

Stereospecificity of ginsenoside Rg3 in promotion of the immune response to ovalbumin in mice

Xiaojie Wei^{1,2}, Jian Chen¹, Fei Su¹, Xiaoyan Su³, Tingjun Hu² and Songhua Hu¹

¹Department of Veterinary Medicine, College of Animal Science, Zhejiang University, Hangzhou, Zhejiang 310058, China

²Department of Veterinary Medicine, College of Animal Sciences and Technology, Guangxi University, Nanning 530005, China

³Department of Veterinary Medicine, College of Animal Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China

Correspondence to: S. Hu; E-mail: songhua@zju.edu.cn

Received 4 January 2012, accepted 2 February 2012

Abstract

Our previous investigation demonstrated that ginsenoside Rg3 was active in promotion of the immune response. In this study, two epimers, 20(R)-Rg3 and 20(S)-Rg3, were compared for their adjuvant effects on the immune response against ovalbumin (OVA). BALB/c mice were immunized subcutaneously with 10 µg of OVA alone or with 10 µg of OVA mixed in 20(R)-Rg3 (50 µg) or 20(S)-Rg3 (50 µg) on days 1 and 15. Two weeks after the last immunization, blood was sampled to test IgG and the IgG subclasses as well as IFN-γ and IL-5; splenocytes were prepared to measure proliferative responses to stimulations with Con A, LPS and OVA and mRNA expressions of cytokines and transcription factors by reverse transcription-PCR. Results indicated that both 20(R)-Rg3 and 20(S)-Rg3 exhibited the adjuvant effect on OVA-induced immune responses. 20(R)-Rg3 promoted significantly higher serum-specific IgG and the IgG isotype responses in association with highly up-regulated serum IFN-γ and IL-5 than 20(S)-Rg3. In addition, 20(R)-Rg3 significantly enhanced splenocyte proliferative responses to Con A, LPS and OVA as well as mRNA expression of IFN-γ, IL-12, IL-4 and IL-10 and transcription factors T-bet and GATA-3 by splenocytes when compared with the 20(S)-Rg3. Therefore, ginsenoside Rg3 is stereospecific in stimulation of the immune response, and 20(R)-Rg3 has more potent adjuvant activity than 20(S)-Rg3.

Keywords: adjuvant, ginseng, ginsenoside Rg3, (R)- or (S)-stereoisomers, T_H1/T_H2

Introduction

Vaccination is one of the most cost-effectively preventive approaches against infectious diseases. The recombinant DNA derived subunit vaccines offer significant advantages over the traditional vaccines in term of safety and production cost. Nevertheless, they are poorly immunogenic due to the lack of innate immune stimulus and require addition of adjuvant to improve their efficacy (1). Therefore, searching for new adjuvants has become a hot topic (2).

Ginseng, the root of *Panax ginseng* C. A. Meyer (Araliaceae), has been utilized as a traditional medicine in China for thousands of years (2, 3) and is officially recorded in the Chinese Pharmacopoeia as well as in the Chinese Veterinary Pharmacopoeia (4, 5). Ginseng saponins, i.e. ginsenosides, are believed to be the main pharmacologically active constituents in *P. ginseng* (6). Currently, >40 ginsenosides have been identified and are classified into several types by their special chemical structures (protopanaxadiol, propanaxatriol and oleanolic acid) (7–9). Recent investigations showed that ginsenosides were biologically active in anti-tumor (10), anti-oxidant (11), anti-inflammatory (12) and adjuvant actions

(13–17). After comparing the various types of ginsenosides, Sun *et al.* (18) found that ginsenoside Rg3 was one of the potent vaccine adjuvants. In the ginseng root, two optical isomers of 20(R)-Rg3 and 20(S)-Rg3 were found (19). They are epimers of each other depending on the position of the hydroxyl (OH) group on carbon-20 (Fig. 1), and this epimerization is known to be produced by the selective attack of the OH group after elimination of the glycosyl residue at carbon-20 (19). Stereospecificity of Rg3 was observed for their pharmacological actions. 20(S)-Rg3 was reported to lower blood pressure by endothelium-dependent relaxation in rat aorta (20), induce Hela cell apoptosis (21) and inhibit metastasis of B16 melanoma in the lung of mice (22). 20(R)-Rg3 was found to inhibit the proliferation of hepatocellular carcinoma in SD rats (23) and have anti-fatigue effect in rats by increase in the storage of hepatic glycogen and decrease in the accumulation of metabolites such as lactic acid and serum urea nitrogen (24). It was also found that 20(R)-Rg3 had stronger anti-tumor activity than 20(S)-Rg3 (25). However, it is not yet known whether the adjuvant activity of Rg3 is stereospecific.

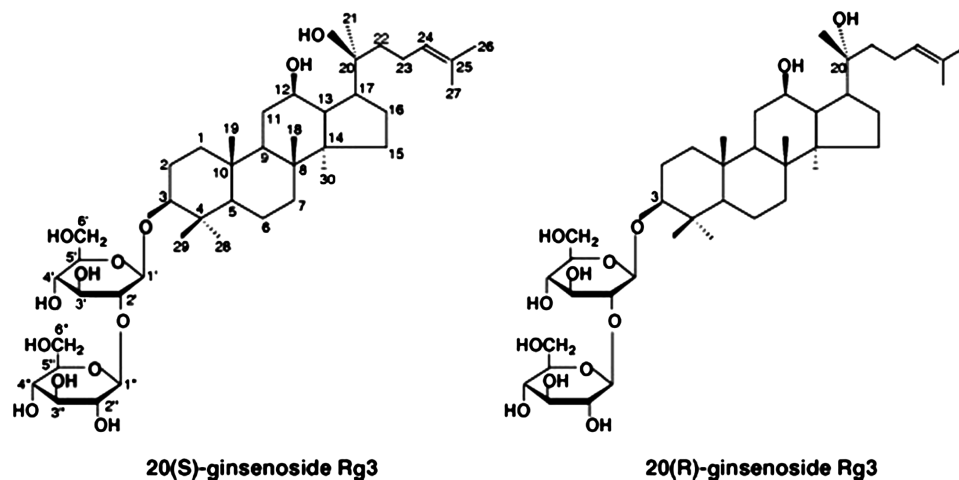


Fig. 1. Chemical structures of ginsenoside Rg3. (a) 20(S)-ginsenoside Rg3; (b) 20(R)-ginsenoside.

In this study, we compared 20(R)-Rg3 and 20(S)-Rg3 for their adjuvant effect on T_H1/T_H2 immune responses to ovalbumin (OVA) in mice. OVA was used as it is a model antigen commonly used to test the adjuvant effect of chemicals (26).

Methods

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Con A and LPS were the products of Sigma Chemical Co., Saint Louis, MO, USA; goat anti-mouse IgG peroxidase conjugate was from Kirkegaard & Perry Laboratories, Inc., USA; goat anti-mouse peroxidase conjugate IgG1, IgG2a, IgG2b and IgG3 were from Southern Biotech. Assoc., Birmingham, AL, USA; RNAisoTM plus was from TaKaRa Biotechnology (Dalian) Co., Ltd. China; revert AidTM M-MuLV reverse transcriptase was from Fermentas, USA; diethylpyrocarbonate (DEPC) and ribonuclease inhibitor was from Biobasic, Canada; Oligo (dT) 18 was from Sangon, China. 20(R)-ginsenoside Rg3 and 20(S)-ginsenoside Rg3 extracted from the root of *P. ginseng* were from Tauto Biotech (Shanghai) Co., Ltd. China. Both types of Rg3 were white powder at a purity of 98% with molecular weight of 785.01328 and molecular formula of $C_{42}H_{72}O_{13}$ (Fig. 1).

Animals care

Thirty-two female BALB/c mice (Grade II, 5 weeks old) weighing 16–18 g were purchased from Zhejiang Experimental Animal Center (Certificate No. 22-2001001, Hangzhou, China) and acclimatized for 1 week prior to use. The animals were housed in polypropylene cages with sawdust bedding in hygienically controlled environment with a temperature of $24 \pm 1^\circ\text{C}$, humidity of $50 \pm 10\%$ and a 12/12-h light/dark cycle. Feed and water were supplied *ad libitum*. Experiments on animals were performed based on animal ethic guidelines of Institutional Animal Ethics Committee.

Immunization

Thirty-two female BALB/c mice were divided into four groups, each consisting of eight mice. Animals were subcu-

taneously immunized with OVA 10 μg alone or OVA 10 μg mixed with 20(R)-Rg3 (50 μg) or 20(S)-Rg3 (50 μg); mice injected with saline alone served as a control. After 2 weeks, a boosting injection was administered. Blood samples were collected 2 weeks after the booster immunization for detection of OVA-specific antibody and western blot analysis. Splenocytes were harvested for determination of lymphocyte proliferation and cytokines mRNA expression.

Measurement of OVA-specific antibody

Serum OVA-specific IgG and the IgG subclasses were measured by an indirect enzyme-linked immunosorbent assay. The wells of polyvinyl 96-well microliter plates were coated with 100 μl of OVA solution (25 $\mu\text{g ml}^{-1}$ in 50 mM carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C . After washing with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 3% FCS in PBS and incubated for 2 h at 37°C . After another washing, 100 μl of series of diluted sera sample or 1% FCS as control were added in triplicate. After washing, aliquots of 100 μl of HRP conjugate of rabbit anti-mouse IgG (1:2000), goat anti-mouse IgG1 (1:500), IgG2a (1:500), IgG2b (1:500) or IgG3 (1:500) with 1% FCS were added and incubated at 37°C for 2 h. Plates were washed again with PBST. After that, 100 μl of 3,3',5,5'-tetramethyl benzidine solution (100 $\mu\text{g ml}^{-1}$ of 0.1 M citrate-phosphate, pH 5.0) was added and incubated for 15 min at 37°C . The reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. The optical density was measured by an automatic ELISA plate reader at 450 nm.

Splenocyte proliferation assay in vitro

Spleen was collected 2 weeks after the booster immunization under aseptic conditions and rinsed with HBSS (Sigma). The organs were minced and passed through a fine steel mesh to obtain a homogenous cell suspension, then added to 0.83% NH_4Cl in 0.01 M Tris-HCl (pH 7.2) to the suspension to lyse contaminated erythrocytes. After centrifugation ($380 \times g$ for 10 min at 4°C), a white cloud-like lymphocytes' band was collected and washed twice with RPMI 1640 media. Finally, the splenic lymphocytes were re-suspended

to 5×10^6 cells ml^{-1} of RPMI 1640 medium supplemented with 0.05 mM 2-mercaptoethanol, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% heat inactivated FCS. Cell viability was estimated according to the trypan blue exclusion method and the purity of splenic lymphocytes was >95% (27). After that, 100 μl of the cell suspension was added to a 96-well flat bottom microliter plate (Corning Costar Inc., USA). Thereafter, Con A (final concentration 5 μg ml^{-1}), LPS (final concentration 7 μg ml^{-1}), OVA (final concentration 100 μg ml^{-1}) or medium were added to give a final volume of 200 μl . The plates were incubated at 37°C in a humid atmosphere with 5% CO_2 for 48 h. At the 44 h, 50 μl of MTT solution (2 mg ml^{-1}) were added to each well. The plates were centrifuged ($1400 \times g$, 5 min) and the untransformed MTT was carefully removed with the use of a pipette. Dimethyl sulfoxide (DMSO) working solution (144 μl DMSO with 6 μl 1 M HCl) (150 μl) was added to each well. After 15 min for reaction, the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen cultures/the absorbance value for nonstimulated cultures.

Quantification of target genes by real-time PCR

Splenocytes were prepared as described above, and the cells were stimulated by incubation with OVA (final concentration at 100 μg ml^{-1}) in 24-well culture plates at 37°C in 5% CO_2 . After 15 h, the plate was centrifuged at $380 \times g$ for 5 min and washed with PBS, and splenocytes were collected for RNA extraction. Splenocytes were lysed in 1 ml of RNAiso™ Plus reagent and the total RNA was isolated according to the manufacture's protocol. The concentration of total RNA was quantified by determining the optical density at 260 nm. The total RNA was used for reverse transcription by mixing 2 μg of RNA with 0.5 μg Oligo(dT)₁₈ primer in a sterile tube. Nuclease-free water was added giving a final volume of 12.5 μl . This mixture was incubated at 70°C for 5 min and chilled on ice for 2 min. The solution containing 4 μl of M-MuLV 5 $\times$ reaction buffer, 2 μl of 10 mM dNTP and 20 U of ribonuclease inhibitor, and DEPC-treated water was then added to give a final volume of 19 μl and were incubated for 5 min at 37°C. After addition of 200 U of M-MuLV reverse transcriptase, the tubes were incubated for 60 min at 42°C. Finally, the reaction was stopped by heating at 70°C for 10 min and stored at -20°C until further use.

Amplification was carried out in a total volume of 20 μl containing 10 μl of SYBR Premix EX Taq™ II (Takara, China), 0.4 μl of ROX Reference Dye (Takara, China), 2 μl of cDNA template, 6 μl of DDwater, and 0.8 μl (10 μM) of each cytokine- or transcription factor-specific primers (Sangon Co., Ltd. Shanghai, China) (Table 1). PCR parameters were as follows: 95°C for 2 min, 50 cycles at 95°C for 15 s and 65°C for 30 s, followed by dissociation stage at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s. Relative quantification between samples was achieved by the $2^{-\Delta\Delta\text{CT}}$ method (28) and calculated by software REST 2005 (gifted by Eppendorf company) and was reported as the n -fold difference relative to target gene mRNA expression in the calibrator group (in the group, mice were administered saline only).

Table 1. Sequences of primer used for reverse transcription-PCR

Gene	Primer sequence	Product size (bp)
β -actin	5'-CTGCCTGACGGCCAGGTC-3' 5'-TTTACGGATGTCAACGTCACACT-3'	150
IL-12	5'-CAAGCTCAGGATCGCTATTACAATT-3' 5'-GGGGACTCTTCCATCCTGAG-3'	149
IFN- γ	5'-GCTTTGCAGCTCTTCTCATG-3' 5'-CTTCCACATCTATGCCACTTGAG-3'	112
IL-4	5'-AGAGACTCTTTCGGGCTTTTC-3' 5'-GCATGGTGGCTCAGTACTAC-3'	144
IL-10	5'-GGGTTGCCAAGCCTTATCG-3' 5'-GCTTCTCACCCAGGGAATTG-3'	113
T-bet	5'-CGAGGACTACGCATTGCC-3' 5'-GACAGGAATGGGAACATTGCG-3'	147
GATA-3	5'-TGGAGGAGGAACGCTAATG-3' 5'-ATGACATGTGTCTGGAGAGAGC-3'	223

Determination of serum cytokines by western blot

Serum IFN- γ and IL-5 were measured by western blot. The samples were diluted with the Laemmli sample buffer 1:10 (IFN- γ) or 1:20 (IL-5), heated in water bath at 100°C for 5 min. The diluted sample (30 μl) was subjected to 12% (w/v) SDS-PAGE (polyacrylamide gel electrophoresis), and the proteins were transferred onto the immobilon-p transfer membrane (Millipore Corporation, USA). The membrane was then washed and blocked in a Tris-buffered saline (TBS) containing 5% skimmed milk for 1 h at 37°C in the incubator shaker. After three washes in TBST (TBS containing 0.1% Tween-20), the membrane was incubated with anti-IFN- γ (1:800) monoclonal antibody (sc-5992; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-IL-5 (1:800) monoclonal antibody (sc-7887; Santa Cruz Biotechnology Inc.) for 1 h at room temperature on the rocker platform. The membrane was washed three times in TBST, followed by incubating with diluted HRP-conjugated goat anti-mouse IgG (sc-2004; Santa Cruz Biotechnology Inc.) (1:2000) for 1 h at room temperature. After the final wash, the immunoblot was examined by BeyoECL Plus (Beyotime Biotechnology, China) according to the manufacturer's instructions. The membrane was exposed to an X-ray film, which was later developed. The intensity of the resulting bands was quantified by the Quantity One® 1-D Analysis Software Version 4.6.2 (BioRad, Hercules, CA, USA).

Statistical analyses

Data were expressed as means $\pm$ standard deviations. Duncan's test was used to compare the parameters between groups by using SPSS 13.0. P -values of <0.05 were considered statistically significant.

Results

Effect of 20(R)-Rg3 and 20(S)-Rg3 on splenocyte proliferation

The effects of ginsenosides 20(R)-Rg3 and 20(S)-Rg3 on splenocyte proliferation in OVA-immunized mice are shown in Fig. 2. Mice immunized with OVA + 20(R)-Rg3 had significantly higher splenocyte response to the stimulation by Con A, LPS or OVA than the mice immunized with OVA + 20(S)-

Rg3 ($P < 0.05$) while both groups had significantly higher splenocyte response than the mice immunized with OVA alone ($P < 0.05$).

Effect of 20(R)-Rg3 and 20(S)-Rg3 on the serum IgG and the IgG subclasses

OVA-specific IgG levels in the sera of OVA-immunized mice are shown in Fig. 3. Mice immunized with OVA + 20(R)-Rg3 had significantly higher IgG response than the mice immunized with OVA + 20(S)-Rg3 ($P < 0.001$) while both groups had significantly higher IgG levels than the mice immunized with OVA alone ($P < 0.001$). The IgG subclasses IgG1, IgG2a, IgG2b and IgG3 in the sera of OVA-immunized mice are also shown in Fig. 3. Injection of OVA together with 20(R)-Rg3 or 20(S)-Rg3 induced significantly higher IgG isotype responses than OVA was injected alone ($P < 0.05$). The higher IgG isotype responses were found in the mice immunized with OVA plus 20(R)-Rg3 than OVA plus 20(S)-Rg3 ($P < 0.001$), except IgG3.

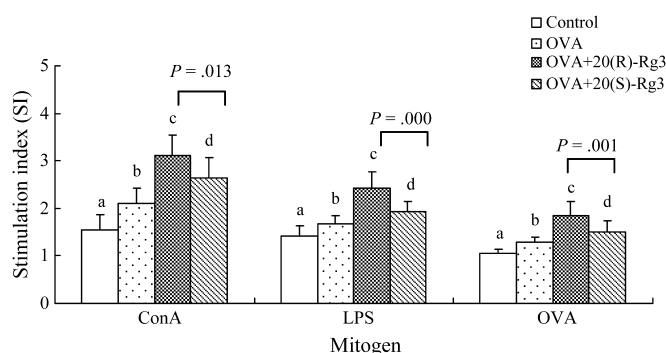


Fig. 2. Effect of 20(R)-Rg3 and 20(S)-Rg3 on mitogen- and OVA-stimulated splenocyte proliferation in mice subcutaneously administered saline (control), OVA alone or OVA mixed with 20(R)-Rg3 or 20(S)-Rg3 on days 1 and 15. Splenocytes were prepared 3 weeks after the last immunization and cultured with Con A ($5 \mu\text{g ml}^{-1}$), LPS ($7 \mu\text{g ml}^{-1}$), OVA or RPMI 1640. Splenocyte proliferation was measured by the MTT method as described in the text and shown as a SI. The values are represented mean $\pm$ SD ($n = 8$). Bars with different letters are statistically different ($P < 0.05$).

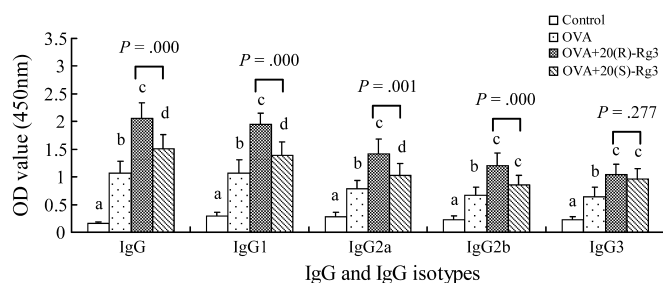


Fig. 3. Serum OVA-specific IgG and the IgG isotypes of mice subcutaneously administered saline (control), OVA alone or OVA mixed with 20(R)-Rg3 or 20(S)-Rg3 on days 1 and 15. Blood samples were collected 3 weeks after the last immunization for measurement of OVA-specific IgG and the IgG isotypes by an indirect ELISA. The values are represented mean $\pm$ SD ($n = 8$). Bars with different letters are statistically different ($P < 0.05$).

Effect of 20(R)-Rg3 and 20(S)-Rg3 on the expression of cytokines, GATA and T-bet mRNA by splenocytes

Cytokines and transcription factors mRNA expressed by splenocytes are shown in Fig. 4. OVA plus 20(R)-Rg3 or 20(S)-Rg3 induced significantly higher mRNA expression of cytokines IL-4, IL-10, IFN- γ and IL-12 as well as the transcription factors GATA-3 and T-bet than OVA alone ($P < 0.001$); 20(R)-Rg3 stimulated higher cytokines and transcription factors expressed by splenocytes than 20(S)-Rg3 ($P < 0.05$).

Effect of 20(R)-Rg3 and 20(S)-Rg3 on serum IFN- γ and IL-5

Serum cytokines IFN- γ and IL-5 analyzed by western blot are shown in Fig. 5. OVA together with 20(R)-Rg3 or 20(S)-Rg3 stimulated significantly higher expression of IFN- γ and IL-5 than OVA alone ($P < 0.01$); 20(R)-Rg3 stimulated significantly higher expression of IFN- γ and IL-5 than 20(S)-Rg3 ($P < 0.05$).

Discussion

Ginseng is one of the most popular traditional Chinese medicines. As a general tonic, the root has been officially written in the Chinese Pharmacopoeia (4). With the expanding demands of ginseng, the global production of this herbal medicine was 3500 tons in 2004 and was estimated to

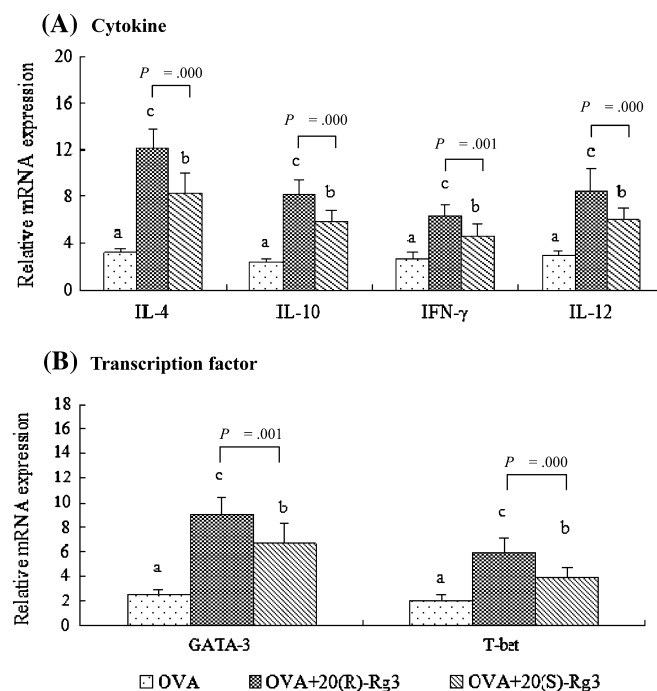


Fig. 4. mRNA of (A) cytokines IL-4, IL-10, IFN- γ , IL-12 and (B) transcription factors GATA-3 and T-bet expressed by splenocytes of mice subcutaneously administered OVA alone or OVA mixed with 20(R)-Rg3 or 20(S)-Rg3 on days 1 and 15. Mice administered with saline alone as a calibrator group. Splenocytes were prepared 3 weeks after the last immunization and cultured with OVA ($100 \mu\text{g ml}^{-1}$) for 15 h. Production of cytokine mRNA was analyzed by reverse transcription-PCR. The values are represented mean $\pm$ SD ($n = 8$). Bars with different letters are statistically different ($P < 0.05$).

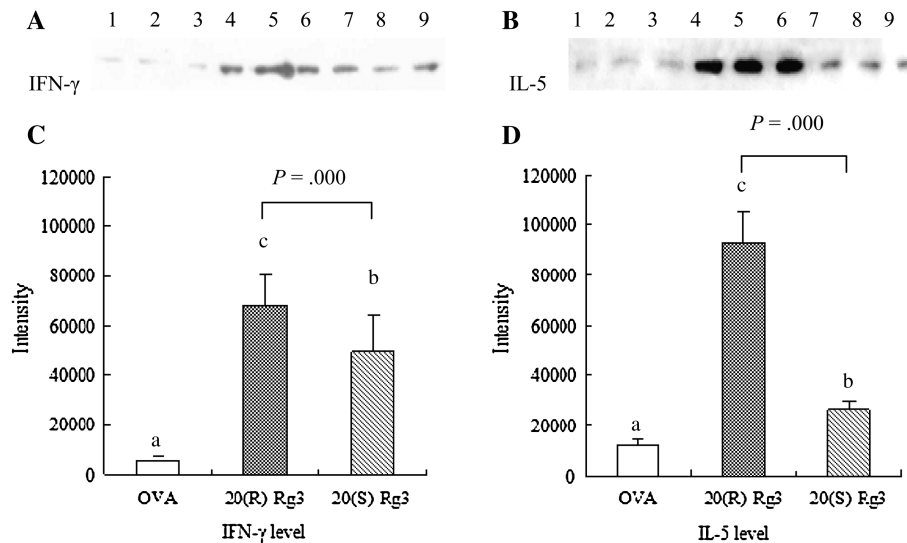


Fig. 5. Serum IFN- γ and IL-5 levels of mice subcutaneously administered OVA alone or OVA mixed with 20(R)-Rg3 or 20(S)-Rg3 on days 1 and 15. Blood samples were collected 3 weeks after the last immunization for measurement of IFN- γ and IL-5 by western blot. (A) 30 μ l of each diluted serum sample (1:5) was used for the determination of IFN- γ , lanes 1–3, OVA; lanes 4–6, OVA + 20(R)-Rg3; lanes 7–9, OVA + 20(S)-Rg3. (B) 30 μ l of each diluted serum sample (1:10) was used for the determination of IL-5, lanes 1–3, OVA; lanes 4–6, OVA + 20(R)-Rg3; lanes 7–9, OVA + 20(S)-Rg3. IFN- γ (C) and IL-5 (D) were quantified by the Quantity One Software. Intensity was graphed as the mean $\pm$ SD. Bars with different letters are statistically different ($P < 0.05$).

increase to 6000 tons in 2010, accounting for the second-highest selling herbal supplement in the United States and Europe (29). Because of its diverse pharmacological activities, ginseng has been reported to have therapeutic effects on the central nervous system disorders, cardiovascular diseases, endocrine secretion, aging and immune function (30–33). Recently, we have found that ginseng extracts have adjuvant properties to enhance the immune responses to vaccines in mice, guinea pigs, cattle, pigs and chickens (13–15, 34–36).

In order to improve its pharmacological activities, ginseng is usually processed into red ginseng by steaming the root at high temperature for hours (37). During the processing, many new constituents are produced because steaming induces changes in chemical composition. For example, ginsenosides Rg3, Rg5, Rh2 and Rk1 are found only in red ginseng, and they are believed to be transformed from diol or triol types of ginsenosides through hydrolysis (38–40). As Rg3 has multiple pharmacological activities, it has attracted much attention. There are two types of ginsenoside Rg3, 20(R)-Rg3 and 20(S)-Rg3. They are epimers of each other depending on the position of the hydroxyl (OH) group on carbon-20, and this epimerization is known to be produced by the selective attack of the OH group after elimination of the glycosyl residue at carbon-20 during the steaming process (41). In weak acid condition, protopanaxadiol such as Rb1, Rb2, Rc and Rd is bio-converted into 20(S)-Rg3, and the S configuration is then transformed into R form (42, 43). The newly formed ginsenosides were reported to include 20(S)-Rg3 32.8%, 20(R)-Rg3 7.3%, Rk1 15.7% and Rg5 18.6% in the *n*-BuOH fraction of a red ginseng (38).

Two forms of Rg3 are different in their pharmacological actions. 20(S)-Rg3 caused a large attenuation of Na⁺ channel activity in a reversible and dose-dependent manner, whereas

20(R)-Rg3 had little effect (44). 20(S)-Rg3 epimer exhibited higher pharmacological effects on insulin secretion and 5' adenosine monophosphate-activated protein kinase (AMPK) activation than 20(R)-Rg3 (45). 20(R)-Rg3 showed more stronger anti-tumor activity than 20(S)-Rg3 (46, 47). These indicate that the precise structural characteristics of ginsenosides such as side chains, glycosyl elimination and epimerization of carbon-20 are required for their biological activity.

In this study, Rg3 was found stereospecific in stimulating the immune responses. Immunity to different infectious agents requires distinct types of immune responses. T_H1 immune response is characterized by production of the cytokines IL-12 and IFN- γ and an enhanced production of IgG2a and IgG3 in mice. T_H2 response is characterized by production of the cytokines IL-4 and IL-10 and an enhanced production of IgG1 (48). Compared with the group without adjuvant, 20(R)-Rg3 and 20(S)-Rg3 significantly enhanced specific IgG, IgG1, IgG2a, IgG2b and IgG3 responses in OVA-immunized mice (Fig. 3) with the highest IgG and subclasses found in 20(R)-Rg3-adjuvanted group. In a T-dependent immune response, T cells and their cytokines are involved in the production of the specific antibody. In mice, IL-4, IL-5 and IL-10 stimulate production of IgG1 isotype; IFN- γ and IL-12 enhances Ig2a and IgG3 responses (48). Increased IgG and the isotype responses to OVA matched the up-regulated T_H1 cytokines (IL-12, IFN- γ mRNA and serum IFN- γ) as well as the T_H2 cytokines (IL-4, IL-10 mRNA and serum IL-5, Figs. 4 and 5) and also associated with simultaneously increased mRNA expression of transcription factors T-bet and GATA-3 (Fig. 4).

The capacity to elicit an effective cellular immunity can be measured by lymphocyte proliferation (49). The lymphocyte proliferative response varied depending on the mitogen used. 20(R)-Rg3 and 20(S)-Rg3 both significantly enhanced

splenocyte proliferation induced by Con A, LPS and OVA, suggesting that T and B cells were activated. But 20(R)-Rg3 group showed higher responses than 20(S)-Rg3 group as indicated in Fig. 2. In order to induce antibody production, activated B lymphocytes are required for clone expansion. The enhanced splenocyte response to OVA stimulation paralleled the increased serum IgG response detected in the mice injected with OVA plus Rg3.

In conclusion, 20(R)-Rg3 and 20(S)-Rg3 significantly enhanced both T_H1 and T_H2 immune responses to OVA in mice. The adjuvant effects of ginsenoside Rg3 are stereospecific. 20(R)-Rg3 induced significantly higher serum-specific IgG and the IgG isotype responses than 20(S)-Rg3 in association with up-regulated serum IFN- γ and IL-5 than 20(S)-Rg3. In addition, 20(R)-Rg3 significantly enhanced splenocyte proliferative responses to Con A, LPS and OVA as well as mRNA expression of IFN- γ , IL-12, IL-4 and IL-10 and transcription factors T-bet and GATA-3 by splenocytes when compared with the 20(S)-Rg3. Therefore, R configuration should be used when Rg3 is considered to be used as a vaccine adjuvant.

Funding

National Scientific Foundation of China (project No. 30771592).

References

- O'Hagan, D. T., MacKichan, M. L. and Singh, M. 2001. Recent developments in adjuvants for vaccines against infectious diseases. *Biomol. Eng.* 18:69.
- Song, X. and Hu, S. 2009. Adjuvant activities of saponins from traditional Chinese medicinal herbs. *Vaccine* 27:4883.
- Li, C. P. and Li, R. C. 1973. An introductory note to ginseng. *Am. J. Chin. Med.* 1:249.
- Commission, C. P. 2010. Ren Shen (Radix et Rhizoma Ginseng). In Commission, C. P., ed., *Pharmacopoeia of the People's Republic of China, Part I*, p. 7. Chemical Industry Press, Beijing, China.
- Commission, C. V. P. 2010. Ren Shen (Radix et Rhizoma Ginseng). In Commission, C. V. P., ed., *Veterinary Pharmacopoeia of the People's Republic of China, Part II*, p. 11. Chinese Agricultural Publishing House, Beijing, China.
- Surh, Y. J., Na, H. K., Lee, J. Y. and Keum, Y. S. 2001. Molecular mechanisms underlying anti-tumor promoting activities of heat-processed *Panax ginseng* C.A. Meyer. *J. Korean. Med. Sci.* 16 (suppl):S38.
- Kowalski, R. 2007. Studies of selected plant raw materials as alternative sources of triterpenes of oleanolic and ursolic acid types. *J. Agric. Food Chem.* 55:656.
- Wurglics, M., Tawab, M. A., Bahr, U., Karas, M. and Schubert-Zsilavecz, M. 2003. Degradation of ginsenosides in humans after oral administration. *Drug. Metab. Dispos.* 31:1065.
- Yuan, C. S., Wu, J. A. and Osinski, J. 2002. Ginsenoside variability in American ginseng samples. *Am. J. Clin. Nutr.* 75:600.
- Zhang, Q. Y., Kang, X. M. and Zhao, W. H. 2006. Antiangiogenic effect of low-dose cyclophosphamide combined with ginsenoside Rg3 on Lewis lung carcinoma. *Biochem. Biophys. Res.* 342:824.
- Yuan, C. S., Shao, Z. H., Xie, J. T. et al. 2004. Antioxidant effects of American ginseng berry extract in cardiomyocytes exposed to acute oxidant stress. *Biochim Biophys Acta* 1670:165.
- Park, E. K., Choo, M. K., Han, M. J. and Kim, D. H. 2004. Ginsenoside Rh1 possesses antiallergic and anti-inflammatory activities. *Int. Arch. Allergy Imm* 133:113.
- Rivera, E., Daggfeldt, A. and Hu, S. 2003. Ginseng extract in aluminium hydroxide adjuvanted vaccines improves the antibody response of pigs to porcine parvovirus and Erysipelothrix rhusiopathiae. *Vet. Immunol. Immunopathol.* 91:19.
- Rivera, E., Hu, S. and Concha, C. 2003. Ginseng and aluminium hydroxide act synergistically as vaccine adjuvants. *Vaccine* 21:1149.
- Hu, S., Concha, C., Lin, F. and Waller, K. P. 2003. Adjuvant effect of ginseng extracts on the immune responses to immunisation against *Staphylococcus aureus* in dairy cattle. *Vet. Immunol. Immunopathol.* 91:29.
- Hu, Y. L., Kong, X. F., Rui, R., Wang, D. Y. and Li, X. G. 2004. Effects of Chinese herbal medicinal ingredients on peripheral lymphocyte proliferation and serum antibody titer after vaccination in chicken. *Int. Immunopharmacol.* 4:975.
- Rivera, E., Pettersson, F. E., Inganas, M., Paulie, S. and Gronvik, K. O. 2005. The Rb1 fraction of ginseng elicits a balanced Th1 and Th2 immune response. *Vaccine* 23:5411.
- Hu, S., Sun, J. H. and Song, X. M. 2007. Adjuvant effects of protopanaxadiol and protopanaxatriol saponins from ginseng roots on the immune responses to ovalbumin in mice. *Vaccine* 25:1114.
- Chang, K. H., Jee, H. S., Lee, N. K., Park, S. H., Lee, N. W. and Paik, H. D. 2009. Optimization of the enzymatic production of 20(S)-ginsenoside Rg(3) from white ginseng extract using response surface methodology. *N. Biotechnol.* 26:181.
- Kim, N. D., Kang, S. Y., Park, J. H. and Schini-Kerth, V. B. 1999. Ginsenoside Rg3 mediates endothelium-dependent relaxation in response to ginsenosides in rat aorta: role of K⁺ channels. *Eur. J. Pharmacol.* 367:41.
- Chen, D., Cui, J. S. and Liu, X. X. 2008. Study on Hela cell apoptosis induced by 20(S)-ginsenoside Rg3 in cervical cancer. *Chin. J. Lab. Diagn.* 12:463.
- Xin, Y., Ni, J. S., Wang, X. R., Shi, B., Wang, Y. H. and Wu, J. X. 2004. Inhibitory effect of 20(S)-ginsenoside Rg3 on B16 melanoma metastasis. *J. Jilin Univ.* 30:540.
- Li, X., Guan, Y. S., Zhou, X. P. et al. 2005. Anticarcinogenic effect of 20(R)-ginsenoside Rg3 on induced hepatocellular carcinoma in rats. *J. Sichuan Univ.* 36:217.
- Gao, S., Tang, W., Zhang, Y., Gao, J. and Ding, X. 2008. The Anti-fatigue Effect of 20(R)-ginsenoside Rg3 in mice by intranasally administration. *Biol. Pharm. Bull.* 31:2024.
- Wang, H. and Lin, Y. 2009. Advances in anti-tumor effects of ginsenoside Rg3. *Med. Rec.* 15:323.
- Larsen, S. T. and Nielsen, G. D. 2007. The adjuvant effect of di-(2-ethylhexyl) phthalate is mediated through a PPAR alpha-independent mechanism. *Toxicol. Lett.* 170:223.
- Song, X. M., Zang, L. M. and Hu, S. H. 2009. Amplified immune response by ginsenoside-based nanoparticles (ginsomes). *Vaccine* 27:2306.
- Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 25:402.
- Ding, L. W. 2010. Investigation of ginseng markets. *Mod. Chin. Med.* 12:44.
- Liu, C. X. and Xiao, P. G. 1992. Recent advances on ginseng research in China. *J. Ethnopharmacol.* 36:27.
- Seong, Y. H., Shin, C. S., Kim, H. S. and Baba, A. 1995. Inhibitory effects of ginseng total saponins on glutamate-induced swelling of cultured astrocytes. *Biol. Pharm. Bull. Commun.* 18:1776.
- Kitts, D. D., Wijewickreme, A. N. and Hu, C. 2000. Antioxidant properties of a North American ginseng extract. *Mol. Cell. Biochem.* 203:1.
- Joo, S. S., Yoo, Y. M., Ahn, B. W. et al. 2008. Prevention of inflammation-mediated neurotoxicity by Rg3 and its role in microglial activation. *Biol. Pharm. Bull.* 31:1392.
- Song, X. M., Chen, J., Sakwivatkul, K., Li, R. and Hu, S. H. 2010. Enhancement of immune responses to influenza vaccine (H3N2) by ginsenoside Re. *Inter. Immunol.* 10:351.
- Zhai, L. J., Li, Y. T., Wang, W. Y., Wang, Y. M. and Hu, S. H. 2011. Effect of oral administration of ginseng stem-and-leaf saponins (GSLs) on the immune responses to Newcastle disease vaccine in chickens. *Vaccine* 29:5007.
- Zhai, L., Li, Y. T., Wang, W. Y. and Hu, S. H. 2011. Enhancement of humoral immune responses to inactivated Newcastle disease and

- avian influenza vaccines by oral administration of ginseng stem-and-leaf saponins in chickens. *Poul. Sci.* 90:1955.
- 37 Li, X. and Li, L. 1991. Pharmacological changes of ginseng resulting from the processing. *Zhongguo Zhong Yao Za Zhi* 16:3.
 - 38 Kwon, S. W., Han, S. B., Park, I. H., Kim, J. M., Park, M. K. and Park, J. H. 2001. Liquid chromatographic determination of less polar ginsenosides in processed ginseng. *J. Chromatogr. A* 921:335.
 - 39 Lau, A. J., Seo, B. H., Woo, S. O. and Koh, H. L. 2004. High-performance liquid chromatographic method with quantitative comparisons of whole chromatograms of raw and steamed Panax notoginseng. *J. Chromatogr. A* 1057:141.
 - 40 Park, J. H., Kim, W. Y., Kim, J. M. *et al.* 2000. Steaming of ginseng at high temperature enhances biological activity. *J. Nat. Prod.* 63:1702.
 - 41 Kang, K. S., Kim, H. Y., Yamabe, N. and Yokozawa, T. 2006. Stereospecificity in hydroxyl radical scavenging activities of four ginsenosides produced by heat processing. *Bioorg. Med. Chem. Lett.* 16:5028.
 - 42 Liu, Z. H., Li, L. and Qian, F. 2009. Process optimization of ginsenoside Rg3 prepared by hydrolysing Panax Notoginseng saponins by acetic acid. *Chin. J. Hosp. Pharm.* 29:881.
 - 43 Li, X., Wan, H. G., Lu, D. Q. and Wei, P. 2003. Advance of research on antitumour activity of ginsenosides. *Chin. J. Bioprocess Eng.* 1:13.
 - 44 Kang, D. I., Lee, J. Y., Yang, J. Y. *et al.* 2005. Evidence that the tertiary structure of 20(S)-ginsenoside Rg(3) with tight hydrophobic packing near the chiral center is important for Na(+) channel regulation. *Biochem. Biophys. Res. Commun.* 333:1194.
 - 45 Chung, S. H., Park, M. W. and Ha, J. 2008. 20(S)-ginsenoside Rg3 enhances glucose-stimulated insulin secretion and activates AMPK. *Biol. Pharm. Bull.* 31:748.
 - 46 Mochizuki, M., Yoo, Y. C., Matsuzawa, K. *et al.* 1995. Inhibitory effect of tumor-metastasis in mice by saponins, ginsenoside-Rb2, 20(R)-ginsenoside-Rg3 and 20(S)-ginsenoside-Rg3, of red-ginseng. *Biol. Pharm. Bull.* 18:1197.
 - 47 Li, Y. X., Qu, X. B., Zhao, Y., Song, Y., Zhang, W. and Zhao, B. 2008. Raman spectroscopy study on the structure of ginsenoside Rg3. *Guang Pu Xue Yu Guang Pu Fen Xi* 28:569.
 - 48 Feldman, M. J. 2001. Cell cooperation in the antibody response. In Roitt, I., Brostoff, J. and Male, D., eds, *Immunology*, 6th edn. Mosby Publication, London, UK.
 - 49 Marciiani, D. J., Press, J. B., Reynolds, R. C. *et al.* 2000. Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine* 18:3141.