RG-II from Panax ginseng C.A. Meyer suppresses asthmatic reaction

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INTRODUCTION

Asthma is a chronic inflammatory disease affecting the airways that is characterized by recurring symptoms, including reversible airflow obstruction and bronchospasm (1). Asthma episodes are thought to be caused by a combination of genetic and environmental factors such as allergens, tobacco smoke, and emotional stress (2, 3). As a model, ovalbumin (OVA)-induced asthma is characterized by airway hyperresponsiveness (AHR) and airway inflammation (4), and is closely associated with the accumulation of eosinophils, neutrophils, and lymphocytes in the bronchial lumen and lung tissues (4). These cellular infiltrates release various chemical mediators capable of inducing AHR (5, 6). Additionally, recruitment of these inflammatory cells from the blood to sites of inflammation is regarded as a central event in the development and prolongation of airway inflammation (7).

The roots of P. ginseng are precious since the plant requires 4-6 years to harvest, whereas the leaves can be harvested every year. If the leaves of P. ginseng had similar pharmacological activity as the roots, much more of the therapeutic compounds could be available for clinical use. Previous studies have reported that polysaccharides from the leaves of P. ginseng possess potent anti-complementary (8) and anti-ulcer activities (9), indicating the potential clinical value of the leaves.

Antigen-activated CD4+ T cells are able to differentiate into different types of effector cells, each with distinct functional properties conferred by cytokines (10, 11). The T helper 2 (Th2)-type cytokines interleukin (IL)-4, IL-5, and IL-13 are produced by activated CD4+ T cells. Dendritic cells played an important role in determining the fate of naïve T cells into either Th1 or Th2 cells. We determined whether RG-II regulates the Th1/Th2 immune response by using an ovalbumin-induced murine model of asthma. RG-II reduced IL-4 production but increased interferon-gamma production, and inhibited GATA-3 gene expression. RG-II also inhibited asthmatic reactions including an increase in the number of eosinophils in bronchoalveolar lavage fluid, an increase in inflammatory cell infiltration in lung tissues, airway luminal narrowing, and airway hyperresponsiveness. This study provides evidence that RG-II plays a critical role in ameliorating the pathogenic process of asthmatic inflammation in mice. These findings provide new insights into the immunotherapeutic role of RG-II in terms of its effects in a murine model of asthma. [BMB reports 2012; 45(2): 79-84]
by binding directly to the promoters of IL-5 and IL-13, as well as by affecting chromatin remodeling, resulting in opening of the IL-4 locus (18). The T-box transcription factor T-bet (Tbx21) has emerged as a key regulator of dendritic cells as well as the type 1 immune response, playing an essential role in establishing effector cell fate in T and B lymphocytes (19). T-bet expression is induced in T<sub>1</sub> cell<sub>s</sub> but not T<sub>2</sub> cells, upon signal transduction, and acts as a potent transactivator of the IFN-γ gene in T<sub>1</sub>, NK, and B cells (20). Therefore, we investigated the effects of RG-II on T-bet and GATA-3 expression in a murine model of asthma.

In this study, administration of RG-II before the final airway OVA challenge resulted in significant inhibition of asthmatic reactions, suggesting that RG-II could play a critical role in the improvement of the pathogenic processes of asthma in mice.

**RESULTS**

**RG-II inhibits AHR, lung inflammation, and inflammatory cell infiltration**

Airway responsiveness was measured as a Penh value in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of the Penh value was shifted to the left compared to that of control mice (Fig. 1A). In addition, the Penh value produced by methacholine administration (at doses ranging from 2.5-50 mg/ml) was significantly higher in the OVA-sensitized and -challenged mice compared to controls. In OVA-sensitized and -challenged mice treated with RG-II, the dose-response curve of the Penh value was shifted to the right compared to that of untreated OVA-sensitized and -challenged mice. Moreover, the shift was dose-dependent.

Histological analyses revealed the typical pathological features of asthma in OVA-exposed mice compared to control mice, with the OVA-exposed mice displaying numerous inflammatory cells, including infiltrated eosinophils around the bronchioles (Fig. 1B). Mice treated with RG-II showed a marked decrease in inflammatory cell infiltration in the peribronchiolar and perivascular regions (Fig. 1B). Therefore, the increases in total lung inflammation and cell infiltration were significantly inhibited by administration of RG-II. These results suggest that RG-II inhibits OVA-induced airway hyper-responsiveness and antigen-induced inflammation in the lungs, including the influx of eosinophils.

**RG-II reduces TH2 cytokine levels in lung tissues of OVA-sensitized and -challenged mice**

BAL fluids were obtained 24 hours after the final airway challenge. In OVA-sensitized mice, the levels of IL-4, IL-5, and IL-13 were significantly higher than those in control mice. Administration of RG-II reduced the secretion of IL-4 (Fig. 2A), IL-5 (Fig. 2B), and IL-13 (Fig. 2C). However, the levels of the Th1 cytokines IL-2, IL-5, and IL-13, as well as those of the Th1 cytokines IFN-γ (Fig. 2D) and IL-12 (Fig. 2E) were higher in OVA-sensitized and OVA-challenged mice compared to cytokine levels in saline-sensitized and -challenged control mice. These results indicate that RG-II treatment reduces Th1 cytokine levels, such as IL-4, IL-5, and IL-13, in BAL fluids.

**RG-II decreases IgE levels in the serum and the number of inflammatory cells in BAL fluid**

Since Th1 cytokines promote airway inflammation in asthma...
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by increasing IgE levels, which in turn favors the Th2 inflammatory pathway, we measured how much RG-II modulates serum IgE levels in OVA-induced mice. As shown in Fig. 3, serum IgE levels in OVA-induced mice were significantly higher compared to those in control mice. RG-II significantly decreased serum IgE levels (Fig. 3A), but not serum IgG2a levels (Fig. 3B). These data indicate that RG-II modulates the Th1/Th2 balance towards Th1 in an OVA-induced asthma model. The numbers of total cells, eosinophils, lymphocytes, and macrophages in BAL fluid increased significantly 24 hours after OVA challenge compared to the numbers of cells after saline inhalation (Fig. 3C). These increases in the numbers of inflammatory cells were significantly inhibited by RG-II administration.

RG-II reduces GATA-3 expression in lung tissues of OVA-sensitized and -challenged mice
Western blot analysis showed that GATA-3 and T-bet expression was significantly increased in lung tissues 24 hours after OVA challenge compared to control tissues. Administration of RG-II significantly inhibited this increase in GATA-3 expression (Fig. 4A). On the other hand, RG-II administration induced an increase in T-bet expression (Fig. 4B).

DISCUSSION
This is first study to demonstrate that RG-II ameliorates airway

Fig. 2. Effect of RG-II on Th1 and Th2 cytokine production. OVA-sensitized mice were treated as described in Materials and methods. BAL fluid was obtained 7 days after the final airway challenge as described by the manufacturer. IL-4 (A), IL-5 (B), IL-13 (C), IFN-\(
\gamma\) (D), and IL-12 (E) levels in the BAL fluid were measured by ELISA.

Fig. 3. Effect of RG-II on IgE and IgG2a levels in serum of OVA-treated mice and the number of total cells and different cell types in BAL fluids of OVA-treated mice. Blood was collected by cardiac puncture, and serum IgE (A) and IgG2a (B) levels were measured. IgE and IgG2a levels were analyzed by using ELISA (n=5). (C) Mice were sensitized with OVA on days 0 and 14 by i.p. injection of OVA emulsified in 1 mg of aluminum hydroxide. Three days later, mice were treated with vehicle (CON), OVA (OVA), OVA plus a low dose of RG-II (20 mg/kg, RG-II/L), or OVA plus a high dose of RG-II (100 mg/kg, RG-II/H). The mice were challenged for 30 minutes with a 5% (w/v) OVA aerosol in saline (or saline alone as a control) using an ultrasonic nebulizer. The BAL cells were collected 24 hours after OVA challenge. The results shown are from a single representative experiment of the total five experiments performed.

Fig. 4. Effect of RG-II on expression of GATA-3 and T-bet in the lungs of OVA-treated mice. Samples were obtained 7 days after challenge from mice treated with control (CON), OVA (OVA), OVA plus a low dose of RG-II (20 mg/kg, RG-II/L), or OVA plus a high dose of RG-II (100 mg/kg, RG-II/H) and then subjected to Western blot analysis with α-GATA-3 (A) and α-T-bet (B) antibody.
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RG-II profoundly inhibited asthmatic reactions, including leukocyte recruitment into the airway and lung inflammation. We also observed that RG-II regulates the Th1/Th2 balance by mediating the levels of T-bet and GATA3.

OVA-induced asthma is recognized as a disease caused by chronic airway inflammation, which is characterized by infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen. Presently, we observed that OVA-induced asthma increased eosinophil infiltration, thickness of the bronchial wall, and surface area of smooth muscle. However, these events were significantly inhibited by administration of RG-II. T-bet, a member of the T-box family of transcription factors, is a master determinant of the Th1 cell lineage (20, 21). Indeed, T-bet-deficient mice lack the Th1 immune response (22), which inhibits allergic responses (23, 24).

Moreover, ectopic expression of T-bet in murine Th2 cells directs activation of IFN-γ as well as upregulation of IL-12Rβ (25, 26). On the other hand, GATA-3 belongs to the GATA family of transcription factors. Six members (GATA-1 to GATA-6) of this family have been identified in birds, and homologues have been found in both mammals and birds. Based on their expression profiles and structures, GATA proteins may be classified as either hematopoietic (GATA-1 to GATA-3) (27) or non-hematopoietic (GATA-4 to GATA-6). Naïve CD4+ T cells express low levels of GATA-3. However, the expression of GATA-3 is dependent upon T cell lineage; it is markedly upregulated in cells differentiating along the Th2 pathway, whereas it is downregulated in cells differentiating along the Th1 pathway (28). These data demonstrate that RG-II inhibits the increase in GATA3 expression in OVA-sensitized and OVA-challenged mice (Fig. 4). Therefore, we can conclude that RG-II administration is a novel, selective way to simultaneously suppress GATA3 and increase T-bet expression in asthmatic reactions in vivo (29).

In addition, Th1 and Th2 cytokine production was examined in CD4+ T cells. RG-II inhibited the increase in the level of IL-2, a Th2 cytokine produced in OVA-sensitized and OVA-challenged mice. In contrast, RG-II increased the level of IFN-γ, a Th1 cytokine produced in mice administered RG-II. Taken together, these results strongly indicate that RG-II reduces allergic airway inflammation and hyperresponsiveness through modulation of the Th1/Th2 balance by suppressing GATA3 expression and increasing T-bet expression.

In conclusion, these data suggest that RG-II might offer a new therapeutic approach to the treatment of allergic airway diseases.

MATERIALS AND METHODS

Mice
Female, 6 to 8-week-old, BALB/c mice that were certified free of murine-specific pathogens were obtained from Charles River Laboratories (Yokohama, Japan). All experimental animals were maintained under a protocol approved by the Institutional Animal Care and Use Committee of Pusan National University Medical School.

Purification of RG-II
A crude polysaccharide fraction (GL-2) was prepared from the leaves of *Panax ginseng* by hot water extraction, ethanol precipitation, and dialysis (8). GL-2 was fractionated by a Cetavlon (cetyltrimethylammonium bromide) precipitation, and a weakly acidic polysaccharide fraction (GL-4) was obtained. The Fc receptor expression-enhancing polysaccharide (RG-II) was purified from GL-4 by anion-exchange chromatography on DEAE Sepharose CL-6B as described previously (30). To remove the colored materials in the polysaccharide, RG-II was further purified on a Q-Sepharose column (Cl- form). For this, the column was washed with water and eluted sequentially with 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M NaCl. The major fraction, which was eluted with 0.3 M NaCl, was further fractionated by gel filtration on a Bio-Gel P-30 column to obtain the purified GL-IIIb2 (yield: 5.8 mg/kg dry leaves).

Experimental protocol
Mice were sensitized intraperitoneally (i.p.) with 20 μg of OVA (Sigma-Aldrich, St. Louis, MO, USA) emulsified in 25 μl of aluminum hydroxide (Pierce, Rockford, IL, USA) on days 0 and 14. Mice were challenged for 30 min with OVA (5%) via the airway from days 20 to 22. Brochoalveolar lavage (BAL) fluid was obtained 24 hours after the final challenge. At the time of lavage, the mice (five mice from each group) were killed using an overdose of ether. The chest cavity was exposed to allow expansion, after which the trachea was carefully incubated and the catheter secured with ligatures. Pre-warmed saline solution was then slowly infused into the lungs and withdrawn. The aliquots were pooled and stored at 4°C. A portion of each pooled aliquot was then centrifuged, and the supernatants were kept at −70°C until use.

Administration of RG-II
Female BALB/c mice were injected i.p. with 20 mg/kg/day or 100 mg/kg/day of RG-II (in a 200 μl volume) everyday from days 17 to 19.

Total cell counting
Total cell numbers were counted with a hemocytometer. Smears of BAL cells prepared with Cytospin II (Shandon, Runcorn, UK) were stained with Diff-Quick solution (Merck, Darmstadt, Germany) for differential cell counting. Two independent and blinded investigators counted the cells using a microscope. Approximately 200 cells were counted in each of four random locations.

Immunohistochemistry
Twenty-four hours after the final challenge, lungs were removed from the mice following sacrifice. Prior to lung re-
moval, the lungs and trachea were filled by intratracheal administration of a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were then fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 μm-thick sections of fixed and embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and sequentially stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, KalamaZoo, MI, USA). To identify mucus-producing cells, lung sections were stained with periodic acid-Schiff (PAS) and Alcian blue (AB).

Determination of airway responsiveness to methacholine
Airway responsiveness in mice was measured 24 hours after the final challenge in an unrestrained conscious state, as previously described (9). Mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea), and baseline readings were taken for 3 minutes and then averaged. Aerosolized methacholine at various concentrations (2.5 to 50 mg/ml) was nebulized through an inlet of the main chamber. Enhanced pause (Penh), which was calculated according to the manufacturer’s protocol as (expiratory time/relaxation time-1) × (peak expiratory flow/peak inspiratory flow), is a dimensionless value that is a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. In this study, Penh was used as a measure of airway responsiveness to methacholine. Results are expressed as the percent increase in Penh following challenge with each concentration of methacholine, and the baseline Penh (after saline challenge) was set to 100%. Penh values taken for 3 minutes following each nebulization were averaged and evaluated.

Measurement of cytokines
Cytokine levels in BAL fluid was determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits from R&D Systems (Minneapolis, MN, USA) were employed for the measurement of IL-4, IL-5, IL-13, IL-12, and IFN-γ.

Western blot analysis
The lung tissues were homogenized, washed with phosphate-buffered saline (PBS), and incubated in lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich) to obtain extracts of lung and spleen proteins. The samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and the separated proteins were electrotransferred to polyvinylidene difluoride membranes. The blots were incubated with anti-GATA-3 antibody or anti-T-bet antibody overnight at 4°C. After washing, the blots were next incubated with horseradish peroxidase-conjugated secondary antibody. Following three washes with Tris-buffered saline containing Tween-20, immunoreactive bands were visualized using an enhanced chemiluminescence detection system.

Measurement of OVA-specific serum IgE levels
OVA-specific serum IgE levels were determined in samples collected 24 hours after the final OVA challenge using ELISA. Briefly, a 96-well microtiter plate was coated with OVA (10 mg/ml), followed by incubation with mouse sera and then biotin-conjugated rat anti-mouse IgE (Pharmingen, San Diego, CA, USA). Then, avidin-horseradish peroxidase solution was added to each well. Units are shown as optical density readings at 405 nm.

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