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Single Component of Ginsenosides Modulates Immune Responses for Oxazolone-House Dust Mite

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Background: It has been reported that Korean Red Ginseng saponins were effective in increasing the synthesis of serum proteins, the cellular proliferation, the antibody production against sheep red blood cells, and in various cancer. However, it has not been reported yet about immunomodulating activity for allergens.
Objective: This study was aimed to find an immunomodulating activity of a single highly purified ginsenosides, Rb_1 and Rg_1, by using oxazolone-house dust mite (Ox-HDM) as allergen.
Method: BALB/c mice were immunized subcutaneously with Ox-HDM containing the alum or ginsenosides, Rb_1 and Rg_1, twice per 2 wk interval. Allergen-specific antibodies and IgG subclasses were determined from serum recovered by cardiac puncture 2 wk after the second immunization by using ELISA method. Allergen-specific cellular proliferation was quantified from splenic mononuclear cells obtained from spleens immunized. NK cell cytotoxicity was generated by co-culture of splenic mononuclear cells against YAC-1 cells as target cells. Hemolytic activity of Rb_1 and Rg_1 was determined by an *in vitro* assay using sheep red blood cells.
Result: BALB/c mice immunized with Ox-HDM plus Rb_1 or Rg_1 produced significantly higher titers of antibodies than mice immunized with alum-adsorbed allergen. Rb_1 remarkably increased titers in IgG_{2b} subclass but Rg_1 increased in IgG_{2a}. Allergen-specific proliferative response was more significantly increased with the use of Rb_1 than with alum-adsorbed allergen or Rg_1. Rb_1 reduced IL-4 production and increased IL-10 production more than alum-adsorbed HDM, but did not affect the IFN-γ and IL-12 production. However, Rg_1 showed weak responses in the production of cytokines. Both ginsenosides of high concentration increased the splenic mononuclear cells that were capable of killing YAC-1 cells. Rb_1 did not stimulate the production of reaginic antibody (IgE) but Rg_1 and alum induced. Rb_1 and Rg_1 did not show any hemolytic activity up to 500μg/ml.
Conclusion: The data suggest that the highly purified ginsenosides extracted from Korean Red Ginseng Radix, Rb_1, modulates antigen-specific immune responses via IL-10 production, that Rb_1 or Rg_1 may be an immunosurveillance in NK cytotoxic activity caused by allergen-specific immunotherapy. **(Korean J Asthma, Allergy Clin Immunol 2004;24:435-444)**

Key words: Ginsenosides, House dust mite, Splenocytes, NK cells, IgG subclass

INTRODUCTION

Panax ginseng is a precious Asian herb medicine and folk medicine for weakened physical strength and fatigue.[1)] It has been found that ginseng prevents stress reactions, facilitates learning, and improves memory[2)] and the aging animals.[3)] Several investigators reported that Panax ginseng was capable of enhancing the immune response in mice[4)] and preventing against cancer.[5)]

Triterpenoid saponins of damarane lineage, ginsenosides, and polysaccharides are major active ginseng constituents.[6)] The ginsenosides have first been isolated and chemically characterized by Shibata.[7)] Single component of ginsenosides, Rg_1, had immunostimulating activity.[8)] Mizuno et al[9)] reported that the mitogenic activity of wild-panax ginseng was almost equal to Conconavalin A, which was well-known as one of T cell mitogens. Hot water soluble fraction from wild Panax ginseng increased the cytotoxic T cell population in the oral administration. The bioginseng of Panax ginseng was found to increase the content of antibody producing cells developed in response to sheep erythrocyte administration under conditions of acquired immunodeficiency.[10)]

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Various single component and functions of ginsenosides have recently been isolated and reported. Ginsenoside Rb_2 inhibited the stress-induced increase in plasma IL-6 level[11] and LPS-induced IL-6/TNFα production.[12,13] Rb_1 protected lung metastasis by their metabolic component Ml[14] and modulated OVA-specific immune responses.[15] The ginsenosides Rh_1 or Rh_2 reduced the proliferation of prostate cancer via MAP kinases, and induced cell apoptosis.[16] Rg_1 was a functional ligand of glucocorticoid receptor in FT02B cells.[17] Recently, Rc or Rg_3 alleviated pain from noxious chemicals,[18] and has antinociception[19] and a neuroprotective role.[20]

The house dust mite (HDM) allergen is a major source of allergen affecting allergic rhinitis, atopic dermatitis, and bronchial asthma. It has been recently reported that proteases associated with HDM emanations stimulated the release of mediators of the allergic response from mast cell in the absence of allergen-specific IgE.[21] Exposure to HDM allergen (Der p) induced the recruitment and activation of eosinophils,[22] and the production of IL-4, IL-5, IL-8, IL-13 in mite-sensitive asthmatic basophils.[23-25] However, its pathophysiology, prevention, and a perpetual cure have not been completely defined, although most allergic diseases in human are caused by HDM allergen. Recently, allergen-specific immunotherapy has been widely used as a specific treatment of allergic diseases for many years. A variety of changes in immunological parameters have been described but it still remains uncertain as to which of them is responsible for the improvement of symptoms.

Based on described above, ginsenosides possess a broad spectrum but opposite pharmacological activities in some cases. A number of studies have been reported to elucidate the effect of individual ginsenosides on the immune systems and other organ systems. However, the effect of single ginsenoside on the immune responses of HDM allergen causing various allergic diseases and bronchial asthma were not reported yet.

Therefore, this study was aimed to examine the immunomodulating activity of individual ginsenoside, Rb_1, which represents compounds identified from protopanaxadiol, and Rg_1, which represents compounds identified from protopanaxatriol, by mediation of HDM allergen.

MATERIALS AND METHODS

1. Materials

Female BALB/c mice aged about 8 wk were used (Samyeuk animal Co., Osan, Korea). Animals were maintained along with "Principles of Laboratory Animal Care". YAC-1 cells from American Type Culture Collection (Rockville, MA); [6-^{3}H]thymidine (s.a., 24.0 Ci/mmol) from Amersham Pharmacia Biotech (Uppsala, Sweden); [$Na_2{}^{51}CrO_4$] (s.a., 400～1,200 Ci/g, 37 MBq) from New England Nuclear (Bedford, MA); RPMI 1640, Fetal bovine serum (FBS), Penicillin-Streptomycin from GIBCO BRL (Grand island, NY); aluminum hydroxide ($Al(OH)_3$, alum, Alhydrogel), from sigma chemicals (St. Lous, MO); sheep red blood cell from Korea Media (Seoul, Korea); ELISA kit (Mab-based mouse Ig isotyping kit) from Pharmingen (San Diego, CA); oxazolone from Fisher Scientific Co. (Pittsburgh, PA); ginsenosides, Rb_1 and Rg_1, from Korean Tobacco & Ginseng Institute (Taison, Korea).

2. Allergens

We used Ox-HDM as allergen conjugated to hapten. HDM cultured and extracted by the department of internal medicine was supplied from Dr. Hong CS (Yonsei University College of Medicine, Seoul, Korea), and then HDM was conjugated with oxazolone (Ox-HDM).

3. Preparation of oxazolone-protein (HDM) conjugates

Preparation of hapten-protein conjugates was performed as detailed previously.[26] Briefly, 10 ml of HDM protein extract (20 mg/ml) was adjusted to pH 9.0 at 25°C by addition of 5% Na_2CO_3. Then 0.5 ml of 10% oxazolone in dioxane was added dropwise while the pH was maintained at 9.0 by addition of 5% Na_2CO_3. After 2 hr of stirring, the mixture was dialyzed extensively against phosphate buffered saline, pH 7.4 (PBS). The dialyzed material was concentrated by negative pressure filtration. The protein quantity was determined by the Micro-Kjeldahl method.

4. Immunization procedure

Female BALB/c mice, 8 wk of age, were divided in groups

(control, Ox-HDM alone, alum-adsorbed Ox-HDM, Ox-HDM plus Rb_1, and Ox-HDM plus Rg_1 for each treatment). The concentration of allergen used was 50μg of Ox-HDM. The concentration of adjuvant was 125μg alum and 50μg, 100μg, 300μg Rb_1 or Rg_1. Mice were immunized via subcutaneous injection twice at 2 wk intervals. Mice were sacrificed on 2 wk after secondary immunization and blood was collected. Six mice in each group were used.

5. Measurement of allergen-specific antibodies

The blood was collected from the cardiac puncture under ether anesthesia. The serum was separated by centrifugation. Antibody titers in serum were determined by ELISA method. The plates were coated overnight at room temperature with 100μl Ox-HDM (2μg/ml) for antigen-specific IgG or with 50μl isotype-specific rat anti-mouse monoclonal antibody for each isotype antibody (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, IgA, IgE) diluted by coating solution. Plates were washed with 0.05% tween 20 in phosphate-buffered saline (PBST) and blocked with 200μl 1.0% BSA for 2 hr at room temperature. Plates were washed with PBST and added 100μl of diluted serum (400×dilution). The plates were incubated for 1 hr at room temperature, washed with PBST. Rabbit anti-mouse IgG antibody for antigen-specific IgG or 50μl of alkaline phosphate (AKP)-labeled polyclonal rat anti-mouse Igs antibody solution for isotype antibody were added and incubated for 1hr at room temperature. Plates for antigen-specific IgG were washed with PBST and incubated with streptavidin-HRP (horse radish peroxidase) for 1 hr at room temperature and washed with PBST. One hundred microliters of substrate TMB (3,3',5,5'-tetramethyl benzidine) solution was added and incubated at room temperature for 30 min. Reaction was stopped by 50μl of 2.5N H_2SO_4. Plates for isotype antibody were washed five times with PBST. Phosphatase substrate (50μg) dissolved with p-nitrophenyl phosphate (p-NPP) (5 mg phosphatase substrate were dissolved with 5.0 mL p-NPP substrate diluents) was added and incubated at room temperature for 30 min. Absorbance was measured at 490 nm.

6. Isolation of splenic mononuclear cells

Spleens were aseptically taken from the immunized mice after collection of blood and dispersed by pressing through a metal mesh. Dispersed splenocytes were collected into the 50 ml conical tube by passing it through the nylon mesh and then centrifuged at 800×g for 10 min. The residual red blood cells in splenocyte pellets were lysed by using Tris-NH_4Cl (0.17 M Tris and 0.16 M NH_4Cl, pH 7.6) solution and immediately made isotonic solution by adding 2x RPMI-1640 medium. The isolated splenic mononuclear cells were resuspended in RPMI-1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100μg/ml of streptomycin. The cells were used for antigen-specific cellular proliferation and NK cytotoxicity assay.

7. Measurement of allergen-specific cellular proliferation

Isolated splenic mononuclear cells were plated at 1×10^6 cells/100μl/well in round-bottom plates and co-cultured with 0.1μg Ox-HDM for 144 hr at 37°C in a CO_2 incubator. Oneμci/10μl of [^{3}H]thymidine was added to the each well 16 hr prior to the end of culture. At the end of incubation, cells were harvested and then [^{3}H]thymidine incorporation was measured by a liquid scintillation counter.

8. Measurement of interleukin-4 (IL-4), IL-10, IL-12 and IFN-γ production

Immunized splenic mononuclear cells were plated at 1×10^6 cells/100μl/well in plates and cocultured with 0.1μg OVA for 4 days. Cytokines (IL-4, IL-10, IFN-γ, IL-12) secreted into media was measured by ELISA method.

9. Cytotoxicity assay (NK cell activity) for splenic mononuclear cells

NK activity was measured as splenic cytolytic activity against YAC-1 (NK cell-sensitive lymphoma cell line) as a target cells.[27] YAC-1 cells at a concentration 1×10^6 cells were labeled for 2 hr in RPMI-1640 medium containing 100 μCi of [$Na_2{}^{51}CrO_4$]. After incubation time at 37°C in a CO_2 incubator, cells were washed three times with RPMI- 1640 medium and then plated 1×10^4 cells/well. Splenic mononuclear cells and target cells (cell ratio 25 : 1) were

cultivated for 5 hr in RPMI-1640 medium. To determine the low (spontaneous) control release, only YAC-1 cells were incubated in RPMI-1640 medium. The maximum release was determined by incubating target cells with 5.0% triton X-100. The radioactivity releasing in the supernatant after the lysis was measured by a gamma scintillation counter. Percent specific lysis was calculated by following formula. % specific lysis = [(experimental ^{51}Cr release-spontaneous ^{51}Cr release)/(maximum ^{51}Cr release-spontaneous ^{51}Cr release)]×100. Spontaneous release ranged from 8 to 26% of the maximum ^{51}Cr release.

10. Hemolytic activity of Rb_1 and Rg_1

Hemolytic activities of Rb_1 or Rg_1 were determined by an assay using sheep red blood cells (SRBC) *in vitro*.[28] First, the Rb_1 or Rg_1 was diluted by two serial dilution from 500 μg/mL up to 4μg/ml with PBS. Five milliliters of SRBC in Alsever's solution (Korea Medical Co., Korea) were spun at 800×g for 10 min. This pellet was washed three times, and resuspended with PBS to a final 4% suspension (v/v). Fifty microliters of two-fold serial diluted Rb_1 or Rg_1 were added to each well of a 96-well round bottom microliter plate (Beckon Dickinson, U.S.A.). Then, 150μl of the washed SRBC suspension was added to each well, and mixed with the Rb_1 or Rg_1. After incubation for 30 min at 37°C, the plate was centrifuged at 1,000×g for 10 min. One hundred microliters of the supernatant from each well were transferred to the wells of a flat bottom microtiter plate. The absorbance was determined at 405 nm using an Emax microplate reader (Molecular Devices, U.S.A.).

11. Statistic analysis

Experimental data were shown as mean±S.E. An analysis of variance (ANOVA) was used for statistical analysis. Analysis of significance between each control group and experimental group was carried out with the Scheffe method. When *P* values were less than 0.05, 0.01, and 0.001, it was considered significant.

RESULTS

1. Characterization of Rb_1 and Rg_1

Rb_1 and Rg_1 are ginsenosides (saponins), which are glycoside obtained from ginseng. It has chemical structure of triterpenoid damarane backbone different from that of general saponins. The purification procedure and the characteristics of Rb_1 were already shown by Ro et al[26] and Rg_1 was purified by using the similar method. Rb_1 and Rg_1 were characterized by the following characteristics: white powder, purity 98%, melting point 201～202°C for Rb_1 and 194～196.5°C for Rg_1, molecular formula $C_{54}H_{92}O_{23}$ and $C_{42}H_{72}O_{14}$, molecular weight=1108 and 800, glucose/aglycone ratio (%)=648/460 (58.5/41.5%) and 324/476 (40.5/59.5%), respectively.

2. Immune responses of Rb_1, Rg_1, and aluminum hydroxide (alum)

The Rb_1 or Rg_1 was compared for effectiveness as an immunostimulator with aluminum hydroxide (alum), which is suitable for use in human. Rb_1 or Rg_1 was selected over the dose of 50μg, an amount that falls in the plateau of maximum effect. Allergen-specific IgG titers increased significantly with the two immunizations in all groups. The IgG titers increased more in immunization with 50μg Ox-HDM plus either Rb_1 or Rg_1 than with either Ox-HDM alone or alum-adsorbed Ox-HDM. The titers (1.402±0.112 or 1.404±0.103) observed in the group received Rb_1 or Rg_1 (100μg) showed higher response than in either the group received allergen alone (0.781±0.400) or the group received alum-adsorbed Ox-HDM (0.980±0.004) (Fig. 1). The titers observed by alum-adsorbed Ox-HDM did not show higher response than that observed by allergen alone. Rb_1 increased antigen-specific IgG titers in dose-dependent manner, but high concentration of Rg_1 (300μg) did not.

3. Isotype of antibodies augmented by Rb_1 or Rg_1

Rb_1 or Rg_1 that augments allergen-specific total IgG titers may be different considerably in various IgG subclasses. Therefore, after two subcutaneous immunizations with Ox-HDM plus Rb_1, Rg_1 or alum, antibodies were determined in terms of IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3), IgM, IgA, and IgE (Table 1). IgM and IgA antibody titers did not change by the treatment of both ginsenosides (data not shown). After two immunizations with Ox-HDM plus Rb_1 or Rg_1, predominant IgG isotype was IgG_{2b} or IgG_{2a} antibody (Table 1). In contrast, antibodies induced by

alum were predominantly IgG_{2b} and IgE antibodies. The use of Rb_1 did not elicit the reaginic antibody, IgE, while Rg_1 induced IgE antibody (Table 1).

4. Proliferative responses of splenic mononuclear cells from the mice immunized with Rb_1 or Rg_1

It was reported that ginsenoside Rg_1 induced the humoral and cellular immune responses.[14] Thus, we examined the effect of Rb_1 or Rg_1 on the cellular response using the measuring lymphocyte proliferations caused by Ox-HDM allergen. The allergen-specific cellular proliferative response in BALB/c mice immunized is shown in Fig. 2. Ox-HDM alone increased allergen-specific lymphocyte proliferation, but significant proliferation was not observed with alum-adsorbed allergen, compared to Ox-HDM immunization. However, low concentration of Rb_1 showed the highest capacity to induce allergen-specific lymphocyte proliferation when stimulated with Ox-HDM plus Rb_1, but Rg_1 did not.

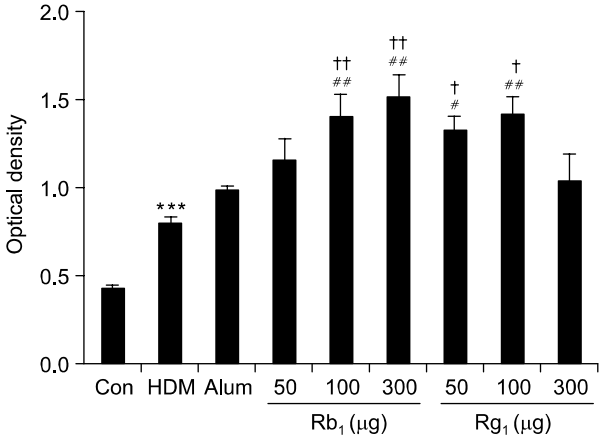

Fig. 1. Allergen-specific IgG titers induced in BALB/c mice by two subcutaneous immunization with Ox-HDM and the indicated dose of Rb_1 or Rg_1. Antibody against Ox-HDM was measured by ELISA using microtiter plates coated with Ox-HDM allergen. Sera were diluted by 1 : 400. Results are expressed as means±S.D. Con, non-immunized; HDM, Ox-HDM. ***, $P<0.001$ by comparison with Con. #, $P<0.05$; ##, $P<0.01$ by comparison with Ox-HDM. †, $P<0.05$; ††, $P<0.01$ by comparison with alum-adsorbed allergen.

5. The production of cytokines in allergen-induced responses ex-vivo

We examined whether Rb_1 or Rg_1 modulated the production of cytokines secreted from Th1 and Th2 cells. Rb_1 inhibited IL-4 production and remarkably increased IL-10 production, but did not affect the production of IFN-γ production, compared to control or alum (Table 2). Rg_1 weakly stimulated IL-10 production and weakly reduced the production of IL-4 (Table 2). However, IL-12 was not detected in the experimental condition.

6. Assessment of splenic NK cell cytotoxicity from the mice immunized with Rb_1 or Rg_1

Splenic mononuclear cells from mice immunized with Rb_1 or Rg_1 were capable of killing YAC-1 cells, which were known as NK-sensitive lymphoma cell line. The percentage

Table 1. IgG subclasses and IgE antibody titers of sera obtained after active immunization of BALB/c mice with each adjuvant adsorbed-house dust mite (HDM) allergen[a]

	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgE
None	1.50±0.04[b]	1.48±0.10	1.67±0.10	1.27±0.02	0.49±0.18
HDM	1.78±0.17	1.51±0.10	1.55±0.51	1.47±0.21	0.92±0.12
Ox-HDM+Alum	1.76±0.19	1.47±0.11	2.45±0.14*	1.45±0.19	1.87±0.13*
Ox-HDM+Rb_1					
50μg	1.81±0.19	1.52±0.08	3.48±0.19†	1.39±0.31	0.89±0.38†
100μg	1.68±0.12	1.52±0.04	3.15±0.13†	1.43±0.23	0.91±0.44†
300μg	1.48±0.10	1.38±0.09	2.40±0.46	1.40±0.18	0.73±0.24†
Ox-HDM±Rg_1					
50μg	1.64±0.07	2.29±0.01†	2.26±0.39	1.35±0.25	1.19±0.05*
100μg	1.70±0.04	1.49±0.14	2.40±0.55	1.38±0.17	0.85±0.17
300μg	1.61±0.08	1.43±0.11	2.60±0.46	1.40±0.13	1.47±0.06*

[a]Sera were obtained at 6 wk from Ox-HDM immunization in the absence or presence of alum and Rb_1 or Rg_1. Antibodies against Ox-HDM were measured by ELISA method. [b]The data (n=6) indicates optical density (O.D.) at 490 nm. *, $P<0.05$ by comparison with none (non-immunized mice); †, $P<0.05$ by comparison with alum-adsorbed Ox-HDM.

Table 2. Effects of Rb_1, Rg_1 or aluminum hydroxide on cytokine production by splenic mononuclear cells from Ox-HDM-immunized mice[a]

	IL-4 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
Control	4.2±0.12	6.5±0.25	6.2±3.71
Rb_1 alone	10.3±1.00	11.3±1.05	7.3±3.55
Rg_1 alone	11.0±1.19	10.9±0.99	25.4±4.75
Ox-HDM	65.5±9.14*	65.1±4.96*	28.3±8.51
Ox-HDM+Alum	95.4±5.18*	90.5±7.65*	27.2±7.27
Ox-HDM+Rb_1			
50μg	47.5±5.24†	110.7±5.22	29.2±5.12
100μg	37.6±4.72††	162.8±8.55†	25.4±6.01
300μg	35.7±9.91†	176.2±9.87††	20.4±9.67
Ox-HDM+Rg_1			
50μg	57.3±8.76	108.2±4.31	23.4±11.21
100μg	49.1±3.12†	115.4±6.77	27.6±9.14
300μg	55.1±7.45	125.4±10.20†	24.7±10.90

[a]Six BALB/c mice per group were immunized subcutaneously twice per 2 wk interval. Splenic mononucrear cells (1×10^6 cells/100μl/well) incubated 0.1μg of Ox-HDM, and were cultured for 4 days. Cytokines (IL-4, IL-10, IL-12, and IFN-γ) secreted into the media were measured by ELISA method. Control, nom-immunized. *, $P<0.05$ compared with control; †, $P<0.05$; ††, $P<0.01$ compared with control and alum-adsorbed Ox-HDM.

Table 3. The hemolytic activity of Rb_1 or Rg_1 on sheep red blood cells[a]

Dose (μg)	Rb_1	Rg_1
Saline	0.158±0.011[b]	0.138±0.007
16	0.123±0.012	0.134±0.011
32	0.123±0.012	0.131±0.0125
125	0.161±0.015	0.138±0.011
250	0.162±0.011	0.143±0.012
500	0.188±0.014	0.147±0.010

[a]Serial dilution of Rb_1 were made, added with 4% SRBC (150μl), incubated for 30 min at 37°C, and the released hemoglobin was determined at 405 nm (n=8). [b]The data indicates optical density (O.D.) at 405 nm.

of specific lysis of Ox-HDM was 5.53±1.42%, and non-stimulated mice was 3.54±0.77% (Fig. 3). However, the splenic NK cell cytotoxicity induced by the high concentration of Rb_1 or Rg_1 (300μg) augmented remarkably (23.85±3.84% for Rb_1; 19.66±2.60% for Rg_1), but the NK cell cytotoxic responses of Rg_1 was lower than those of Rb_1. The splenic NK cell cytotoxicity was not produced significantly in mice immunized with only alum-adsorbed allergen (Fig. 3).

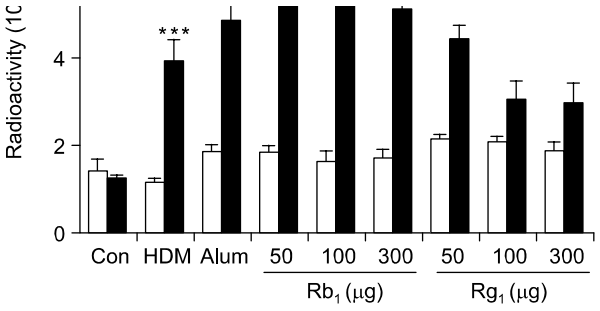

Fig. 2. Effects of Rb_1, Rg_1, and aluminum hydroxide on the proliferative response of splenic mononuclear cells to Ox-HDM. Six BALB/c mice per group were immunized subcutaneous twice per 2 wk interval. Splenic mononuclear cells (1×10^6 cells/100μl/well) included the 0.1μg Ox-HDM, and cultured for 144 hr. OneμCi of [^{3}H]thymidine was added to all wells 16 hr prior to harvesting the cultures. The cultured cells without Ox-HDM were used as negative control (None). Con, non-immunized; HDM, Ox-HDM. ***, $P<0.001$ by comparison with Con. $^{\#}$, $P<0.05$; $^{\#\#}$, $P<0.01$ by comparison with Ox-HDM. †, $P<0.05$ by comparison with alum- adsorbed Ox-HDM.

7. Toxic and hemolytic activities

Toxicity (assessed by lethality) has been associated with the use of various ginsenosides. However, Rb_1 and Rg_1 are

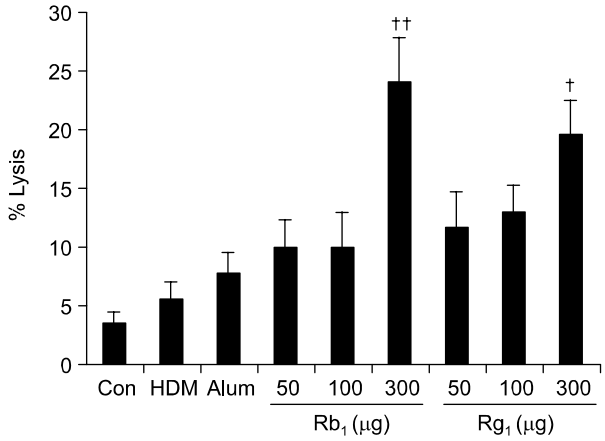

Fig. 3. NK cytotoxicity measured using YAC-1 as target cells. Splenic mononuclear cells as effector cells from BALB/c mice after two immunizations with Ox-HDM mixed with Rb_1, Rg_1, alum were co-cultured with YAC-1 for 5 hr. Responses were shown as percentage specific lysis using a titration of E/T ratio used, 25 : 1. Con, non-immunized; HDM, Ox-HDM. †, $P<0.05$; ††, $P<0.01$ by comparison with Ox-HDM and alum-adsorbed Ox-HDM.

apparently non-lethal up to 1.0 mg (data not shown).

The hemolytic activities of Rb_1 and Rg_1 were also assessed by using SRBC. The Rb_1 and Rg_1 did not show any hemolytic activity up to 500μg/ml (Table 3).

DISCUSSION

In this study, we demonstrated that Rb_1 or Rg_1 increased significantly allergen-specific immune responses to allergen (HDM) conjugated to carrier (oxazolone). We also demonstrated that Rb_1 or Rg_1 increased NK cell cytotoxic activity. Rb_1 did not have any monosaccharides galactose, rhamnose, fucose, arabinose, xylose, and glucuronic acid except for glucose. Rb_1 has only four glucoses and one aglycone, and Rg_1 has two glucoses and one aglycone. The various fractions purified from Quillaja Saponaria Molina bark contained the various monosaccharides although the fractions are consisted predominantly of one component.[28,29] Hence, Rb_1 or Rg_1 is structurally distinct from the previously identified other saponins.

Rb_1 and Rg_1 induced similar increase in allergen-specific IgG titers when used at doses ranging from 50 to 300μg except for the high concentration of Rg_1 (Fig. 1). The similar results have been reported by investigators who used the different antigens.[9] Rb_1 or Rg_1 was more effective than alum for augmentation of responses to Ox-HDM allergen. The strong antibody response elicited by allergen plus Rb_1 or Rg_1 may be due to the interaction between allergen and ginsenosides in the immune responses. It has also been supported that Rb_1 or Rg_1 has no cytotoxicity *in vitro* due to its non-lethality and no hemolytic activity (Table 3).

The effect of single component of ginsenosides on the allergen-specific isotype profile were not reported yet although there are many reports about QS 21 (saponin) purified from Quillaja Saponaria Molina bark as adjuvant for antibody formation.[28-32] We observed that Rb_1 or Rg_1 also influenced the allergen-specific isotype profile (Table 1). Isotypes produced by mice immunized with Ox-HDM plus Rb_1 showed induction of the major IgG subclasses, IgG_{2b}, but isotypes produced by Rg_1 showed IgG_{2a} (Table 1). Viral infections in mice induce an Ig response in which IgG_{2a} accounts for 65 to 92% of total specific antibody.[37] In mice, the antibody isotype associated with T-helper (Th) cell (IgG_{2a} and IgG_{2b}) is much more potent in inducing protection from challenge by *E. Coli*.[34] The isotype antibody profile induced by ginsenosides was similar to that induced by natural immunity arising from a viral or bacterial infection. Therefore, these results indicate that Rb_1 or Rg_1 enhances the immunogenicity to the conjugated allergen as well as induction a shift of IgG subclass produced.

Allergen-specific IgE was not detected by the immunization of Ox-HDM plus Rb_1, but alum-adsorbed-Ox-HDM or Ox-HDM plus Rg_1 was responsible for the production of reaginic antibody (Table 1). It can be inferred from this different result that Rb_1 releases slowly in site of administration because it is hardly adsorbed, and prolongs immune responses of allergen, but Rg_1 is rapidly adsorbed. However, the significance of this finding remains to be determined.

Rb_1 augmented the allergen-specific cellular proliferative response in BALB/c mice immunized with OX-HDM plus Rb_1, but Rg_1 did not (Fig. 2). The most significant proliferation by Ox-HDM plus Rb_1 was found in the low concentration of Rb_1. This finding suggests that Rb_1 induces highly numbers of allergen-primed lymphocytes in vivo, particularly Th cells or the responding cells have high affinity allergen receptors. These same mechanisms could also account for the increases in antibody responses, in which

the induction of Th cells has been shown to augment antigen-specific antibody response.[8] However, our result for Rg_1 is not in agreement with the reports by other researchers.[8,9] This different result can be suggested to be due to the differences of glucose binding site in Rb_1 and Rg_1 structures. However, it remains to be confirmed.

Since Rb_1, but not Rg_1, enhanced T cell proliferation, we examined whether Rb_1 modulates antigen Ox-HDM response. Rb_1 significantly inhibited IL-4 level without decrease of IFN-γ level, but enhanced the production of IL-10 (Table 2). There are a lot of reports that IL-10 is anti-inflammatory cytokine[35-38] and immunomodulating cytokine[39,40] in several diseases and cells, especially allergic airway inflammatory cells. It has also been reported that IL-10 strongly inhibits cytokine production and proliferation of $CD4^+$ T cells and T cell clones via its down-regulatory effects on antigen presenting cells (APC) function,[41] and inhibits the marked Th2 cytokine profile.[42] These effects were neither mediated by IFN-γ, nor involved with a Th2 to Th1 switch.[43] However, they were affected by the induction of $CD4^+CD25^+$ T cells as the suppressive immunoregulatory mechanism controlling antigen-specific T cell responses and the increased suppressive activity of $CD4^+$ $CD25^+$ T cells was associated with production of IL-10.[37-40] Therefore, our data can be inferred that Rb_1 increases the suppressive activity of $CD4^+CD25^+$ T cells via IL-10 production, and then IL-10 inhibits IL-4 production. We did not examine directly the function of $CD4^+CD25^+$ T cells. It needs further study.

There are many reports indicating that noxious stimulation or stress diminishes NK cell activity. NK cells are thought to play a significant role in immunosurveillance against viruses and tumors.[44] A decrease in NK cell cytotoxic activity was also found in various diseases.[44] The splenic cytotoxic effect by Rb_1 showed relatively high concentration of Rb_1 (Fig. 3). This supports the finding by See et al that NK cell activity is increased by ginseng.[45] We assume that NK cells are produced by Ox-HDM allergen containing Rb_1 or Rg_1, but its mechanisms are not yet found. The data suggest that Rb_1 or Rg_1 could be a valuable immunosurveillance to the immunotherapy of various allergic diseases.

CONCLUSION

Based on the results, it is suggested that the highly purified ginsenosides extracted from Korean Red Ginseng Radix, Rb_1 modulates antigen-specific immune responses via IL-10 production. It also suggests that Rb_1 or Rg_1 may be an immunosurveillance in the decrease in NK cytotoxic activity caused by allergen-specific immunotherapy.

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