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Ginsenoside-Rb2 and 20(S)-Ginsenoside-Rg3 from Korean Red Ginseng Prevent Rotavirus Infection in Newborn Mice

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It is well known that Korean red ginseng has various biological activities. However, there is little knowledge about the antiviral activity of Korean red ginseng and its ginsenosides. In this study, we addressed whether oral administration of ginsenoside-Rb2 and -Rg3 is able to protect against rotavirus (RV) infection. The protective effect of ginsenosides against RV infection was examined using an in vivo experiment model in which newborn mice (10-day-old) were inoculated perorally (p.o.) with 1.5×10^6 plaque-forming units/mouse of RV strain SA11. When various dosages of ginsenoside-Rb2 (25–250 mg/kg) were administered 3 days, 2 days, or 1 day before virus challenge, treatment with this ginsenoside at the dosage of 75 mg/kg 3 days before virus infection most effectively reduced RV-induced diarrhea. In addition, consecutive administration of ginsenoside-Rb2 (75 mg/kg) at 3 days, 2 days, and 1 day before virus infection was more effective than single administration on day −3. The consecutive administration of ginsenoside-Rb2 also reduced virus titers in the bowels of RV-infected mice. In an experiment to compare the protective activity between ginsenoside-Rb2 and its two hydrolytic products (20(S)- and 20(R)-ginsenoside-Rg3), 20(S)-ginsenoside-Rg3, but not 20(R)-ginsenoside-Rg3, prevented RV infection. These results suggest that ginsenoside-Rb2 and its hydrolytic product, 20(S)-ginsenoside-Rg3, are promising candidates as an antiviral agent to protect against RV infection.

Keywords: *Panax ginseng*, ginsenoside-Rb2, ginsenoside-Rg3, rotavirus, diarrhea

Introduction

Rotaviruses (RVs) are an important causative source of viral gastroenteritis in the immature hosts of various animal species, such as human infants and young children <5 years of age [1]. Since RV brings out 114 million cases of diarrhea annually, RV-induced acute diarrhea is regarded as a significant health problem in the world [1]. In fact, this virus causes 24 million hospital visits and 2.4 million hospitalizations every year [1]. In addition, RV gives rise to more than 527,000 deaths annually, mainly in developing countries [2]. Therefore, the morbidity and mortality caused by RV infection emphasize the importance of its prophylaxis and cure. The present methods to treat RV-associated diarrhea consist mainly of symptomatic therapies; that is, oral rehydration to prevent dehydration. Although, at present, two attenuated live vaccines were shown to elicit an anti-RV effect in humans, they are neither effective enough to prevent severe diarrhea in some cases nor globally distributed [3]. Therefore, the development of an effective approach to prevent RV-induced severe diarrhea is urgently needed.

Saponins belong chemically to the glycosides with a triterpenoid, steroidal aglycone, or sapogenin, and they possess diverse biological functions, such as immuno-modulatory effects and adjuvant activities [4–8]. In addition, it was found that oral administration of saponins augmented antigen-specific immune responses orally as well as parenterally [8, 9], and their immunomodulating activity might be due to potentiation of mucosal immunity.

Ginsenosides, saponin preparations isolated from *Panax ginseng*, are classified into two groups of saponins according to chemical structure: dammarane-type having a 20(S)-protopanaxadiol/triol skeleton, and oleanolic acid type having an oleanolic acid skeleton. Many investigators have reported that ginsenosides possess a diversity of biological activities, including anticancer [10–13] and immunopotentiating [7–9, 14] effects. However, only little knowledge of the protective effect by ginsenosides against viral infection has been reported. Recently, Kim *et al*. [15] reported that oral administration of Korean red ginseng protected against influenza A (H1N1) virus. Moreover, Yoo *et al*. [16] demonstrated protective activity of Korean red ginseng extract against two types of influenza viruses, H1N2 and H3N2, in animal models. Furthermore, Lee *et al*. [17] clearly showed that Korean red ginseng extract and ginsenosides have antiviral activity to protect against murine norovirus and feline calicivirus as surrogates for human norovirus.

Ginsenoside-Rb2, a dammarane-type saponin, is known to have a variety of biological activities, especially immunomodulating activity to regulate the proliferation of lymphocytes [14]. Additionally, this ginsenoside was shown to possess an inhibitory effect on metabolic syndromes such as diabetes and hyperlipidemia in mice [18]. Previously we suggested that treatment of ginsenoside-Rb2 inhibited lung metastasis of B16-BL6 melanoma cells and angiogenesis produced by tumor cells in mice [11]. We also reported that oral administration of ginsenoside-Rb2 protected the host against the lethal respiratory infection of haemagglutinating virus of Japan (HVJ), which causes a severe acute respiratory infection in mice [19]. These findings led us to a possibility that oral administration of ginsenoside-Rb2 can augment the host resistance to prevent RV-induced gastrointestinal infections.

In this study, we examined the application of ginsenoside-Rb2 as a mucosal immunostimulant to enhance nonspecific resistance against RV infection in an infection model using immature mice. We also compared the protective activity between ginsenoside-Rb2 and its hydrolytic products 20(S)- and 20(R)-ginsenoside-Rg3.

Materials and Methods

Reagents

The ginsenosides used in this study were kindly provided by the Korean Ginseng Corporation (Korea). All ginsenosides were isolated from the roots of 6-year-old *Panax ginseng* C. A. Meyer in Korea as described previously [19], and their purity was above 99.9% as estimated by high-performance liquid chromatography [20]. The chemical structures of these ginsenosides are shown in Fig. 1. Each ginsenoside was suspended in phosphate-buffered saline before use.

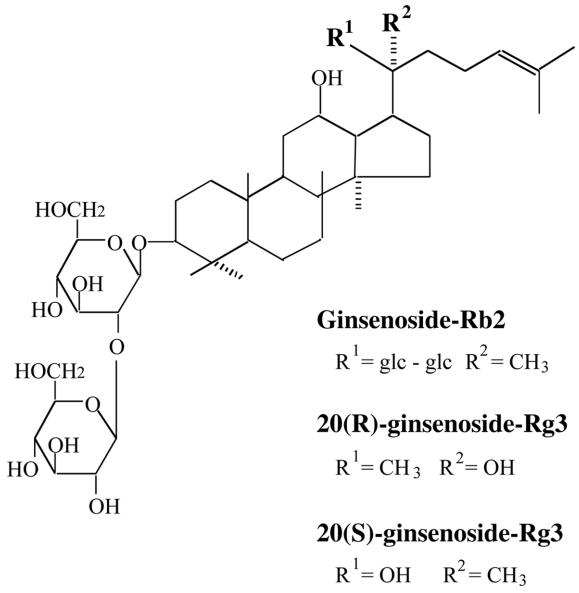

Fig. 1. Chemical structures of ginsenoside-Rb2, 20(S)-ginsenoside-Rg3, and 20(R)-ginsenoside-Rg3.

Animals

Specific pathogen-free pregnant BALB/c mice were purchased from Raon Bio Ltd. (Korea). The mice were housed in plastic cages in vinylfilm isolators. All mice had water and pelleted diets *ad libitum*. All animal experiments were carried out according to the Laboratory Animal Control Guidelines of IACUC of Konyang University (Approval No. P-16-01-A-01).

Virus and Cell Line

Rotavirus strain SA11 (RV-SA11) was kindly supplied by Dr. J. Arikawa of Hokkaido University in Japan. MA-104 cells, a cell line derived from fetal rhesus monkey, were cultivated in Eagle's minimum essential medium (EMEM, Korea) supplemented with 5 mM glutamine, 0.1% sodium bicarbonate, 50 μg/ml of gentamicin, 3 μg/ml of amphotericin B, and 10% fetal calf serum. RV-SA11 was replicated in MA-104 cells as described previously [21]. The titer of virus stocks used in this study was 2.5×10^8 plaque-forming units (PFU)/ml.

Protection against RV Infection

The newborn litters of 10-day-old BALB/c mice were inoculated perorally (p.o.) with various doses of RV, 1.5×10^6 to 1.5×10^7 PFU/50 ml/mouse. The mice were fasted for 4 h before virus infection. Ginsenosides were administered p.o. on the indicated days prior to virus infection. A clinical score for RV-induced diarrhea was measured by the severity of diarrhea per

mouse every 24 h after virus infection, as described previously [21]. The scores were determined by the following criteria: point 2, serious; point 1, moderate; point 0, cured. The diarrhea score of each group was determined by a calculation of (the number of mice under serious diarrhea) × 2 + (the number of mice under moderate diarrhea) × 1/total number of mice. The total severity of diarrhea was expressed as a cumulative number of diarrhea scores obtained from the whole observation period.

Isolation of RV from the Bowels

The bowels harvested from RV-infected mice were homogenized by a glass homogenizer in 1 ml of EMEM. After centrifugation, the supernatants were massed up to 5 ml with EMEM, and stored at −80°C. Virus titer of the bowel homogenates was measured by plaque formation test using MA-104 cells [21]. Briefly, the monolayers of MA-105 cells were incubated with 1,000-fold diluted homogenates (0.5 ml/well) in 6-well tissue culture plates for 1 h at 37°C. After washing with EMEM, the cells were overlaid with an overlay medium (2.5 ml/well) consisting of 0.7% purified agar (SeaKem ME Agarose; FMC Bio Products, USA) and 0.0001% trypsin in EMEM, and incubated for 5 days at 37°C in 5% CO_2. Thereafter, the cells were treated with a second overlay medium (2 ml/well) containing 0.7% purified agar and 0.005% neutral red for 48 h, and the plaques formed in each well were counted.

Statistical Analysis

Statistical significance was determined by Student's two-tailed *t* test.

Results and Discussion

In Vivo Titration of RV-SA11 in Newborn Mice

To establish an animal model, 10-day-old newborn mice were inoculated p.o. with various doses of RV-SA11, and the severity of diarrhea was calculated. Prominent symptoms of diarrhea were observed in all of the RV-infected mice during the infection period of 2–5 days (Table 1). The severity of diarrhea was dependent upon the titer of RV challenged. On the basis of the results of Table 1, we carried out the following experiments using an infection model in which 10-day-old newborn mice were inoculated with 1.5×10^6 PFU/mouse of RV-SA11.

Table 1. Titration of rotovirus (RV)SA11 in newborn mice.

Doses of inoculum (PFU/mouse)	Duration of diarrhea (days)		Total diarrhea score[c]
	Total[a]	Severe[b]	
1.5×10^5	2–3	ND[d]	0.25
1.5×10^6	2–5	2–3	4.50
1.5×10^7	2–5	2–4	5.00

Groups of five BALB/c newborn mice (10-day-old) were inoculated perorally with the indicated doses of RV-SA11. All mice were deprived of food for 4 h before virus infection, being isolated from their maternal mice.

[a]The total duration of diarrhea observed.

[b]The partial duration of severe diarrhea showing more than 1.0 value of diarrhea score.

[c]Accumulative diarrhea score per group during the whole observation period.

[d]Not detected.

Protective Effect of Ginsenoside-Rb2 on RV Infection

Although many investigators have tried to find effective substances available for potentiating the protective effect of the host on mucosal pathogens, there is few scientific evidence of beneficial stimulants that can enhance host resistance against mucosal infections [21–23]. Saponin preparations from red ginseng have been widely recognized to possess immunomodulating activity to enhance cytokine production from immune cells, natural killer cell activity, and cellular immune responses, even though the precise mechanisms associated with their biological activities is unclear [22–25]. In a series of previous studies, we clearly demonstrated that oral administration of this ginsenoside enhanced nonspecific resistance against tumor cells, inhibited lung metastasis of B16-BL6 melanoma cells [13], and prevented the lethal infection of HVJ [19]. Herein, we addressed a possibility that oral administration of ginsenoside-Rb2 enhances the resistance of immature host against RV that causes severe gastrointestinal disease in the bowels.

Table 2. Protective effect of oral administration of ginsenoside-Rb2 on rotovirus (RV) infection in newborn mice.

Treatment of ginsenoside-Rb2		Duration of diarrhea (days)		Total diarrhea score (Inhibition %)
Doses	On day	Total	Severe	
Infection only	-	2–5	2–3	4.40
75 mg/kg	-3	2–5	3	2.80 (36.4)
	-2	2–5	2–3	2.95 (33.0)
	-1	2–4	2–3	3.85 (12.5)

Groups of five BALB/c newborn mice were inoculated perorally (p.o.) with RV-SA11 (1.5×10^6 PFU/mouse) and administered p.o. with 75 mg/kg of ginsenoside-Rb2 on the indicated days before virus infection.

Table 3. Effects of multiple oral administrations and dose-dependent activity of ginsenoside-Rb2 on RV infection in newborn mice.

Treatment of ginsenoside-Rb2		Duration of diarrhea (days)		Total diarrhea score (Inhibition %)
Doses	On day	Total	Severe	
Experiment-1				
Infection only	-	2–5	2–4	5.80
250 mg/kg	-3	1–5	2–4	4.80 (17.2)
75 mg/kg	-3	2–5	2–4	4.00 (31.0)
25 mg/kg	-3	2–5	2–4	4.40 (24.1)
Experiment-2				
Infection only	-	2–5	3–5	5.00
75 mg/kg	-3	2–5	2–4	3.35 (33.0)
25 mg/kg	-1,-2,-3	2–4	4	2.25 (54.8)

Groups of five BALB/c newborn mice were inoculated perorally (p.o.) with RV-SA11 (1.5×10^6 PFU/mouse) and administered p.o. with the indicated doses of ginsenoside-Rb2 on the indicated days. RV, rotovirus.

In order to examine the protective effect of ginsenoside-Rb2 on RV infection, newborn mice were treated orally with 75 mg/kg of this ginsenoside at 3 days, 2 days, or 1 day before RV infection, and the severity of diarrhea of RV-infected mice was calculated. As seen in Table 2, all mice treated with ginsenoside-Rb2 were protected against RV infection regardless of the timing of administration, and the highest activity was observed in mice treated 3 days before RV infection. In addition, in an experiment in which various doses of ginsenoside-Rb2 ranging from 25 to 250 mg/kg were administered to mice 3 days before RV infection, ginsenoside-Rb2 at the dose of 75 mg/kg elicited higher protective activity than either of the dose of 25 or 250 mg/kg (Table 3). This implied that the protective effect of ginsenoside-Rb2 against RV infection was not dose-dependent. Of particular significance was the finding that ginsenoside-Rb2 (75 mg/kg) administered consecutively for 3 days before infection was more active in protecting against RV infection than that administered one time 3 days before virus infection (Table 3). These data indicate that oral administration of ginsenoside-Rb2 is active in preventing RV infection, and the optimal administration condition for its prophylactic effect on RV infection was multiple administrations at the dose of 75 mg/kg for 3 days before virus infection in newborn mice. In a previous study, we reported that the preventive effect of ginsenoside-Rb2 on HVJ was strictly dependent upon the dose and administration frequency; however, multiple administrations of ginsenoside-Rb2 at the dosage of 75 mg/kg were not active in preventing HVJ infection in adult mice [19]. This discrepancy in the protective activity of ginsenoside-Rb2 against two different types of viruses, HVJ and RV, may result from the characters of virus infected and the maturity of the host.

Effects of 20(S)- and 20(R)-Ginsenoside-Rg3 on RV Infection

Two types of epimeric ginsenosides, 20(R)- and 20(S)-ginsenoside-Rg3, are generated from the hydrolysis of ginsenoside-Rb2 [11]. Our previous study showed that both types of ginsenoside-Rg3 suppressed lung metastasis of B16-BL6 tumor cells, and their antitumor activity was almost the same as that of ginsenoside-Rb2 [11]. However, in the protective effect against virus infection, oral administration of 20(S)-ginsenoside-Rg3, but not 20(R)-ginsenoside-Rg3, showed significant protection against HVJ [19]. Since multiple administrations of ginsenoside-Rb2 effectively prevented RV infection (Table 3), we next addressed whether consecutive administrations of its hydrolytic products, 20(R)- and 20(S)-ginsenoside-Rg3, could elicit protective effect against RV infection in newborn mice. In oral administration at 3 days, 2 days, and 1 day

Table 4. Comparison of protective effect against RV infection among ginsenoside- Rb2, and 20(S)- and 20(R)-ginsenoside-Rg3 in newborn mice.

Treatment	Duration of diarrhea (days)		Total diarrhea score (Inhibition %)
	Total	Severe	
Infection only	2–6	2–4	5.30
Ginsenoside-Rb2	2–4	0	2.00 (62.3)
20(S)-Ginsenoside-Rg3	2–5	3–4	2.90 (45.3)
20(R)-Ginsenoside-Rg3	2–6	2–4	6.7

Groups of five BALB/c newborn mice were inoculated perorally (p.o.) with RV-SA11 (1.5×10^6 PFU/mouse) and consecutively administered p.o. with 75 mg/kg of ginsenoside-Rb2, or 20(S)- or 20(R)-ginsenoside-Rg3 at 3 days, 2 days, and 1 day before virus infection. RV, rotovirus.

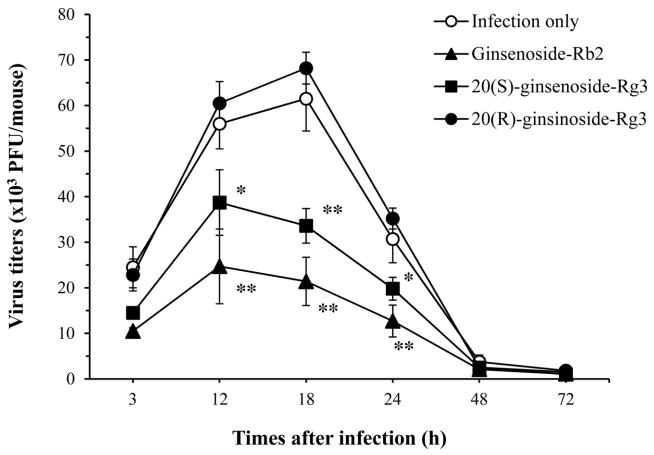

Fig. 2. Inhibitory effect of ginsenosides on rotovirus (RV) growth in the bowels of mice.

Three BALB/c newborn mice per group were administered orally with 75 mg/kg of each ginsenoside at 3, 2, and 1 days prior to RV infection. Virus titers in the bowels were measured by counting the number of PFU on MA-104 cells in 6-well plastic tissue culture plates as described in the Materials and Methods. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with non-treated (infection only) group (by Student's two-tailed *t*-test).

before virus infection, 20(S)-ginsenoside-Rg3 (75 mg/kg) significantly protected against RV infection, although its inhibition of total diarrhea score was lower than that of ginsenoside-Rb2 (Table 4). However, 20(R)-ginsenoside-Rg3 as an epimeric structure of 20(S)-ginsenoside-Rg3 was not active. These results strongly suggest that the protective effect against RV infection by ginsenosides administered orally varies according to the chemical structures of these ginsenosides.

Growth Inhibition of RV in the Bowels by Ginsenoside Administration

We performed an experiment to compare the growth of RV in the bowels between untreated and ginsenoside-treated mice. Since RV is known to cause an acute diarrhea symptom in the intestines at the early time after virus exposure [1, 19], we isolated RV from the bowels in the early time after infection. As shown in Fig. 2, mice inoculated with RV showed the maximal virus titer (about 6.5×10^4 PFU/mouse) at 18 h after infection, and decreased thereafter. Consecutive oral administration of ginsenoside-Rb2 (75 mg/kg) at 3 days, 2 days, and 1 day before infection significantly reduced the growth of RV in the bowels in RV-infected mice. Similarly, multiple administrations of 20(S)-ginsenoside-Rg3, a hydrolytic product of ginsenoside-Rb2, at the dose of 75 mg/kg also significantly inhibited the growth of RV in the bowels, even though its inhibitory effect was lower than that of ginsenoside-Rb2. However, 20(R)-ginsenoside-Rg3, an epimeric type of 20(S)-ginsenoside-Rg3, had no effect in inhibiting the growth of RV in the bowels. Collectively, these results indicate that oral administration of ginsenoside-Rb2 and 20(S)-ginsenoside-Rg3 significantly protected against RV infection via reduction of the virus titer in the bowels, and also suggest that, in comparison of the protective activity between ginsenoside-Rb2 and its hydrolytic products, ginsenoside-Rb2 was the most effective.

In the present study, we demonstrated that ginsenoside-Rb2 and its hydrolytic product, 20(S)-ginsenoside-Rg3, are potent mucosal stimulants to potentiate nonspecific resistance against severe infection of RV in newborn mice, and that their protective effect against RV was associated with the inhibition of virus growth in the bowels at the early period of RV infection. Further study to examine the mechanisms involved in the activation of mucosal immune systems in the gut by ginsenoside-Rb2, and the relationship between the chemical structures and biological activities of ginsenoside-Rb2 and its hydrolytic products is now under way.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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