

## Research Article

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# Protective effect of ginsenoside-Rb2 from Korean red ginseng on the lethal infection of haemagglutinating virus of Japan in mice

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Korean red ginseng has been shown to possess a variety of biological activities. However, little is known about antiviral activity of ginsenosides of Korean red ginseng. Here, we investigated the protective effect by oral administration of various ginsenosides on the lethal infection of haemagglutinating virus of Japan (HVJ) in mice. In a lethal infection model in which almost all mice infected with HVJ died within 15 days, the mice were administered orally (per os) with 1 mg/mouse of dammarane-type (ginsenoside-Rb1, -Rb2, -Rd, -Re, and -Rg2) or oleanolic acid-type (ginsenoside-Ro) ginsenosides 3, 2, and 1 d before virus infection. Ginsenoside-Rb2 showed the highest protective activity, although other dammarane-type and oleanolic acid-type ginsenosides also induced a significant protection against HVJ. However, neither the consecutive administration with a lower dosage (300 µg/mouse) nor the single administration of ginsenoside-Rb2 (1 mg/mouse) was active. In comparison of 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, elicited a partial protection against HVJ. The protective effect of ginsenoside-Rb2 and 20(S)-ginsenoside-Rg3 on HVJ infection was confirmed by the reduction of virus titers in the lungs of HVJ-infected mice. These results suggest that ginsenoside-Rb2 is the most effective among ginsenosides from red ginseng to prevent the lethal infection of HVJ, so that this ginsenoside is a promising candidate as a mucosal immunoadjuvant to enhance antiviral activity.

**Keywords:** *Panax ginseng*, Ginsenoside-Rb2, Red ginseng, Haemagglutinating virus of Japan, Mucosal adjuvant

## INTRODUCTION

Saponins are heterogeneous fractions of natural products that are chemically belonged to glycosides with a triterpenoid, steroidal aglycone or sapogenin. During decades, a great number of biological activities of these saponins have been investigated and their immuno-regulatory effects and adjuvant activities have been reported [1,2]. Furthermore, oral administration of saponins was found to enhance immune response to antigens both parenterally and non-parenterally [3,4], although the precise mechanism of enhancement of mucosal immunity by

saponins is not understood.

Ginsenosides derived from *Panax ginseng* involve two groups of saponins having a distinct structure: 20(S)-protopanaxadiol/triol skeleton (dammarane-type) and oleanolic acid skeleton (oleanolic acid type). The relationship with the structure and biological functions between the two ginsenosides is unclear. Many investigators have demonstrated that ginsenosides have a variety of biological activities including immunomodulatory [5,6] and antitumor [7-9] effect. However, to the best of

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our knowledge, there is no report on the effect of ginsenosides on the lethal infection by pathogenic viruses in *in vivo* models. Recently, it was demonstrated that Korean red ginseng and its ginsenosides have beneficial effects on prevention of influenza A (H1N1) virus [10], H1N2 and H3N2 influenza viruses in mice [11], and murine norovirus and feline calicivirus *in vitro* [12].

Ginsenoside-Rb2, extracted from the root of *P. ginseng* Meyer, is a dammarane-type saponin which does not induce hemolysis, and possesses diverse biological activities such as regulation of lymphocyte proliferation [13], anti-diabetic, and anti-hyperlipidemic effect [14]. Previously we reported that ginsenoside-Rb2 had antitumor activity to inhibit tumor metastasis and tumor-induced angiogenesis in mice [15].

Haemagglutinating virus of Japan (HVJ) which belongs to the parainfluenza viruses (type 1) and is called Sendai virus, is known to cause a lethal acute respiratory infection in mice, giving rise to complications characteristic of mild rhinitis, moderate tracheitis and severe bronchopneumonia [16]. Immunological studies showed that target tissue of the virus is confined to the epithelial mucosa of the upper respiratory tract, tracheobronchi and bronchioles [17]. In the previous study, we demonstrated that MDP-Lys(L18), a stearyl derivative of muramyl dipeptide, administered through mucosal routes, i.e. oral and intrarectal administration, augmented non-specific resistance against HVJ, and significantly suppressed the lethal infection of this virus in mice [16].

Considering that ginsenosides have immunomodulatory activity [18] and oral administration of ginsenosides can augment immune responses [19], it is possible that oral administration of ginsenoside-Rb2 enhances the host immunity to protect against the lethal infection of HVJ. In the present study, to examine whether oral administration of ginsenoside-Rb2 is able to enhance host resistance against virus infection, we investigated its activity to protect against the lethal infection of HVJ using an experimental model in mice. And we partially compared the protective activity between ginsenoside-Rb2 and other ginsenosides from Korean red ginseng.

## MATERIALS AND METHODS

### Reagents

Authentic ginsenosides used in this study were kindly provided by the Korea Ginseng Corporation (Daejeon, Korea). All ginsenosides were obtained from the roots of 6-year old *P. ginseng* Meyer in Korea. The purity of these ginsenosides was above 99.9% as estimated by high

performance liquid chromatography [20]. The chemical structures of ginsenosides used in this study are shown in Fig. 1. An appropriate amount of each ginsenoside was suspended in phosphate-buffered saline (PBS) before use.

### Animals

Specific pathogen-free BALB/c mice were purchased from SLC (Hamamatsu, Japan) and used at the age of 7 to 8 wk. The mice were housed in plastic cages in vinyl-film isolators. Water and pelleted diets were supplied *ad libitum*. The mice were treated according to the Laboratory Animal Control Guidelines of Institutional Animal Care and Use Committee of Konyang University.

### Virus and cells

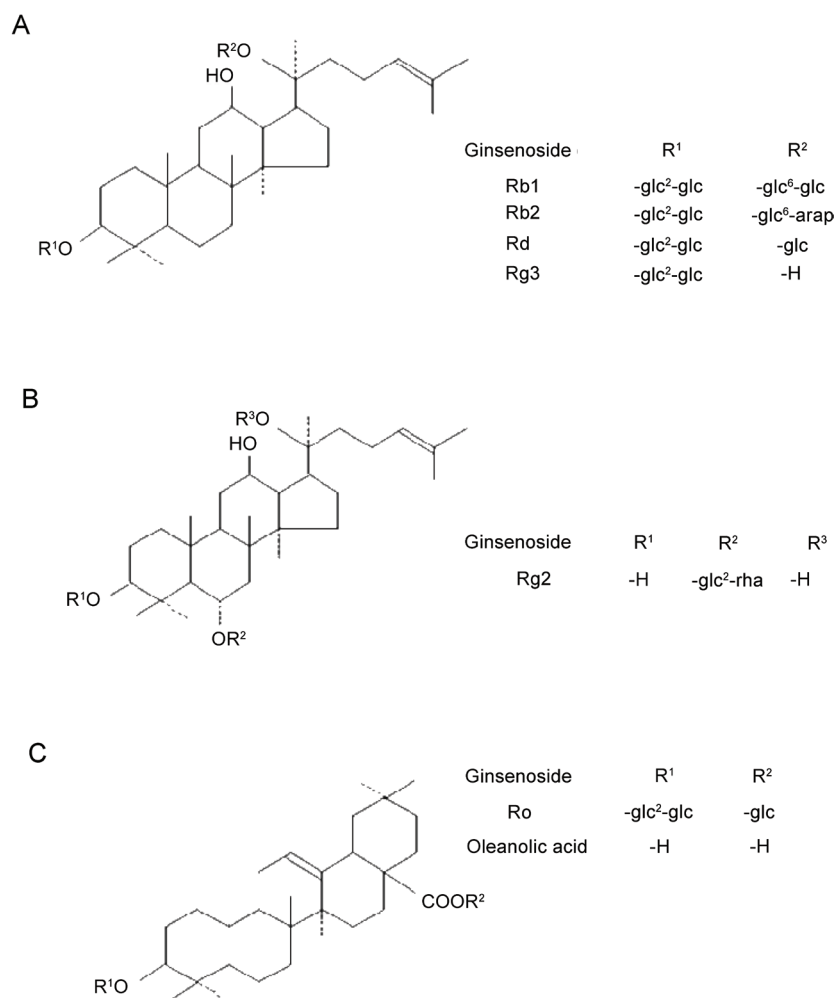
HVJ was purchased from Flow Laboratories (Rockville, MD, USA). After 13 passages of this virus in C3H/He mice, the lungs were homogenized in PBS to make a 20% suspension as described previously [16]. The supernatant was dispersed in ampoules of 0.5 mL amounts, and stored as seed virus suspension at  $-80^{\circ}\text{C}$ . The titer of this seed virus was determined by the hemadsorption (HAD) test as described ( $1.7 \times 10^6$  HAD/mL). The 50% lethal dose ( $\text{LD}_{50}$ ) of the seed virus in BALB/c mice was calculated as  $1.3 \times 10^4$  HAD/ $\text{LD}_{50}$ /0.03 mL by intranasal (i.n.) inoculating the virus into the mice. The LLCMK-2 cell line, derived from a rhesus monkey kidney cell, was cultivated in Eagle's minimum essential medium (EMEM) supplemented with 3 mM glutamine, 0.07% sodium bicarbonate and 5% fetal calf serum.

### Protection against haemagglutinating virus of Japan infection

For each experiment, ampoules of seed virus were thawed and 2  $\text{LD}_{50}$  ( $2.6 \times 10^4$  HAD/0.03 mL/mouse) of the virus suspension was inoculated i.n. under anaesthesia. In this model, almost all mice infected with HVJ died within 15 d after infection. Ginsenosides was administered per os (p.o.) various days before HVJ infection. The survival rates were monitored for up to 20 d after infection.

### Isolation of haemagglutinating virus of Japan from the lungs

Groups of three BALB/c mice were inoculated i.n. with HVJ ( $2.6 \times 10^4$  HAD/mouse). The lungs and tracheae removed from the mice 5 d after infection, were added to 0.5 mL of RPMI-1640 medium with 5% fetal bovine serum and homogenized as described [16]. After centrifugation at 650 g ( $2000 \text{ revs min}^{-1}$ ) for 10 min, the supernatants were massed up to 1 mL with RPMI-1640 and



**Fig. 1.** The chemical structures of dammarane-type and oleanolic acid-type ginsenosides. (A) Dammarane type ginsenosides: 20(S)-protopanaxadiol. (B) Dammarane type ginsenosides: 20(S)-protopanaxatriol. (C) Oleanolic acid-type ginsenosides.

stored at  $-80^{\circ}\text{C}$ . The virus titer of each homogenate was determined by plaque forming units (PFU) on the monolayer of LLCMK-2 cells. The monolayer of LLCMK-2 cells on 24-well spot slides was infected with 1,000-fold diluted each homogenate ( $20\ \mu\text{L}/\text{well}$ ) for 1 h. After wash, the cells were incubated for 1 d under an overlay medium containing 1.5% carboxymethyl cellulose sodium salt and EMEM. Each well was reacted with 200-fold mouse immune serum to HVJ which was prepared in our laboratory, and then 500-fold fluorescein isothiocyanate-conjugated goat anti-mouse IgG+A+M (H+L) (Zymed, CA, USA) for 1 h. The number of foci of each well was counted under a fluorescent microscope.

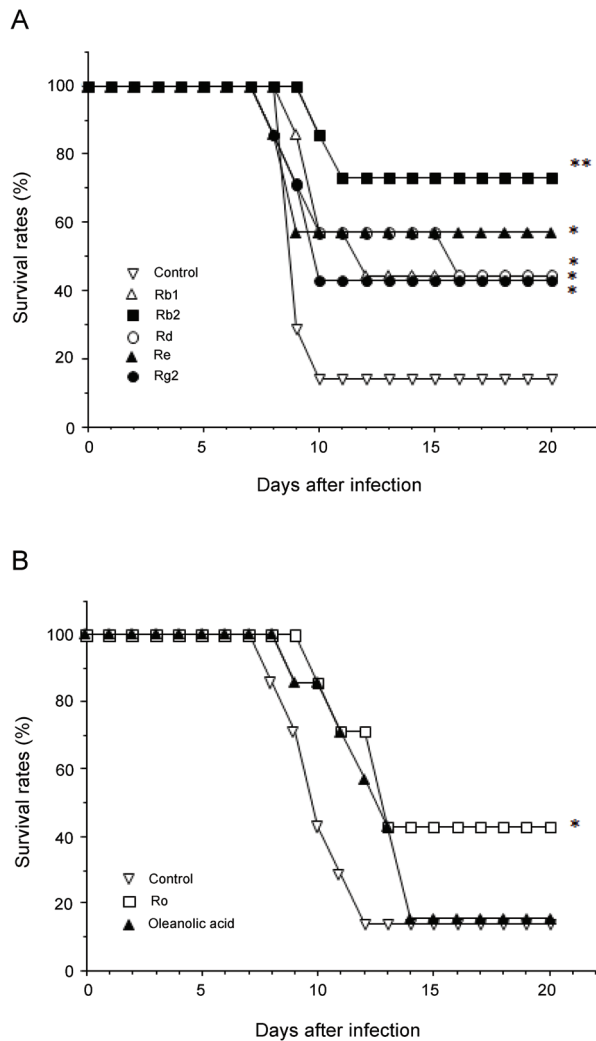
#### Statistical analysis

Statistical significance was determined by Mann-Whitney *U*-probability test or Student's two-tailed *t*-test.

## RESULTS

### Effect of various ginsenosides on haemagglutinating virus of Japan infection

To examine the protective effect of oral administration of various types of ginsenosides on HVJ infection, we investigated their activity to prevent the lethal infection of this virus that causes a severe pneumonitis in mice. Five dammarane-type (ginsenoside-Rb1, -Rb2, -Rd, -Re, and -Rg2) and one oleanolic acid-type (ginsenoside-Ro) saponin fractions (1 mg/mouse) were administered orally into the mice 3, 2, and 1 d before the lethal infection of HVJ, and the survival rates of ginsenoside-treated mice were compared with that of HVJ-infected control mice. As shown Fig. 2A, the mice treated with all dammarane-type ginsenosides protected against HVJ, showing a higher survival rates ranging from 41.8% to 71.4% com-

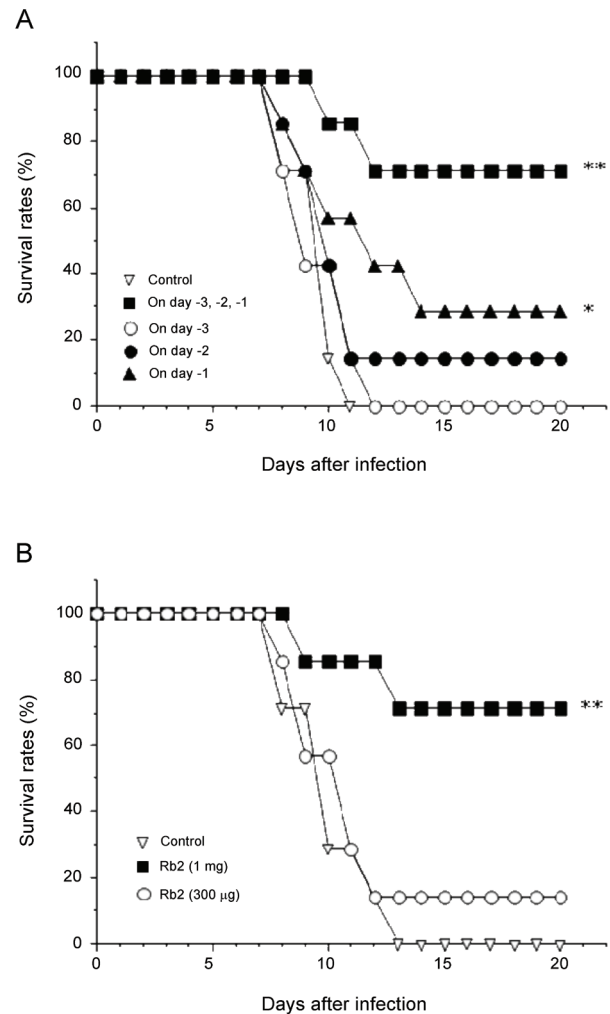


**Fig. 2.** Protective effect of oral administration of various types of ginsenosides on haemagglutinating virus of Japan (HVJ) infection. Seven BALB/c mice per group were administered per os with dammarane-type (A) or oleanolic acid-type (B) ginsenosides (1 mg) 3, 2 and 1 d before intranasal inoculation of HVJ ( $2.6 \times 10^4$  hemadsorption/mouse). The survival rates were monitored for up to 20 d after infection. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with untreated control (infection only) by Mann-Whitney *U*-test.

pared with the control (14.3%). Similarly, oleanolic acid-type saponin ginsenoside-Ro, but not oleanolic acid which is its aglycone, was also active, even though its survival rate was relatively low (42.8%) (Fig. 2B). Of particular significance was the finding that ginsenoside-Rb2 showed the highest survival rate (71.4%) among dammarane-type as well as oleanolic acid-type ginsenosides.

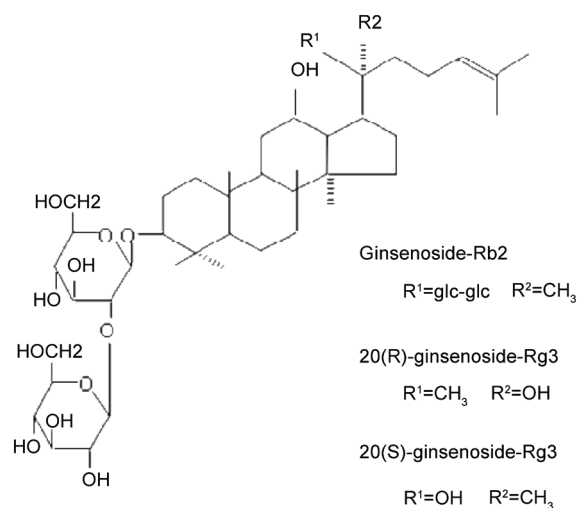
**Protective effect ginsenoside-Rb2 on haemagglutinating virus of Japan infection**

Since the consecutive administration of ginsenoside-Rb2 was the most effective to prevent HVJ infection, we



**Fig. 3.** Administration frequency and dosage of ginsenoside-Rb2 to induce the effective protection against haemagglutinating virus of Japan (HVJ). In analysis for the number of times being administered, the mice were administered per os with 1 mg of ginsenoside-Rb2 on the indicated days before HVJ infection (A). Optimal dosage of ginsenoside-Rb2 to induce its protective effect on HVJ infection was examined in an experiment in which the mice were administered p.o. with 300 µg or 1 mg of ginsenoside-Rb2 3, 2, and 1 d before virus infection (B). The survival rates were monitored for up to 20 d after infection. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with untreated control (infection only) by Mann-Whitney *U*-test.

next examined the relationship between its protective effect and the number of times being administered. Single administration of ginsenoside-Rb2 (1 mg) 3, 2, or 1 d before HVJ infection resulted in a lower survival rate (0% to 28.5%) than that of the multiple administration for 3 d before infection (71.4%) (Fig. 3A). According to the results from Fig. 2A and Fig. 3A that treatment with ginsenoside-Rb2 (1 mg) 3, 2, and 1 d before infection manifested the most prophylactic effect on HVJ infection, we examined whether a relatively lower dosage (300 µg) of



**Fig. 4.** The chemical structures of ginsenoside-Rb2 and its hydrolytic products.

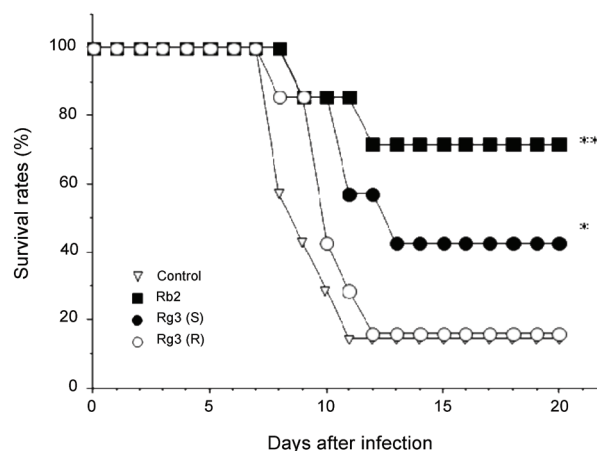
ginsenoside-Rb2 was also active at the same administration schedule. Unfortunately, multiple administration of ginsenoside-Rb2 at the dosage of 300  $\mu\text{g}$  had no effect (Fig. 3B). These results suggest that oral administration of ginsenoside-Rb2 is effective for the prevention of HVJ infection, and that its prophylactic effect on HVJ infection is dependent upon the dosage and the number of times being administered.

#### Effect of 20(R)- and 20(S)-ginsenoside-Rg3 on haemagglutinating virus of Japan infection

In the previously study, we demonstrated that two epimeric saponin preparations such as 20(R)- and 20(S)-ginsenoside-Rg3 which are hydrolytic products of ginsenoside-Rb2 (Fig. 4) inhibited tumor metastasis in mice, and that their antitumor activity was almost same with that of ginsenoside-Rb2 [8]. Here, to address whether oral administration of 20(R)- and 20(S)-ginsenoside-Rg3 induce the protective effect on virus infection as ginsenoside-Rb2 did, we assessed their activity to protect against the lethal infection of HVJ in mice. In oral administration 3, 2, and 1 d prior to HVJ infection, 20(S)-ginsenoside-Rg3 showed a significant protective activity, even though its survival rate was much lower than that of ginsenoside-Rb2 (42.8% and 71.4%, respectively) (Fig. 5). However, 20(R)-ginsenoside-Rg3 in a epimeric structure of 20(S)-ginsenoside-Rg3 was not active.

#### Growth inhibition of haemagglutinating virus of Japan by ginsenoside-Rb2 and 20(S)-ginsenoside-Rg3 in vivo

Next, we tried to compare the growth of HVJ in the

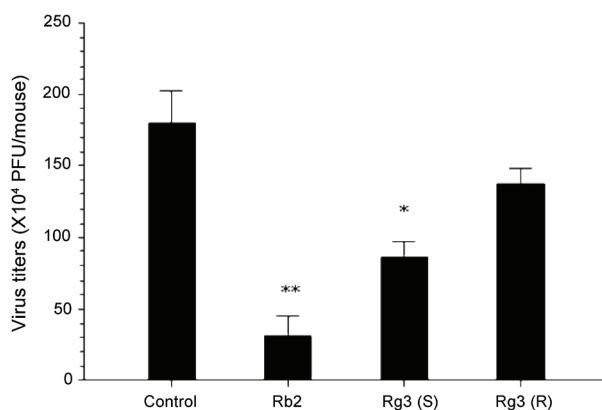


**Fig. 5.** Comparison of the protective effect on haemagglutinating virus of Japan (HVJ) between ginsenoside-Rb2 and its hydrolytic products. Seven BALB/c mice per group were administered per os with 1 mg of ginsenoside-Rb2, 20(R)- or 20(S)-ginsenoside-Rg3 3, 2, and 1 d before HVJ infection. The survival rates were monitored for up to 20 d after infection. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with untreated control (infection only) by Mann-Whitney *U*-test.

lungs between non- and ginsenoside-treated mice. In the previous study, we found that the mice infected i.n. with 2  $\text{LD}_{50}$  ( $2.6 \times 10^4$  HAD/mouse) of HVJ showed the highest virus titers in the lungs 5 d after infection. Therefore, we collected the lungs of HVJ-infected mice 5 d after virus infection, and determined virus titers in the lungs of the mice by counting the number of PFU on LLCMK-2 cells. The mice administered with ginsenoside-Rb2 as well as 20(S)-ginsenoside-Rg3 significantly suppressed the growth of HVJ in the lungs (Fig. 6). Especially the inhibitory activity of ginsenoside-Rb2 was higher than that of 20(S)-ginsenoside-Rg3. However, 20(R)-ginsenoside-Rg3 that had no effect on the lethal infection of HVJ did not inhibit the growth of this virus in the lungs. These results suggest that oral administration of ginsenoside-Rb2 and 20(S)-ginsenoside-Rg3 significantly protects against HVJ infection through inhibition of viral growth in the lungs, and also indicate that, among ginsenoside-Rb and its hydrolytic products such as 20(R)-ginsenoside-Rg3 and 20(S)-ginsenoside-Rg3, ginsenoside-Rb2 has the highest protective activity to inhibit the lethal infection of HVJ.

## DISCUSSION

A variety of approaches to enhance specific immunity against mucosal pathogens have been tried by administration of various types of antigens [16]. However, there are few reports on enhancement of nonspecific protection against mucosal infections by mucosal immunoadjuvant.



**Fig. 6.** Inhibitory effect of ginsenoside-Rb2 and 20(S)-ginsenoside-Rg3 on the growth of haemagglutinating virus of Japan (HVJ) in the lungs in mice. Three BALB/c mice per group were administered per os with each of the indicated ginsenosides (1 mg) 3, 2, and 1 d before HVJ infection. Mice were killed 5 d after virus infection. Virus titers in the lungs of mice were determined by counting the number of plaque forming units (PFU) on LLCMK-2 cells. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with untreated control (infection only) by Student's two-tailed  $t$ -test.

Considering the distinctive feature of mucosal immune system against foreign antigens in the host, it may be possible that the stimulation of mucosal tissues by a potent immunoadjuvant effectively augments nonspecific resistance against mucosal pathogens. Since many studies have shown that the antigen stimulation of a mucosa-associated lymphoid tissue (MALT) induced a potential activation of mucosal immunity of different mucosal tissues, the concept of common mucosal immune system (CMIS) has been introduced [21,22]. Especially, oral administration to stimulate mucosal immunity against infectious diseases has been shown to be convenient, relatively safe, and taking advantage of the mass of MALT.

It has been well known that saponin preparations from red ginseng possess immunomodulatory activities such as the enhancement of cytokine induction, cell-mediated immunity, and natural killer cell activity, although not all studies on the immunomodulatory activities of ginseng-derived saponins have been positive [18,23,24]. In our previous study indicating a possibility that a dammarane-type saponin ginsenoside-Rb2 acts as a mucosal immunoadjuvant, we showed that p.o. administration of this saponin into tumor-bearing mice markedly inhibited the lung metastasis of B16-BL6 melanoma cells, by enhancing nonspecific resistance to tumor cells in mice [8]. In those regards, we tried to investigate the stimulatory effect of an immunoadjuvant on mucosal immunity, using the combination of ginsenoside-Rb2 as an immunoadjuvant and infection model by mucosal infectious HVJ.

Here, we demonstrated that oral administration of gin-

senoside-Rb2 prior to virus challenge enhanced host resistance against HVJ that causes severe acute infection in the lungs. The most effective protection of HVJ infection by ginsenoside-Rb2 was obtained from an administration condition in which mice were consecutively administered 3, 2, and 1 d before virus infection in a dose of 1 mg/mouse (Fig. 2). In addition, ginsenoside-Rb2 was shown to have the highest activity to protect against HVJ infection among dammarane-type (ginsenoside-Rb1, -Rb2, Rd, Re, and Rg2) and oleanolic acid-type ginsenoside (ginsenoside-Ro), even though other types of ginsenosides showed a partial activity (Fig. 1). Interestingly, in comparison between ginsenoside-Rb2 and its hydrolytic products, 20(S)-ginsenoside-Rg3 partially inhibited the lethal infection of HVJ, but 20(R)-ginsenoside-Rg3, an epimer of 20(S)-ginsenoside-Rg3, was not active (Fig. 5). These results suggest that the protective effect on HVJ infection by oral administration of ginsenosides is associated with the characteristic of chemical structures of these saponins.

In the analysis of the inhibition of virus growth *in vivo*, the protective activity acquired from oral administration of ginsenoside-Rb2 was involved in the suppression of virus growth in the lungs. The involvement was clearly supported by the results of Fig. 6 that the mice administered p.o. with ginsenoside-Rb2 prior to virus infection exhibited prominently lower titers of HVJ in the lungs 5 d after virus infection. Considering that the severity of infection caused by HVJ is dependent upon the extent of virus replication in its infection site, it is of note that oral administration of ginsenoside-Rb2 inhibited virus growth in the lungs, the different mucosal route from that of ginsenoside-Rb2 administration. In this regard, it is possible that the protective effect on HVJ infection by oral administration of ginsenoside-Rb2 may result from enhancement of mucosal immunity based on CMIS.

In the present study, we demonstrated that oral administration of ginsenoside-Rb2 is a potent mucosal immunoadjuvant to enhance nonspecific resistance against the lethal infection of HVJ by potentiating mucosal immunity and that its protective effect was associated with the inhibition of virus growth in the lungs. Further study to elucidate the mechanisms involved in activation of mucosal immune cells by ginsenoside-Rb2 and the relationship between the chemical structures and biological activities of ginsenosides derived from red ginseng is now on the way.

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