Those data show that EM-1® treatment suppresses type 2 helper T (Th2), but not type 1 helper T (Th1), cell response. This finding was also supported by serum antibody data showing that IgE and IgG1 levels in EM-1® treated mice were significantly lower than in controls, while IgG2a level was not significantly different between two groups. In conclusion, oral administration of EM-1® attenuates asthmatic manifestations including AHR and airway recruitment of eosinophils in a mouse model and which possibly results from selective inhibition of Th2 cell response to allergen. Our data also suggest that EM-1® may be effectively applied for control of allergic asthma.

Key Words: Effective microorganisms, Airway hyperreactivity, Inflammation, Eosinophils, Cytokine

INTRODUCTION

Asthma is a complex syndrome, characterized by a range of phenotypic markers which include reversible airways obstruction and airway hyperreactivity (AHR) associated with pulmonary inflammation (11,29). Despite intensive studies for pathogenesis as well as therapy, asthma remains an increasingly prevalent disease of industrialized nations (43,48), particularly, in low-income and minority children (31).

Upon exposure to the allergen, inflammatory cells including lymphocytes, macrophages, neutrophils, and eosinophils infiltrate the airway. Among these, eosinophils are predominant effector cells for tissue damage and pulmonary dysfunction (18,41) and they induce various inflammatory changes in the airways caused by a wide variety of immunomodulator molecules (37,41). Recent findings in asthmatics and animal models point to a crucial role of T cells in mediating many manifestations of asthma. Local recruit-

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ment of the helper type 2 T cell (Th2) subset may initiate a response leading to asthma, since airway $CD4^+$ T cells express a Th2 cytokine profile that can increase AHR and induce airway eosinophilia (30,36). These ideas have been supported by those data showing that mice with deficiencies of Th2 cytokine expression, or by treatment with Th2 cytokine antagonists, have diminished AHR and eosinophilic inflammation (14,15,19).

Yet, the current asthma therapies are not cures and symptoms relapse soon after treatment is stopped even after long term treatment (9). Pharmacological control of asthma may be achieved in most asthmatics only with anti-inflammatory agents (controllers), such as inhaled glucocorticoids and theophylline (20,39) or in combination with long-acting bronchodilators (1,33). The leukotriene antagonist has been the only new class of asthma treatments to be licensed by the pharmaceutical industry in the past 30 years (22). Consequently, it has been a long-term goal to develop disease-modifying immunotherapy, that could be introduced in childhood to alter the natural history of asthma (4).

Effective microorganism (EM) developed by Dr. Higa (University of the Ryukyus, Japan) (25) is a complex group of microorganisms consisting of mixed cultures of beneficial naturally-occurring microorganisms such as photosynthetic bacteria, lactobacilli and yeasts (27), and its fermentation extract has been widely used for agricultural and environmental application in South East Asia (26,34).

EM has been emergingly accepted in clinical practice (28). It was recently revealed that mixed ferment cocktail of EM with rice, papaya and sea-weeds inhibits in vitro growth of tumor cells (10) and lots of clinical trials are being carried out for cancer therapy (23,50). Moreover, EM in vivo protects liver and kidney from oxidative stress-dependent damage (2) and showed anti-inflammatory (16) and neuroprotective effects for retinal (3) and dopaminergic neurons (15). Although underlying mechanisms are unclear, accumulated evidences show that anti-oxidant properties displayed by flavonoids, saponins, ubiquinones, lycopene and Vitamin E are most probably associated with those activities of EM (2,16,51). However, effectiveness of EM on asthma has never been described. Hypothesizing that anti-inflammatory activity of EM would effectively reverse allergic lung inflammation and asthma, in the current study, we investigated the potential therapeutic efficacy of EM in a murine model of asthma. Here, we report evidences supporting that EM administration may be applicable to asthma treatment.

MATERIALS AND METHODS

1. Mice

The C57BL/6 mice between 6 and 8 weeks of age were supplied from the Korean branch of Taconics, SamTaco (Osan, Korea). The mice were housed in the animal facility at Jeonju University using an environmentally controlled chamber.

2. Antigen sensitization and airway challenge

Mice were sensitized with 10 μg of OVA (Sigma Chemical Co., St. Louis, MO. USA) and 1 mg of alum intraperitoneally on days 0 and 7. Sham-immunized mice received alum alone. On days 15, 16 and 18, mice were anesthetized by i.p injection of Avertin (2.5% wt/vol in PBS) and intranasally challenged with 2% OVA in PBS (50 μl/mouse). A control group was exposed to PBS alone with no OVA in an identical fashion.

3. EM cocktail and treatment

EM-1® was kindly supplied by EM Korea (Jeonju, Korea) and 0.1 ml of 8-fold diluted preparation was orally given from day 14 for 5 consecutive days.

4. Measurement of airway hyperreactivity

One day after final aerosol challenge, bronchoconstriction in response to inhaled methacholine (MCh) was determined from changes in enhanced pause (Penh) that were measured by barometric plethysmography in conscious mice as described previously (21). Mice were placed in whole-body plethysmographs (All-Medicus, Seoul, Korea), exposed to PBS aerosol for 30 s, and the average Penh value was calculated during the next 5 min. After a 10-min recovery period, mice were challenged with increasing concentrations of MCh (6.25~50 mg/ml) by aerosol for 30 s at intervals of 20 min. The average Penh value for the 5 min after challenge was calculated.

5. Bronchoalveolar lavage (BAL)

Immediately after measurement of AHR, mice were ane-

sthetized by i.p injection of Avertin (2.5% wt/vol in PBS). For bronchoalveolar lavage, the trachea was cannulated and the lungs were lavaged with three 0.4-ml aliquots of PBS. The live cells (excluded by trypan blue) recovered were counted in a hemocytometer and differential counts were determined on Diff Quik (Baxter Healthcare Corp., Miami, FL. USA)-stained cytocentrifuge preparations.

6. Collection of serum and lung homogenate

After mice were anesthetized, blood was taken by cardiac puncture. Blood was allowed to clot at room temperature for 30 min and serum recovered by centrifugation (10,000 rpm, 5 min at 4℃) was stored at -70℃ until use.

To collect lung homogenates, lungs were homogenized in PBS using a homogenizer (Janke & Kunkel GMBH, Germany).

7. Lymph node (LN) cell isolation

Peribronchial LN were obtained by dissection and placed in chilled RPM1 1640 medium (Life Technologies, Gaithersburg, MD. USA). Single-cell suspensions were created by teasing with curved needles and erythrocytes were lysed by hypotonic shock. After cell suspensions were washed by centrifugation at 1,800 rpm for 5 min at 4℃, the supernatant was discarded, and the cells were filtered through nylon mesh (70 μm) and subsequently resuspended in IMDM (HyClone Laboratories, Logan, UT. USA) with 10% fetal bovine serum (HyClone), 5×10^{-5} M 2-ME, 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, and 2 mM L-glutamine (Sigma). Lymphocytes were counted with a hemacytometer.

8. LN cell culture for proliferation and cytokine assays

LN cells were harvested on day 19 and T cells were prepared by adherence to plastic dishes and negative selection using anti-B220- and anti-NK cell (DX5)-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Syngeneic B cell-enriched splenocytes were used as APCs that were prepared by plastic attachment and subsequent treatment with mitomycin C for 20 min. 5×10^5 T cells together with 5×10^5 APCs were cultured with OVA (200 μg/ml) in complete IMDM medium supplemented with 10 % FBS. Cultures were incubated for 3 days and number of viable cells were counted with trypan blue staining and supernatants were collected for cytokine analyses.

9. Cytokine and Ig assays (ELISA)

Protein levels of IL-2, IL-4, IL-5, IL-13 and IFNγ in BALF and culture supernatants and Ag-specific IgE in serum were determined by ELISA according to the manufacturer's recommendation. Antibody pairs and standards were purchased from BD PharMingen (IL-5; San Diego, CA. USA), Biosource International (IL-4, IFNγ; Camarillo, CA. USA) or R & D Systems (IL-13; Minneapolis, MN. USA). The lower limits of detection for the cytokines were: IL-4, 5 pg/ml; IL-5, 5 pg/ml; IFNγ, 15 pg/ml; and for the IL-13, 3 pg/ml.

Anti-OVA Ig serum levels were also measured by ELISA according to the manufacturer's recommendation. Plate wells were precoated with 100 mg/ml of OVA overnight at 4℃ and antibody conjugates against mouse IgG1, IgG2a and IgE were purchased from PharMingen.

10. Statistical analysis

Statistical analysis and graphical presentation was done using SigmaPlot 5.0 (SPSS Inc.). Values are given as means ± SE and group means were compared with Student's t test in which $p<0.05$ was considered significant.

RESULTS

1. EM-1® administration attenuates AHR in a mouse model of asthma

Intraperitoneal OVA sensitization followed by intranasal challenge has been employed as an established model consistantly leading to allergic response and AHR in mice. Mice that were sensitized according to this protocol developed significant AHR to inhlaed methacholine. As illustrated in Fig. 1, Penh values in response to increasing doses (12.5, 25 and 50 mg/ml) of methacholine in control mice were 1.81±0.14, 2.69±0.16 and 3.52±0.19, respectively. However, those in EM-1® treated mice were significantly lower (1.39±0.10, 1.89±0.15 and 2.48±0.24, respectively; p=0.029, 0.002 and 0.003, respectively, n=10) (Fig. 1).

2. EM-1® administration reduces airway inflammation

For evaluation of airway inflammation, airway recruit-

ment of leukocytes were enumerated. Total number in BALF of EM-1® treated mice (2.4±0.24 cells/ml) was considerably decreased by 53% compared with control mice (4.5±0.43 cells/ml, p=0.008, n=7) (Fig. 2A) and distributions of eosinphils (20.1±2.8%) was also significantly diminished by EM-1® treatment when compared to controls (37.7±3.2%, p=0.001), while those of macrophages were relatively elevated from 49.7±4.3% to 69.7±5.6% (p=0.015) (Fig. 2B). There was no significant differences in numbers of neutrophils and lymphocytes between EM-1® treated and control mice.

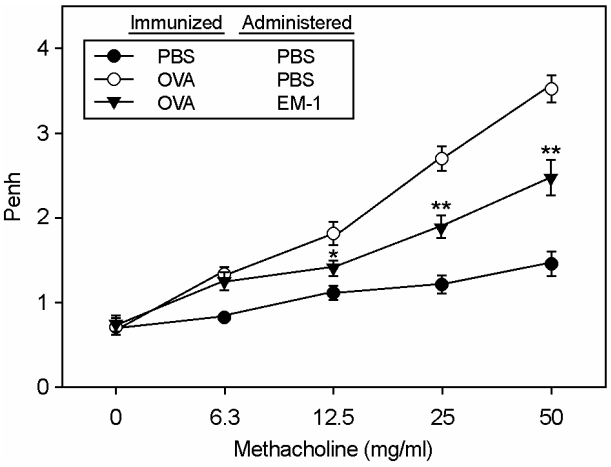

Figure 1. Effect of oral administration of EM-1® on airway hyperreactivity in a mouse model of asthma. Mice were sensitized with OVA at days 0 and 7, and then challenged with aerosolized OVA from day 15 to day 20 and EM-1® was orally given from day 14 for 7 consecutive days. One day after last challenge, Penh was determined in response to increasing doses of methacholine. Data are means ±SE of 10 mice from 3 separate experiments. *, p<0.05, **, p<0.005 vs each control.

3. Cytokine levels in BALF and lung tissues.

To account for EM-1® mediated control of asthmatic manifestations, we assessed in vivo secretion of relevant cytokines. Levels of IL-4, IL-5 and IFNγ in BALF of controls were 334.3±30.5, 741.7±51.6 and 123.7±23.3 pg/ml, respectively, and which were not significantly different from those of EM-1® treated mice (316.8±36.1, 665.7±92.0 and 187.5±42.0 pg/m, respectively) (Fig. 3). However, IL-13 level was significantly lower in EM-1® treated mice (45.0±17.6 pg/ml) than in control mice (206.0 ±32.6 pg/ml) (p=0.012, n=6).

Levels of IL-4, IL-5, IL-13 and IFNγ in lung tissues of controls (199.5±44.3, 390.8±100.1, 15.7±2.3 and 138.3± 23.6 pg/ml) were not significantly different from those of EM-1® treated mice, either (180.5±38.5, 372.8±125.6, 12.3 ± 1.5 and 174.5±20.1 pg/ml, respectively).

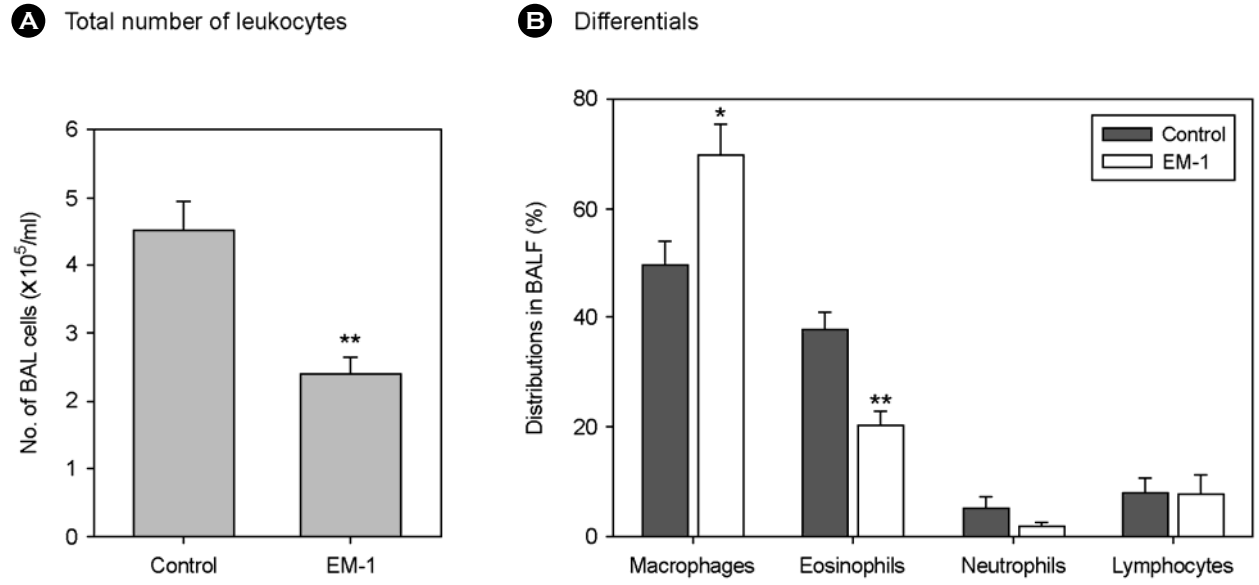

Figure 2. Effect of oral administration of EM-1® on airway inflammation in a mouse model of asthma. Mice were immunized and EM-1® was orally given as described in Materials and Methods. After examination of AHR, the trachea was cannulated and the lungs were lavaged. Total number of live leukocytes in recovered BALF **(A)** was enumerated (p=0.008, n=7), and differential cell counts **(B)** were performed on Diff Quik-stained cytocentrifuge preparations. *p=0.015; **p=0.001 vs control (n=7).

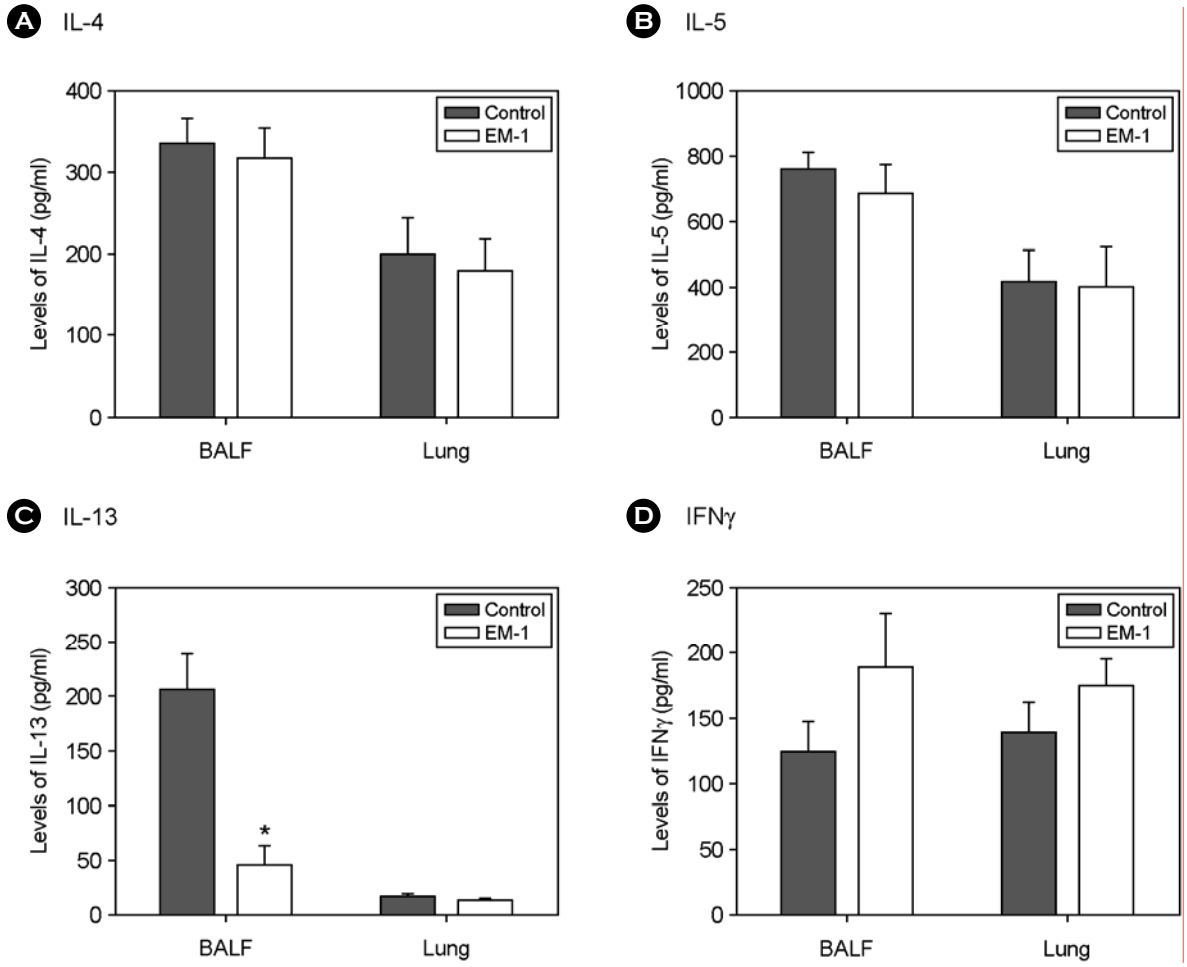

Figure 3. Effect of oral administration of EM-1® on cytokine levels in inflammatory sites. Mice were immunized and EM-1® was orally given as described in Materials and Methods. Levels of cytokines in BALF and homogenized lung tissues were determined by ELISA. *, p=0.012 vs control (n=6).

4. EM administration downregulates type 2 cytokine production by draining lymph node cells

For a further insight into the immune modulation by EM-1® treatment, Ag-specific cytokine productivity was compared using draining lymph node cells. Production of type 2 (IL-4 and IL-5) and type 1 (IFNγ) cytokines was analyzed in culture supernatants of peribronchial lymph node cells stimulated with OVA. While their proliferation was comparable (Fig. 4A), IL-4 (364.7±44.4 vs 115.3± 11.6 pg/ml; p=0.0003, n=6), IL-5 (968.8±105.7 vs 336.5± 67.8 pg/ml; p=0.0005) and IL-13 production (1809.0±158.4 vs 397.7±98.2 pg/ml; P=0.002) was markedly downregulated by EM-1® treatment. In contrast, IFNγ production was slightly depressed, but not significantly different between treated and control groups (631.7±188.8 vs 413.0±141.9 pg/ml; p=0.37) (Fig. 4B).

5. EM administration inhibits IgE secretion.

Finally, Ag-specific serum Ig levels was evaluated for comparison. As shown in Fig. 5A, Significantly lower levels of OVA-specific IgE was detected in EM-1® treated mice compared to controls ($p<0.01$ or $p<0.05$, n=5). Moreover, EM-1® treatment also resulted in a significant decrease in the serum level of IgG1 (Fig. 5B, $p<0.05$, n=5), whereas IgG1 level was so low and looked rather elevated although there was no significant difference (Fig. 5C).

DISCUSSION

There are numerous therapies in clinical development that combat the inflammation occurring in asthma, specifi-

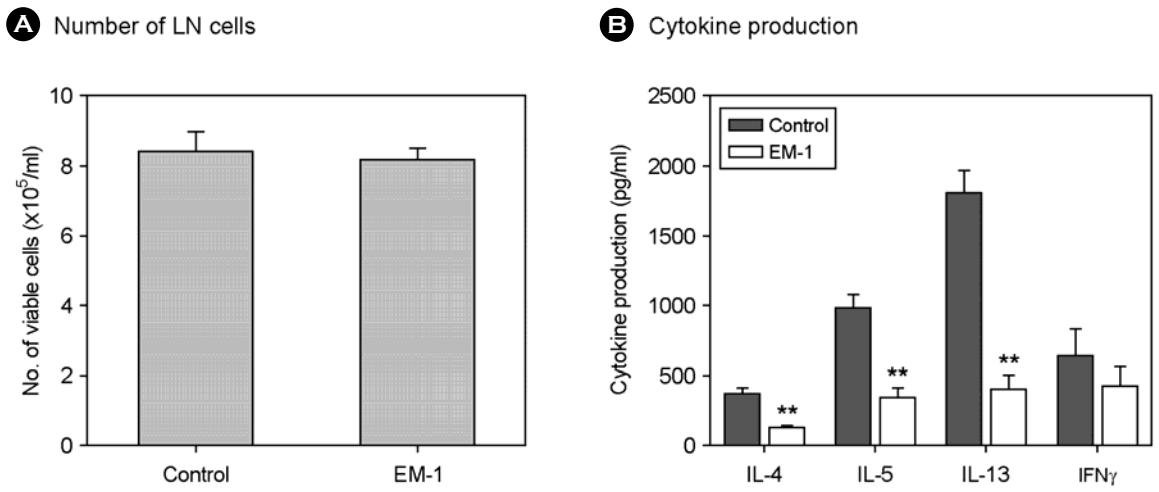

Figure 4. Effect of oral administration of EM-1® on Ag-specific responses of lymph node cells. One day after last challenge, peribronchial LN cells were prepared and stimulated with OVA. At day 3 of culture, number of viable cells **(A)** and cytokine production **(B)** were examined. **p<0.005 vs each control (n=6).

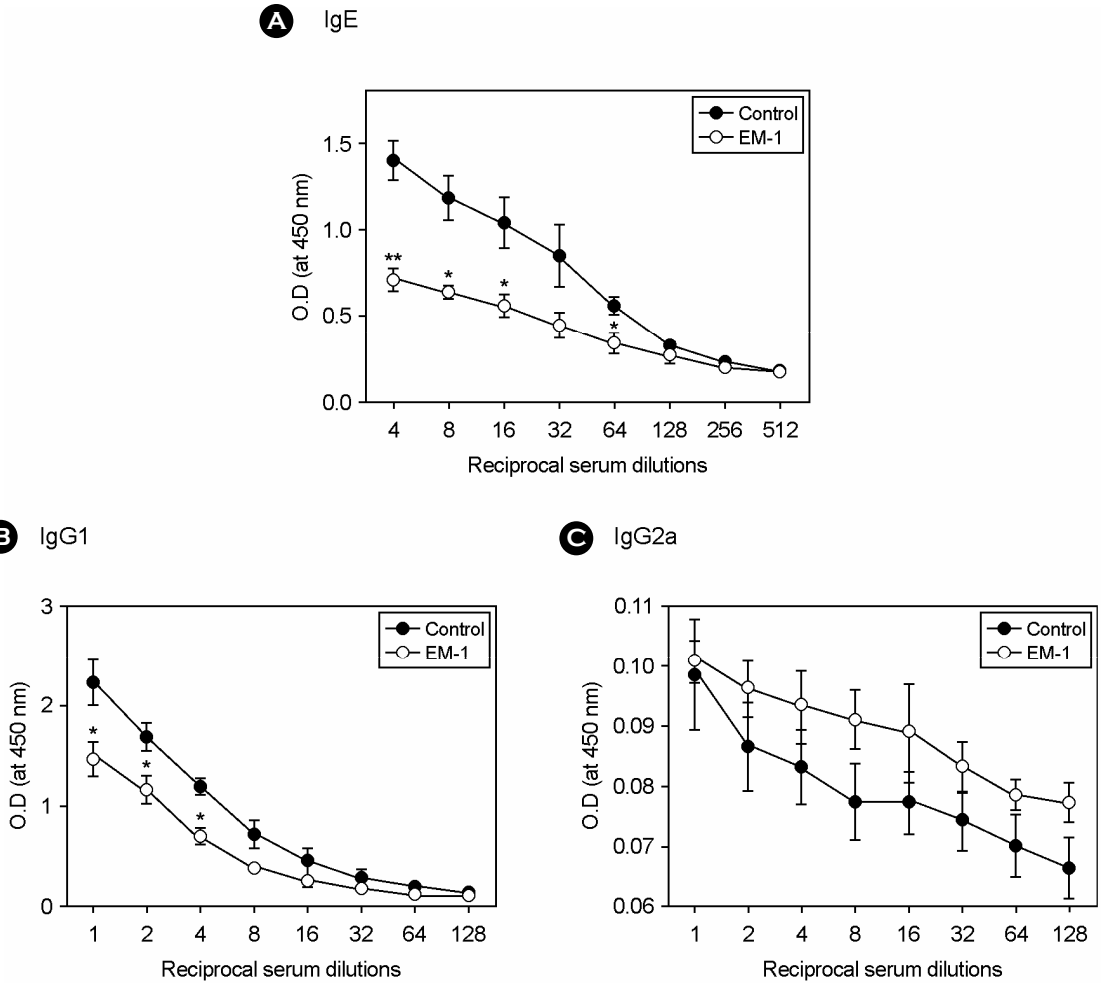

Figure 5. Effect of oral administration of EM-1® on Ag-specific antibody responses in vivo. After examination of airway lavage, blood was taken by cardiac puncture and serum was recovered. Ag-specific serum IgE **(A)**, IgG1 **(B)** and IgG2a **(C)** levels was evaluated by ELISA. *, p<0.05; **, p<0.01 vs each control (n=6).

cally targeting eosinophils, IgE, adhesion molecules, cytokines and chemokines, inflammatory mediators and cell signalling. In particular, there is the obvious need for new therapy for severe asthma that is poorly controlled by high doses of corticosteroids (38), as well as agents to counter acute emergency asthma. A long-term goal is to develop disease-modifying immunotherapy, that could be introduced in childhood to alter the natural history of asthma (4).

Although it has been applied for treatment of cancer, liver and kidney damage (2) and neurologic disease (3), in our knowledge, anti-asthma effect of EM has never been described so far. Thus, to provide a new insight into the possible applicability of EM to control asthma was the purpose of this study.

When AHR was evaluated by enhanced pause (Penh) values, it was significantly reduced by EM-1® treatment (Fig. 1). Analysis of leukocytes infiltrating into airway also showed that oral administration of EM-1® effectively inhibits airway inflammation (Fig. 2). AHR is a hallmark of allergen-induced asthma (7) and, in most cases, AHR is strongly associated with airway inflammation (24,42). Collectively, our present study revealed that oral administration of EM-1® effectively attenuates asthmatic manifestations in a mouse model (Fig. 1 and Fig. 2).

Although the asthmatic inflammatory response is complex and not fully understood, accumulating data have associated the asthma phenotype with the presence of an aberrantly active type 2 immune response in the lungs (12,36,46) and the importance of eosinophils (8). Moreover, Th2-associated cytokines are known to be involved in the development of IgE production and airway eosinophilia.

IL-4 directs the development of Th2-like helper effectors (45) and drives IgE synthesis by B cells (17,35), and resultingly crucial for AHR induction (13). IL-5 is required for eosinophil chemotaxis (47) and airway eosinophilia (44). Another Th2 cytokine, IL-13, is also hypothesized to play a pivotal role in the pathogenesis of asthma by activating B cells, eosinophils, and airway smooth muscle cells (49) and IgE synthesis (52). Oral treatment of EM-1® resulted in a significant reduction in IL-4, IL-5 and IL-13 production of draining lymph node cells, whereas IFNγ production was not influenced. All of these results strongly indicate that EM-1® treatment selectively inhibits allergen-specific Th2 response and which contributes to reduction in asthmatic manifestations.

However, as shown in Fig. 3, EM administration failed to lower the levels of IL-4 and IL-5 in BALF unlike to those data of cytokine production by draining LN cells. This discordance may suggest that the effectiveness of oral treatment of EM-1® is rather systemic but much vulnerable in local response.

Final data of serum antibody was additionally supportive for selective inhibition of Th2 response by EM-1® treatment. As shown in Fig. 5A and 5B, serum levels of IgE and IgG1 were also considerably decreased, whereas IgG2a, a type 1 antibody (32), level was rather higher in EM-1® treated mice, although there was no significant difference (Fig. 5C). Elevated serum IgE levels is undoubtedly important in the development of asthmatic responses (6,40). Thus, these data clearly show that selective inhibition of type 2 response following EM-1® treatment during progress of asthma leads to attenuation of AHR and airway eosinophilia.

Although the exact mode of action of EM-1® is largely unclear, the use of Th1-skewing reagent for immunotherapy in allergic diseases has a great potential. Besides the prevention of asthmatic symptom, EM-1® looks to have a merit to prevent the induction of adverse side effects which can occur after anti-inflammatory therapy. Continuing study to define active ingredient in EM-1® would be necessary to extend clinical use of EM-1®.

Acknowledgements

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