



Alleviation of OVA-Induced Airway Inflammation by Flowers of *Inula japonica* in a Murine Model of Asthma

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The flowers of *Inula japonica* (Inulae Flos) have long been used in traditional medicine for treating inflammatory diseases. The effects on OVA-induced asthmatic mice of an Inulae Flos extract (IFE) were evaluated in this study. The anti-asthmatic effects of IFE were determined by observing eosinophil recruitment, airway hyper-responsiveness (AHR), Th2 cytokine and IgE levels, and lung histopathology. The IFE treatment effectively reduced the percentage of eosinophils and Th2 cytokines in the bronchoalveolar lavage fluid (BALF) when compared to the levels in OVA-induced mice. IFE also suppressed AHR induced by aerosolized methacholine in OVA-induced mice. The results of the histopathological studies indicate that inflammatory cell infiltration and mucus hypersecretion were both inhibited by the IFE administration when compared to the effect on OVA-induced mice. The IFE treatment also suppressed the serum IgE levels and decreased Th2 cytokines in the supernatant of cultured splenocytes. These results suggest that IFE may have therapeutic potential against asthma.

Key words: *Inula japonica*; Inulae Flos extract (IFE); eosinophil; bronchoalveolar lavage fluid (BAL); asthma

Asthma is a chronic inflammatory disease of the lungs characterized by variable airflow obstruction, bronchial eosinophilic inflammation, airway hyper-responsiveness (AHR) and mucus overproduction.^{1,2)} It has largely been accepted that an imbalance between Th1 and Th2 cells leads to the clinical expression of this allergic disease.³⁾ Th2 cells orchestrate the recruitment and activation of mast cells and eosinophils through the release of such cytokines as IL-4, IL-5 and IL-13 which are the primary effector cells of the allergic response. It has also been shown that Th1 cells secreting such a cytokine as IFN- γ were involved in asthma.

The worldwide prevalence and severity of allergic asthma has increased dramatically over the last few

decades.⁴⁾ Current standard medication consists of combined therapy, including inhaled corticosteroids, β_2 -agonists, leukotriene receptor antagonists, and others.⁵⁾ However, this therapy produces side effects and does not consistently ameliorate airway inflammation in many asthmatic individuals. There is therefore a need for new or alternative approaches to improve the treatment for this disease.

The use of herbal therapy or alternative medicine is becoming an increasingly attractive approach for the treatment of various inflammatory disorders. The genus *Inula* consists of more than one hundred species and is primarily found in Mediterranean regions.⁶⁾ The flowers of *Inula japonica* Thunb. (*Inula Britannica* L. var. *chinensis* Regal) have long been used in traditional Chinese medicine for treating digestive disorders, bronchitis and inflammation.⁷⁾ However, the effects of *Inula japonica* on the murine model of asthma have not previously been evaluated.

We investigated in the present study the effects of *Inula japonica* on the treatment of bronchial asthma by measuring the eosinophil infiltration, AHR, Th2 cytokines, and mucus secretion in an ovalbumin (OVA)-induced allergic airway inflammation model.

Materials and Methods

Plant material. The dried flowers of *Inula japonica* Thunb. (Inulae Flos) collected in Anhui province of China were purchased from Omniherb (Yongchun, Korea), and a voucher specimen has been deposited at the Daegu Gyeongbuk Institute for Oriental Medicine Industry (Gyeongsan, Korea). The Inulae Flos was extracted with ethanol at a ratio of 1:10 (w/v) and then refluxed for 24 h at 60 °C. The extracted solution was filtered, and the solvent evaporated under vacuum at 40 °C (Eyela, Tokyo, Japan), before being freeze-dried to obtain a concentrated extract (8% yield, w/w).

Animals and experimental protocol. Six-week-old female BALB/c mice (16–20 g) were obtained from Orient Co. (Seoul, Korea) and fed with laboratory feed (Purina, Seoul, Korea) and water *ad libitum*. The animals were acclimatized in a specific pathogen-free animal facility under conditions of 20–22 °C, 40–60% relative humidity, and a 12 h/

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12 h (light/dark) cycle for at least 7 d. The mice were sensitized by an intraperitoneal (i.p) administration on days 0 and 14 with 20 µg/mL of OVA in PBS mixed with an equal volume of alum (1 mg) as an adjuvant in a total volume of 200 µL. The mice were exposed to aerosolized OVA (1% OVA in PBS) or PBS for 30 min on days 22, 23 and 24. The Inulae Flos extract (IFE), montelukast (Mont, 30 mg/kg) or dexamethasone (Dex, 2 mg/kg) was orally administered 10 times every 12 h from 1 d before the first challenge to the last challenge.

Measurement of the airway resistance. The mice were intraperitoneally anesthetized with ketamine (62.5 mg/kg) and xylazine (25 mg/kg). Pin electrodes were inserted into the paws of the mice to record ECG for monitoring the animal condition. The trachea was then surgically exposed, cannulated (18 G), and connected to a Inspira ventilator (Harvard Apparatus, Holliston, MA, USA) and a pressure transducer. Each mouse was ventilated at 120 breaths/min with a tidal volume of 0.20 mL, and the pressure at the airway opening was measured by a BIOPAC system with Acknowledge software (Biopac, Santa Barbara, CA, USA). The jugular vein was cannulated with a 30 G needle cannula filled with heparine for the delivery of methacholine. Vecuronium bromide (0.2 mg/kg) was administered *via* the jugular vein to eliminate spontaneous respiration. A bolus of PBS (1 µL/g of body weight) was administered to measure the basal airway pressure after it had stabilized. Various concentrations of methacholine chloride (0.03, 0.1, 0.3, and 1 mg/mL in PBS) were administered 2 min later at a dose of 1 µL/g of body weight. The data for airway resistance were continuously collected up to 5 min after each methacholine administration, and maximum values for the airway pressure were selected. The airway resistance is expressed as the percentage of airway pressure provoked by methacholine to the basal pressure (100%) obtained by PBS administration.

Analysis of the bronchoalveolar lavage fluid (BALF). The mice were sacrificed 18 h after the last aerosol challenge. The mice were anesthetized, a tracheal cannula was inserted into each mouse *via* a mid-cervical incision, and the airway of each mouse was lavaged two times with 0.5 mL of PBS to obtain BALF. BALF was immediately centrifuged (2 min, 4°C, 160 g). The cells were resuspended in 0.5 mL of PBS after removing the supernatant. The suspended cells were spun on to a Shandon Cytospin 4 glass microscope slide (Thermo Scientific, Kalamazoo, MI, USA), each slide was air dried for 10 min and then stained with a Diff-Quick solution (Sysmex, Kobe, Japan). The number of eosinophils was determined by counting at least 200 cells in each of four different locations, and data are expressed as the percentage of total leukocytes.

Measurement of cytokines and serum IgE. The levels of IL-4 and IL-5 in BALF and the supernatant isolated from the lung tissue homogenate were quantified by enzyme immunoassays performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Total serum IgE was measured by using OptEIA™ set mouse IL-5 (BD Biosciences, San Diego, CA, USA). The mice were anaesthetized and the spleen excised 18 h after the final challenge to quantify cytokines from the cultured supernatant of the splenocytes. The spleen cells (4×10^6 cells/mL) were suspended in a complete culture medium (RPMI 1640 containing 2 mm l-glutamine, 0.1 mm non-essential amino acids, 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10% fetal calf serum) and cultured in 24-well plates in the presence of OVA (50 µg/mL). The supernatant was collected after a 72-h culture, and the protein level of cytokines in the spleen culture supernatant was determined by ELISA according to the manufacturer's instructions (R&D Systems).

Histological analysis of lung tissue. The lungs were removed and fixed with 10% (v/v) formaldehyde, after which the tissues were dehydrated in various concentrations of ethanol and embedded in paraffin. Sections of fixed paraffin tissues were cut with a microtome to a thickness of 4 µm for a histological examination, after which they were deparaffinized and stained with hematoxylin and eosin (H&E) and the periodic acid Schiff reagent (PAS). Peribronchial inflammation, eosinophil recruitment, and mucus secretion were assessed according to a five-point scale by two independent examiners as previously described.⁸⁾

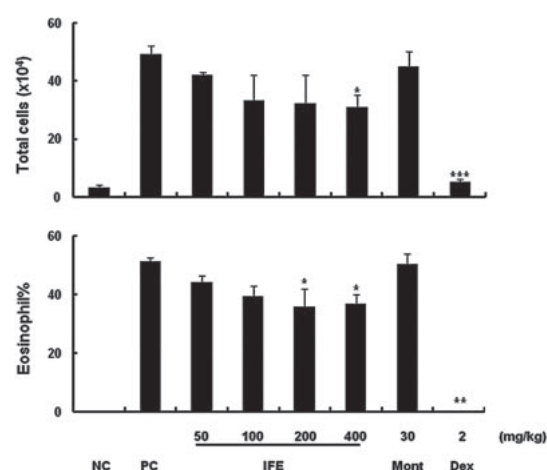


Fig. 1. Effect of IFE on the Recruitment of Leukocytes in the Airways of OVA-Induced Asthmatic Mice.

A murine model of OVA-induced asthma was developed, and BALF was obtained from the lungs of mice. Total leukocytes and eosinophils from BALF were counted (A). The eosinophils are expressed as a percentage of the total leukocytes (B). The data are expressed as the mean $\pm$ SEM ($n = 6$). ** $p < 0.01$, * $p < 0.05$ significantly different from the control as determined by one-way ANOVA.

Statistical analysis. Data are expressed as the mean $\pm$ SEM. One-way ANOVA was used to determine the statistical significance. Differences in the airway resistance between groups were evaluated by two-way ANOVA and subsequent Bonferroni *post hoc* tests for individual group comparisons. A value of $p < 0.05$ is considered significant.

Results

Effects of IFE on OVA-induced total cells and eosinophils in BALF

The reduction in inflammation of OVA-induced asthmatic mice was determined by measuring the total number of cells and the percentage of eosinophils in BALF. As shown in Fig. 1A, the total number of leukocytes in BALF of the OVA-induced lung tissues was significantly higher when compared to the control mice. However, treating the mice with IFE (400 mg/kg) inhibited the OVA-induced recruitment of total leukocytes into the airway. The number of eosinophils in BALF of mice was determined as a percentage of the total number of cells. Again, prior administration of IFE (200 and 400 mg/kg) greatly reduced the percentage of eosinophils recruited into the airways of the OVA-induced mice (Fig. 1B). Dex also strongly inhibited the eosinophil recruitment into the airways of the OVA-induced mice, which was as expected, although Mont did not reduce the infiltration of eosinophils into the airways. These data indicate that IFE effectively inhibited cell filtration into the OVA-induced asthmatic lung.

Effects of IFE on airway hyper-responsiveness

Airway responsiveness to methacholine was examined 18 h after the final OVA challenge to determine the effect of IFE on the airway function in a murine asthma model. As shown in Fig. 2, there was a dose-dependent increase in airway resistance in response to methacholine in all experimental groups. The airway resistance in the OVA-induced mice was significantly higher than

that in control mice at doses of 0.1, 0.3 and 1 mg/kg of methacholine, indicating the development of AHR in the OVA-induced mice. This increase was significantly attenuated by treating with IFE at 200 and 400 mg/kg, but there was no difference between the groups treated with 100 and 200 mg/kg of IFE. Treating the mice with Dex almost completely diminished the increased level of AHR and resulted in a similar dose-response curve for airway resistance to that of the control mice. These findings suggest that administering IFE (200 and 400 mg/kg) effectively reduced AHR in the asthma animal model.

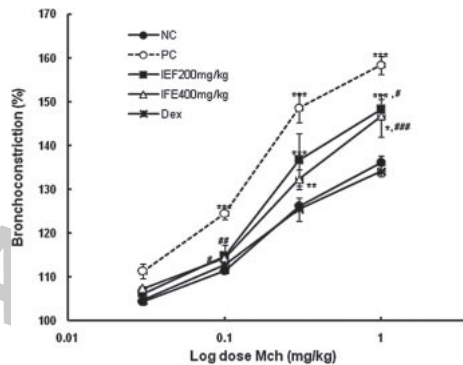


Fig. 2. Effects of IFE on Airway Hyper-Responsiveness.

The airway hyper-responsiveness of PBS in OVA-induced mice was measured after treating with an increasing dose of methacholine chloride. Each group consisted of four to seven mice. Data are expressed as the mean $\pm$ SEM ($n = 6$). ** $p < 0.01$, * $p < 0.05$ (vs. PBS group), ## $p < 0.01$, # $p < 0.05$ (vs. positive group) significantly different from the control as determined by two-way ANOVA.

Effects of IFE on the cytokine and IgE levels

Since airway inflammation is generally accompanied by the generation of inflammatory mediators, the levels of Th2 cytokines (IL-4 and IL-5) were determined in BALF and the lung homogenate supernatant by using ELISA. As shown in Fig. 3A, the levels of IL-4 and IL-5 were higher in the OVA-induced asthmatic mice than in the control mice. The IL-4 level in BALF and the IL-5 level in the lung were decreased significantly in response to the respective treatment with 400 mg/kg and 200 mg/kg of IFE, while treatment with most concentrations of IFE decreased the IL-5 level in BALF and the IL-4 level in the lung. Treatment with IEF therefore attenuated the Th2 cytokine levels in both BALF and the lungs of OVA-induced mice.

IgE favors Th2 in airway inflammation, so we determined whether IFE modulated the level of serum IgE in the OVA-induced mice. Figure 3B shows that the level of serum IgE was significantly upregulated in OVA-induced mice when compared to the control mice. However, treatment with IFE at all concentrations tested led to a significant decrease in the serum level of IgE, these data indicating that IFE modulated the Th1/Th2 levels in OVA-induced asthmatic mice.

Effect of IFE on the T-cell cytokine response in splenocytes

We evaluated the effect of IFE on T-cell cytokine secretion by splenocyte cultures to determine its possible effects on the T-cell response. Figure 4 shows that the IL-4, IL-5 and IL-13 levels were significantly lower in cultures from the IFE-treated group than in those from the OVA-induced group, showing a predominant Th2

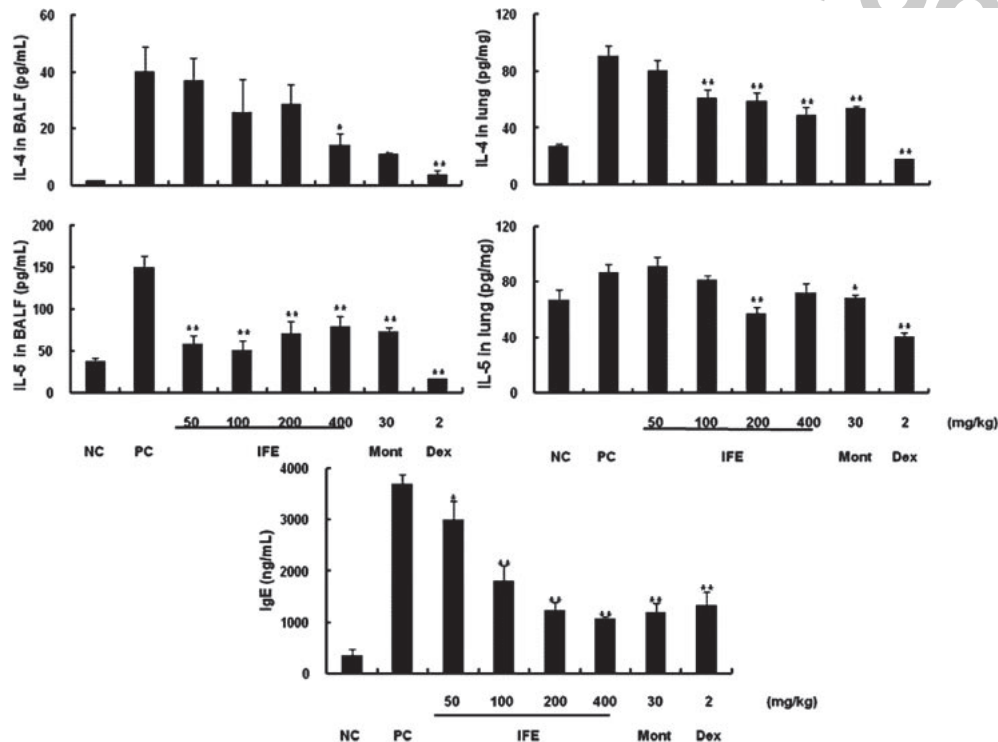


Fig. 3. Effect of IFE on the Protein Expression and Serum IgE Level.

BALF was collected 18 h after the final challenge to determine the protein levels by using ELISA (A). Blood was collected by cardiac puncture to measure the serum IgE (B). Data are expressed as the mean $\pm$ SEM ($n = 6$). ** $p < 0.01$, * $p < 0.05$ significantly different from the control as determined by one-way ANOVA.

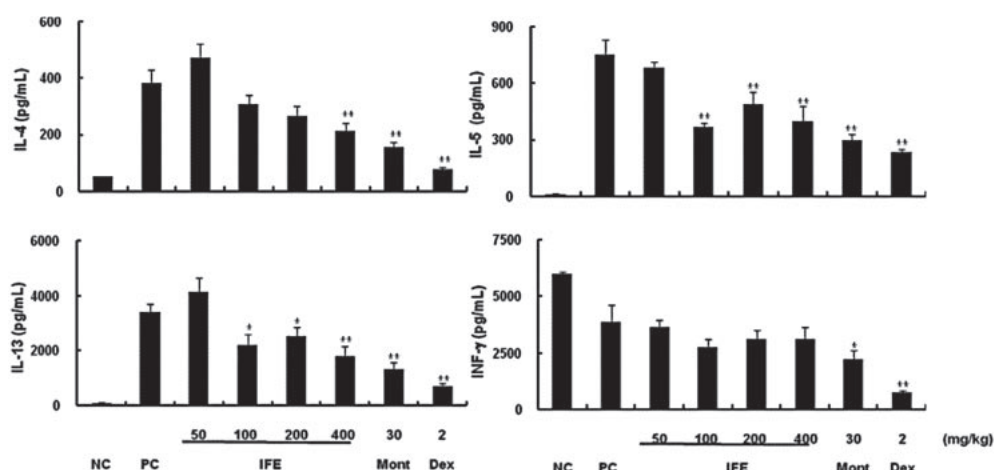


Fig. 4. Effect of IFE on the T-Cell Cytokine Response of Splenocytes.

The splenocytes were isolated and cultured in a medium with OVA (50 µg/mL). Supernatants were collected after 72 h. The levels of IL-4, IL-5, IL-13, and IFN-γ were determined by ELISA. Data are expressed as the mean ± SEM (n = 6). ***p* < 0.01, **p* < 0.05 significantly different from the control as determined by one-way ANOVA.

response. However, IFN-γ secretion by splenocytes from the IFE-treated mice was not significantly different from that of cultures from the OVA-induced mice.

Histological analysis of lung tissue

The infiltration of leukocytes into lung tissue was induced as an index of asthma development. We stained lung tissues with H&E and PAS staining solutions to examine the inhibitory effect of IFE on the histological change in the OVA-induced asthma model. The lungs of the OVA-induced mice showed dense perivascular and peribronchial infiltration of leukocytes and mucus secretion within the bronchi when compared to the control tissue (Fig. 5). IFE suppressed the increased infiltration of leukocytes and the mucus secretion. The inflammation and mucus score revealed that IFE had an inhibitory effect against airway inflammation.

Discussion

Glucocorticoids are currently the most effective drugs available for treating asthma. Their molecules act through several mechanisms,⁹⁾ but they have major side effects that limit their therapeutic efficacy when they are administered for a prolonged period. Although inhaled corticosteroids have greatly reduced the side effects, long-term trials with children have shown that problems still exist, even at low doses.¹⁰⁾ A safe alternative to corticosteroids is therefore needed as an anti-asthmatic agent.

Among the activated cells in the pathogenesis of allergic asthma, eosinophils are the most prominent cell type, and an increased number in the airways is correlated with the severity of asthma;⁵⁾ accordingly, their selective elimination is often a target for the treatment of asthma. The recruitment of eosinophils into BALF was clearly observed as expected in the OVA-induced mice. However, when treated with IFE, the recruitment of leukocytes, particularly eosinophils, into the airways was reduced (Fig. 1). These findings indicate that IFE had a preventive effect on the airway allergic inflammation model.

AHR has long been considered as a potential target for treating asthma.¹¹⁾ The effect of IFE on AHR to

methacholine was investigated in this study by monitoring the airway resistance. Our results reveal that mice showed a significant reduction in AHR in response to treatment with IFE at concentrations of 200 and 400 mg/kg (Fig. 2), implying the possibility of using IFE for asthma intervention as a bronchodilator.

Th2 cytokines activate the inflammatory pathway in the pathogenesis of asthma both directly and indirectly.¹²⁾ Since IL-4 causes class switching in B cells to synthesize IgE production and IL-5 promotes the differentiation to and activation of eosinophils, the effects were determined of IEF on the expression of Th2 cytokines in BALF and the lungs of mice. The results show that the expression of IL-4 and IL-5 were increased by the OVA-challenge, and that this increase was prevented by the IEF treatment (Fig. 3). The decreased IL-4 and IL-5 levels respectively inhibited IgE secretion from B cells and reduced eosinophil infiltration into the airways and lungs of the OVA-induced mice, indicating that the protein expression of the Th2 cytokines was prevented by the IFE treatment. The histological analysis demonstrated the inhibition of leukocyte infiltration and mucus secretion into the lung tissue of the OVA-induced mice (Fig. 5).

Th2 cells lead to an allergic response in the immune response of asthma. Since IFN-γ has been reported to inhibit the synthesis of IgE and the differentiation of precursor cells to Th2 cells, an increased level of IFN-γ has been used to explain the anti-asthmatic effect of therapeutic agents.^{13,14)} We have demonstrated in the present study that IFE reduced Th2 cytokine production (IL-4, IL-5, and IL-13) in cultured splenocytes, whereas it did not increase IFN-γ production in cultured splenocytes when compared with the OVA-induced mice (Fig. 4). In fact, other data have suggested that IFN-γ contributed to the activation of eosinophils and was likely to augment inflammation.^{15,16)} IFE therefore did not influence the IFN-γ production in cultured splenocytes when compared with OVA-induced mice, indicating the minimal effect of IFE on Th1 cells.

We used Dex as a reference drug in this study because it has always strongly inhibited the OVA-induced airway inflammation in a murine model of asthma.

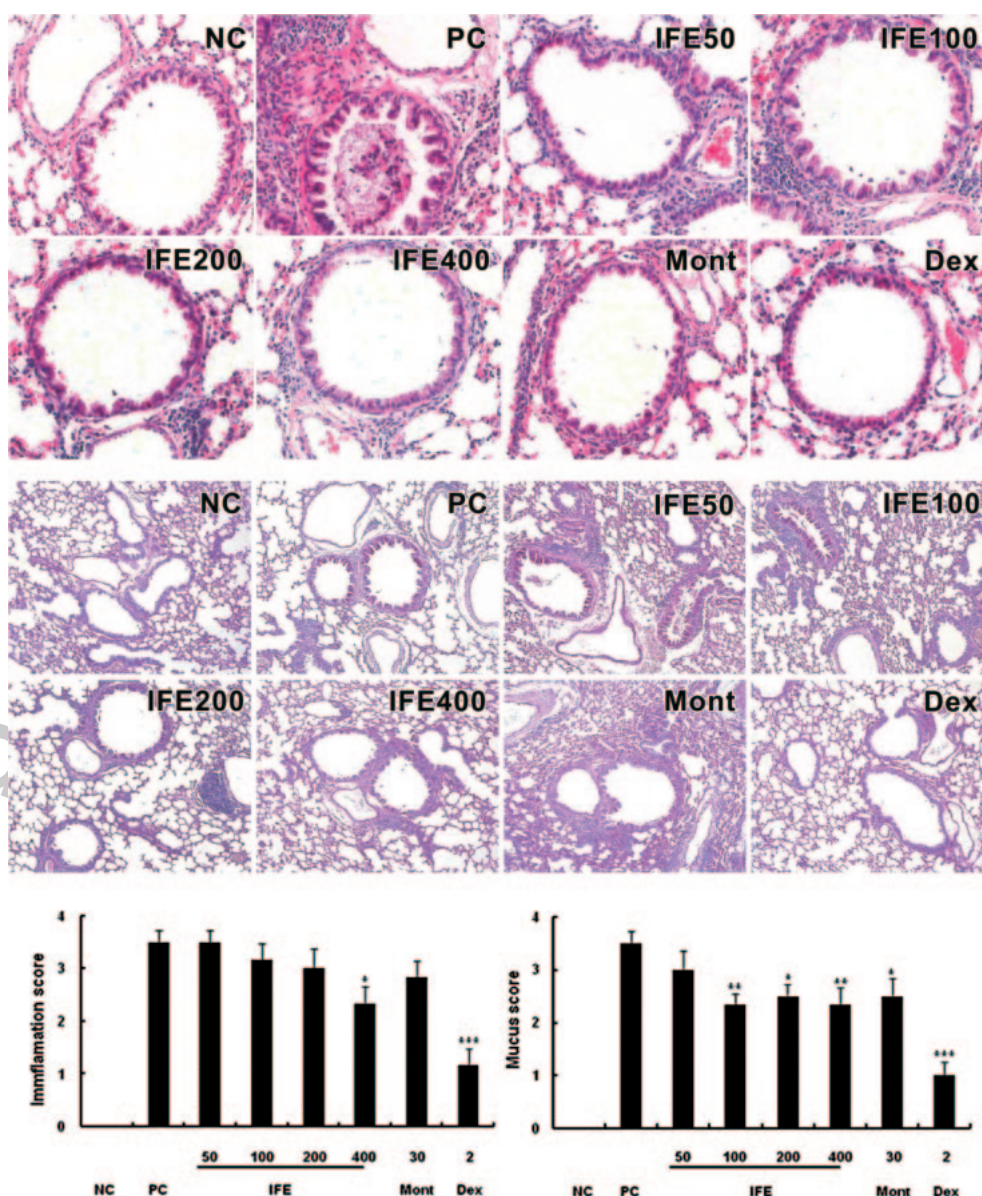


Fig. 5. Histological Analysis of the Lung Tissue.

The lungs were removed 18 h after the OVA challenge, and a histological analysis was conducted. Lung sections of the control (NC, PBS-induced; PC, OVA-induced) mice, and of asthmatic mice treated with IFE, Mont, or Dex were stained with H&E (A, magnification $\times 200$) or PAS (B, magnification $\times 100$). The scored cell infiltration and mucus production in the tissues were calculated as described in the Materials and Methods section (C). Representative photomicrographs of lung sections are shown ($n = 6$).

However, the other reference, Mont was not as effective as Dex and did not always reduce the recruitment of eosinophils into BALF in the murine model of asthma on this occasion. Otherwise, treatment with Mont attenuated the levels of Th2 cytokines in the BALF and lung, serum IgE, Th2 cytokine secretion by the splenocyte culture and mucus secretion into the lung tissue of OVA-induced mice in this experiment.

Although other studies have demonstrated that Mont, a leukotriene receptor antagonist, ameliorated AHR and inflammation in a mouse model of allergic asthma, we could not show any suppression of eosinophil infiltration into the airways.^{17,18} Dex and Mont have generally been used as reference drugs in the OVA-induced asthma model. However, Mont did not reduce the factors related to AHR and inflammation in our laboratory, probably because of drug solubility and technical problems, and we are trying to find out the reason.

Various plant extracts have been used in traditional medicine to treat allergic asthma, and their activities have been demonstrated.^{13,19,20} However, the effects of *Inulae flos* have not previously been elucidated in an asthma model. We have demonstrated for the first time in this study that IFE inhibited the OVA-induced airway inflammation, and that this probably occurred due to decreases in leukocyte infiltration, AHR, Th2 cytokines and IgE production, as well as to mucus secretion in a mouse model of asthma. The effects of the compounds isolated from IFE on their anti-inflammatory activities will be further studied in detail.

Acknowledgments

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