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Immuno-potentiating Effects of the Antler-shaped Fruiting Body of *Ganoderma lucidum* (Rokkaku-Reishi)

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The immuno-potentiating effects of the antler-shaped fruiting body of *Ganoderma lucidum* (Rokkaku-Reishi, RR), which has been used as a traditional supplement for human health, were investigated in mice. BALB/c mice were administered orally with RR for 3 days at a dose of 50 mg/kg or 500 mg/kg, and interferon-gamma (IFN-γ) production by splenocytes in response to lipopolysaccharide (LPS) was examined on day 4. The oral administration of 500 mg/kg of RR resulted in a significant increase ($p < 0.05$) in IFN-γ production. Stimulation of splenic adherent cells from these mice with LPS also resulted in a significant increase ($p < 0.05$) in interleukin-12 (IL-12) production compared with that from the control mice, suggesting that splenic macrophages were activated by RR administration. Furthermore, 500 mg/kg of RR administered for 14 days resulted in a significant increase ($p < 0.05$) in IFN-γ production by splenocytes in response to both LPS and concanavalin A (Con A). These results suggest that not only splenic macrophages but also T cells were activated by the long-term treatment with RR *in vivo*. On the other hand, the production of interleukin-4 (IL-4), which is known as an allergic disease-related cytokine, was not affected by the long-term treatment with RR. Our results suggest that the oral administration of RR resulted in Th1-associated immuno-potentiating activities *in vivo*.

Key words: Rokkaku-Reishi (RR); interferon-gamma (IFN-γ) production; interleukin-12 (IL-12) production; immuno-potentiating activity; *in vivo*

The fruiting body of *Ganoderma lucidum* (*G. lucidum*) has been used as home remedy in Japan, China, Korea and other East Asian countries for many years. A number of studies have indicated that the fruiting body of *G. lucidum* has many biological benefits such as antitumor activity,[1–3] blood glucose-lowering activity,[4] blood pressure-lowering activity[5] and cholesterol synthesis-inhibiting activity.[6] It has recently been shown that *G. lucidum* contains β-D-glucan, free single saccharides, sugar alcohol, oligosaccharides, amino acids, steroids, coumarin derivatives, mannitol, proteins and triterpenoids. Of these components, it has been reported that β-D-(1,3)-glucan, a major component of *G. lucidum*, exhibited immune-enhancing activities *in vivo* and *in vitro*.[7–14] Suzuki *et al.*[11] have reported that an enhancement of phagocytic activity and an increase in interleukin-1 (IL-1) production were clearly apparent in peritoneal macrophages of mice after an oral administration of β-D-(1,3)-glucan produced from a liquid culture of the fungus.

The antler-shaped fruiting body of *G. lucidum*, called Rokkaku-Reishi (RR) in Japan, is a variant type of *G. lucidum*, and is rarely found in the wild. To date, only a few studies have been reported on the biological activities of an RR extract *in vitro* and *in vivo*.[15–18] Furthermore, no information is available on the immuno-regulatory activities of RR *in vivo*. In the present study, we analyzed the glycosyl linkage of β-glucan contained in RR. The data indicate that RR contained 40.1% of β-D-glucan, which reportedly has important immune-enhancing activities. We therefore examined whether an oral administration of RR to normal BALB/c mice could potentiate the immune functions of the mice.

Materials and Methods

Material. Dried powder made from RR was obtained from Sakamoto Bio (Akita, Japan). Murine recombinant IFN-γ (rIFN-γ) was prepared and purified by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). The activity of this rIFN-γ was calibrated against the World Health Organization-National Institute of Allergy and Infectious Diseases International Reference Reagent by a bioassay using L929 cells and a

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Abbreviations: RR, Rokkaku-Reishi; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; Con A, concanavalin A; IFN-γ, interferon-gamma; IL-4, interleukin 4; IL-12, interleukin 12; mAb, monoclonal antibody; FCS, fetal calf serum; SAC, splenic adherent cell

vesicular stomatitis virus. Murine recombinant IL-4 (rIL-4) was purchased from Intergen (Purchase, NY, USA), murine recombinant IL-12/p70 (rIL-12) was purchased from Endogen (Woburn, MA, USA), and LPS (Difco, Detroit, MI, USA) and Con A (Difco) were obtained commercially. The following mAb pairs for ELISA capture and biotinylated detection were purchased from BD PharMingen (San Diego, CA, USA): BVD4-1D11 and BVD6-24G2 for IL-4; XMG1.2 for IFN-γ detection mAb. Rabbit anti-IFN-γ polyclonal antibodies (the IgG fraction) used as the capture antibodies for IFN-γ ELISA were prepared in our laboratories. A mouse IL-12 p70 ELISA Kit was purchased from Endgen (Woburn, MA, USA). Standard curves were generated by using recombinant cytokines.

Determination of the β-D-glucan content. One gram of dried RR was suspended in an 80 mM phosphate buffer (pH 6.0) and then treated with thermostable α-amylase (A-3306; Sigma-Aldrich Japan, Tokyo, Japan) at 100 °C for 30 min. The suspension obtained from this treatment was adjusted to pH 7.5 and subjected to protease digestion (A-5380; Sigma-Aldrich Japan) at 60 °C for 30 min. After adjusting the pH value to 4.3, the suspension was treated with amyloglucosidase (A-9913; Sigma-Aldrich Japan) at 60 °C for 30 min. Four volumes of 95% ethanol (EtOH) in distilled water (DW) were added to the solution, and the resulting precipitate was washed with 80% EtOH in acetone on a glass filter. The precipitate was dried at 105 °C for 6 h and then treated with 72% sulfuric acid in DW at 25 °C for 4 h. After adding 14 volumes of DW, the enzyme-treated RR was hydrolyzed at 100 °C for 2 h. The hydrolyzed RR solution was neutralized and centrifuged, and the amount of glucose contained in the supernatant was determined by the glucose-oxidase method.[19] The amount of β-D-glucan was calculated as β-D-glucan (mg/g) = glucose (mg/g) $\times$ 0.9.

Glycosyl linkage analysis of β-D-glucan contained in RR. The enzyme-treated and dried polysaccharides of RR were methylated by using NaOH and methyl iodide in DMSO.[20,21] The per-methylated polysaccharides were hydrolyzed, reduced and per-acetylated. The partially methylated alditol acetates were analyzed by gas chromatography, using a capillary column (DB-5, J & W, Folsom, CA, USA) equipped with a flame-ionization detector and operating a linear temperature gradient at 5 °C/min from 130 °C to 250 °C.

Animals and experimental design. BALB/c female mice were obtained from Charles River Japan (Kanagawa, Japan) and used at 6–8 weeks of age in this study. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.[22] Five grams of RR powder was suspended in 100 ml or 1000 ml of DW, and 0.2 ml of the suspension was administered orally to mice. The equivalent volume of DW (0.2 ml) was administered as a vehicle control. Mice weighing 18–20 grams were divided into 3 groups and bred under specific pathogen-free conditions in a temperature-controlled room. Sixteen mice were used for the experiment with 3 days of RR administration. RR was administered orally at a dose of 50 mg/kg (n = 6) or 500 mg/kg (n = 6) for 3 days. Four mice served as the control. On the day after the last administration of RR, the mice were sacrificed, and their spleens were aseptically removed. In a separate experiment, sixteen mice were used for 14 days of RR administration. In this experiment, RR was administered orally at a dose of 500 mg/kg (n = 8). Eight mice served as the control. On the day after the last administration of RR, splenocytes were prepared in the same manner as that of the experiment with 3 days of RR administration.

Cell preparation. After removing the spleen from each BALB/c mouse, a single cell suspension was prepared according to the method of Kunikata *et al.*[23] Briefly, the cells were treated with a Tris-ammonium chloride buffer at pH 7.65 on ice to lyse the red blood cells, and the remaining splenocytes were washed three times with an RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FCS (Thermo Trace Ltd., Melbourne, Australia), 60 μg/ml of penicillin, 50 μg/ml of streptomycin and 10 mM HEPES (complete medium). The splenocytes were resuspended in the same medium at a cell density of 1×10^6 cells/ml. The cells were stimulated with 5 μg/ml of LPS or with 1 μg/ml of Con A. After an incubation period of 48 h at 37 °C, each culture supernatant was collected, and the cytokine levels (IFN-γ and IL-4) were determined by specific sandwich ELISA.[24]

Splenic adherent cells (SACs) were prepared according to the method of Dekruyff *et al.*[25] Briefly, splenocytes were suspended in the RPMI 1640 medium supplemented with 10% FCS and incubated in 90-mm plastic dishes for 2–3 h at 37 °C. Non-adherent cells were removed by washing the dishes three times with the warmed medium until visual inspection revealed a lack of lymphocytes. SACs were incubated on ice and then removed by vigorous pipetting with ice-cold PBS. SACs were counted, suspended in the complete medium at 2×10^5 cells/ml, and stimulated with 50 μg/ml of LPS. After incubating for 18 h at 37 °C, the culture supernatant was collected, and the cytokine levels (IL-12) were determined by sandwich ELISA.

Cytokine assays. The culture supernatants were collected and stored at −80 °C until needed for the cytokine assay. The lower limit of detection was 0.0625 IU/ml for IFN-γ, 12.5 pg/ml for IL-4 and 12.35 pg/ml for IL-12.

Statistical analysis. Each result is expressed as the mean ± S.D. An analysis of variance (ANOVA) was used to determinate differences between the control

Table 1. Glycosyl Linkage Analysis of β-Glucan Contained in RR

Aditol acetate	Linkage	Percentage (%)
2,3,4,6-Me_4-Glc	Glc-(β1→	4.2
2,4,6-Me_3-Glc	→3)-Glc-(β1→	79.4
2,3,6-Me_3-Glc	→4)-Glc-(β1→	8.0
2,3,4-Me_3-Glc	→6)-Glc-(β1→	2.5
2,4-Me_2-Glc	→3,6)-Glc-(β1→	5.9

The content of β-D-glucan in RR was determined after sequentially digesting the fruiting body with α-amylase, protease and amyloglucosidase.

mice and treated mice. When statistically significant differences ($p < 0.05$) were found between the groups, unpaired *t*-tests were used to determine the level of significant difference between the control and treatment group.

Results

β-(1,3)-D-glucan content of RR

Since β-D-glucan is a major component of *G. lucidum*, it was expected that RR also would contain a considerable amount of β-D-glucan. We therefore measured the β-D-glucan content of RR. After successive digestion of the fruiting body with α-amylase, protease and amyloglucosidase, the β-D-glucan content in RR was determined to be 401 mg per gram (40.1%) of dried RR. A glycosyl linkage composition analysis of dried RR indicated the total percentage of β-D-(1,3)-glucan, which was composed of linear β-D-(1,3)-glucan (79.4%) and β-D-(1,6)-branched β-D-(1,3)-glucan (5.9%), to be 85.3% (Table 1).

Effects of 3-days RR-administration on IFN-γ production by splenocytes

Since RR contained a high percentage of β-D-(1,3)-glucan, which exhibited immune-enhancing activities *in vitro* and *in vivo*, we examined whether an oral administration of the RR could potentiate the immune function *in vivo*. For this purpose, we administered RR to normal BALB/c mice *via* the oral route for 3 days. To evaluate the cytokine production, splenocytes separated from each mouse were stimulated for 48 h *in vitro* with either LPS as a stimulator for such myeloid cells as macrophages and dendritic cells (DCs), or by Con A as a stimulator for T lymphocytes. As shown in Fig. 1(A), IFN-γ production by LPS-stimulated splenocytes from RR-treated mice increased in a dose-dependent manner. In particular, a significant ($p < 0.05$) increase in IFN-γ production was observed in the 500 mg/kg RR-treated mice compared with that in the control mice.

On the other hand, there was no difference in IFN-γ production by Con A-stimulated splenocytes between the RR-treated group of mice and the control group of mice (Fig. 1(B)). These results suggest that myeloid cells such as macrophages and DCs in the spleen were activated by the oral administration of RR for 3 days.

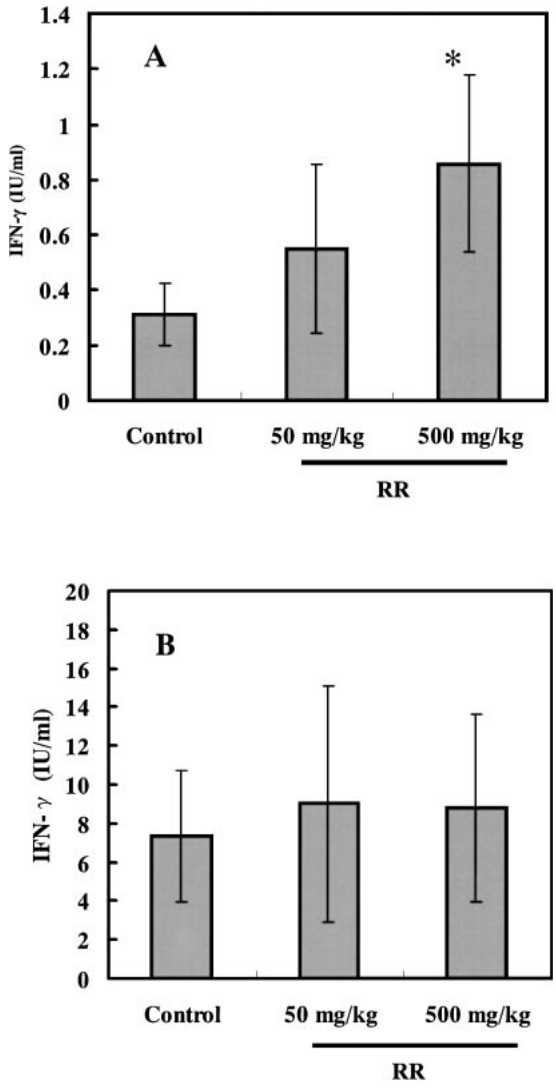

Fig. 1. Effect of the 3-Day RR Administration on INF-γ Production by Splenocytes in Response to LPS or Con A.

After orally administering the RR suspension for 3 days, splenocytes from each mouse were stimulated *in vitro* for 48 h with 5 μg/ml of LPS (A) or with 1 μg/ml of Con A (B). IFN-γ level in each culture supernatant was measured by ELISA, and is expressed in IU per ml. Each result is presented as the mean ± S.D. of 6 mice. * Statistically significant value ($p < 0.05$) compared with the control group.

Effect of 3 days of RR administration on the IL-12 production by SACs

Macrophages and DCs produce IL-12, the most potent inducer of IFN-γ production, in response to LPS.[26–28] We next examined whether IL-12 production by splenic macrophages from RR-treated mice would be enhanced in response to LPS. SACs were therefore prepared from control mice or mice treated with RR for 3 days, and were then stimulated with LPS. As shown in Fig. 2, IL-12 production by LPS-stimulated SACs increased in a dose-dependent manner. In particular, a significant ($p < 0.05$) increase in IL-12 production was observed in the 500 mg/kg RR-treated group of mice compared with the control group of mice. These results suggest

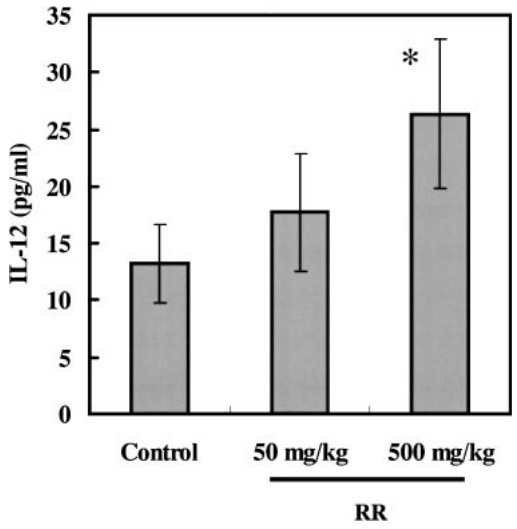

Fig. 2. Augmented IL-12 Production by LPS-Stimulated SACs after 3 Days of RR Administration.

After orally administering the RR suspension for 3 days, SACs were stimulated *in vitro* with 50 μg/ml of LPS for 18 h. The IL-12 level in the culture supernatant was measured by ELISA, and is expressed in picograms per ml. Each result is presented as the mean ± S.D. of 6 mice. * Statistically significant values ($p < 0.05$) compared with the control group.

that the increased production of IL-12 was responsible for the augmentation of IFN-γ production by LPS-stimulated splenocytes from the RR-treated mice.

Effect of 14 days of RR administration on the cytokine production by splenocytes

We subsequently studied the immune-activating effect of a long-term treatment with RR *in vivo*. We treated mice with 500 mg of RR per kg for 14 days and isolated splenocytes as described in the Materials and Methods section. There was no difference in either the initial body weight or body weight gain at the end of the experiment between the RR-treated group of mice and the control group of mice (Table 2). This suggests that the oral administration of RR had no side effect on the general condition of the mice.

As shown in Fig. 3(A), IFN-γ production by LPS-stimulated splenocytes from mice treated with 500 mg of RR per kg for 14 days was significantly ($p < 0.05$) increased in comparison with the control mice (1.17 ± 0.52 IU/ml for the RR-treated group and 0.55 ± 0.25 IU/ml for the control group) after 3 days of the RR treatment. In contrast to the 3-day RR treatment, IFN-γ production by Con A-stimulated splenocytes obtained from 14-day RR-treated mice was also significantly ($p < 0.05$) increased compared with the control mice (10.52 ± 2.40 IU/ml for the RR-treated group and 7.18 ± 3.40 IU/ml for the control group) (Fig. 3(B)). These results suggest that the long-term RR treatment activated not only splenic macrophages but also T cells.

We next investigated whether splenocytes from the RR-treated mice would produce Th2 cytokine, IL-4. In contrast to IFN-γ production, there was no difference in the IL-4 level produced by splenocytes between the RR-treated mice and the control mice (Fig. 3(C)).

Discussion

We have demonstrated in this study that Rokkaku-Reishi, which is a variant type of *G. lucidum*, comprised 40.1% of β-D-glucan. Furthermore, we have demonstrated that RR contained a high percentage (85.3%) of β-D-(1,3)-glucan in the β-D-glucan. A large number of studies have shown the immuno-modulatory effects of β-D-(1,3)-glucan *in vitro* and *in vivo*.[7–14] Sherwood *et al.*[14] have reported that β-D-(1,3)-glucan enhanced the production of IL-1 and interleukin-2 in splenic macrophages of Sprague-Dawley rats after its intraperitoneal injection. These results prompted us to speculate that RR rich in β-D-(1,3)-glucan would exert an immunopotentiating effects *in vivo*. We therefore administered RR to mice and investigated the cytokine production by splenocytes in response to LPS or Con A.

An oral administration of the RR suspension to normal BALB/c mice for 3 days increased the IFN-γ production by LPS-stimulated splenocytes, while the IFN-γ production by splenocytes in response to Con A was not affected. Recent studies have shown that macrophages are capable of producing IL-12, an IFN-γ-inducing cytokine, in response to LPS.[23,27–29] We therefore analyzed the effect of an oral administration of RR on the IL-12 production by LPS-stimulated SACs. As expected, the IL-12 production by SACs from the RR-treated mice increased in a dose-dependent manner. These results suggest that splenic macrophages activated *in vivo* by the RR treatment produced an increased amount of IL-12 in response to LPS, resulting in the up-regulation of IFN-γ production.

We subsequently examined the effect of a 14-day oral administration of RR on the activation of the immune system in mice. We found that the IFN-γ production by splenocytes from the RR-treated mice was increased in

Table 2. Initial Body Weight, Final Body Weight and Body Weight Gain of the Mice

Experimental group	No. of mice	Body weight		
		Initial body weight (g)	Final body weight (g)	Body weight gain (g)
Control (DW)	8	19.6 ± 0.8	19.6 ± 1.0	0.0 ± 0.6
RR (500 mg/kg)	8	20.3 ± 1.2	20.8 ± 0.8	0.4 ± 0.7

Each value is presented as the mean ± S.D. of 8 mice in each group.

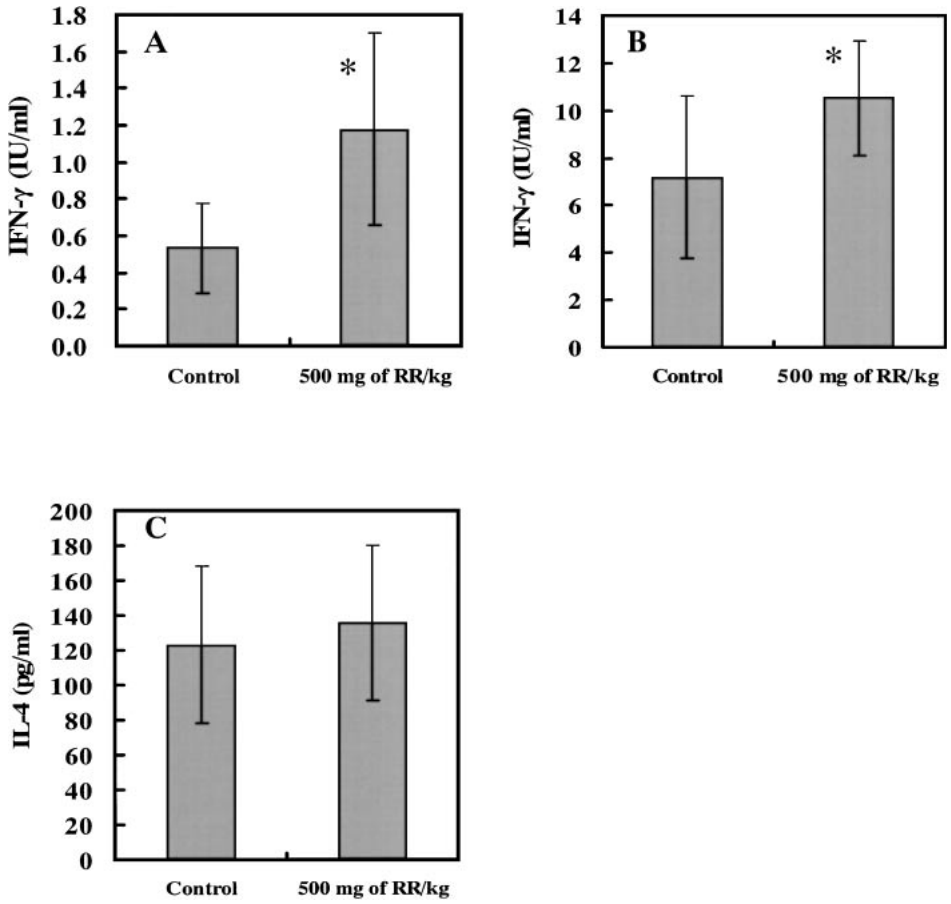

Fig. 3. Effect of the 14-Day RR Administration on Cytokine Production by Splenocytes in Response to LPS or Con A.

After orally administering the RR suspension for 14 days, splenocytes were stimulated *in vitro* with 5 μg/ml of LPS (A) or 1 μg/ml of Con A (B and C) for 48 h. The levels of IFN-γ (A and B) and IL-4 (C) in the culture supernatant were measured by ELISA, and are expressed in IU per m and picograms per ml, respectively. Each result is presented as the mean $\pm$ S.D. of 8 mice. * Statistically significant value ($p < 0.05$) compared with the control group.

response to both LPS and Con A. This suggests that a long-term RR treatment would activate not only splenic macrophages, but also T cells. Bao *et al.*[30] have recently reported that β-D-(1,3)-glucan and its derivatives from *G. lucidum* enhanced the proliferation of T lymphocytes separated from ICR mice in the presence of Con A *in vitro*. It therefore seems likely that β-D-(1,3)-glucan contained in RR was responsible for activation of the T cells. On the other hand, the level of IL-4 produced by Con A-stimulated splenocytes obtained from the RR-treated mice was comparable with that from the control mice. These results, together with the finding that the IFN-γ production was up-regulated by the RR treatment, suggest that the oral administration of RR enhanced the Th1-associated immune response.

The mechanism by which the oral administration of RR resulted in the augmentation of IFN-γ production by splenocytes in response to LPS remains to be elucidated. Sherwood *et al.*[31] reported that a glucan phosphate treatment potentiated LPS-induced IFN-γ expression in mice. They have also shown that the LPS-induced IL-12 p40 mRNA expression was increased in the spleens of glucan-treated mice. In addition, Saito *et al.*[32] have reported that β-D-(1,4)-glucan induced IL-12 p40 and TNF-α production by macrophage cell lines *via* Toll-like receptors (TLRs) *in vitro*. Furthermore, it has been shown that dectin-1, which is expressed on macrophages and DCs, is a lectin family receptor for β-glucans.[33,34] These results suggest that β-D-glucans contained in RR activated the macrophages and DCs by binding to TLRs and dectin-1 on their surface.

The reason why the long-term treatment with RR resulted in the increased IFN-γ production by Con A-stimulated spleen cells also remains unclear. It is well known that interaction between the CD40 antigen on macrophages/DCs and the CD40 ligand (CD154) on antigen- or mitogen-activated T cells induced IL-12 production by macrophages/DCs.[35–37] It is tempting to speculate, therefore, that the long-term treatment with RR may have up-regulated the expression of the CD40 antigen on macrophages/DCs. The increased expression of the CD40 antigen may have facilitated the interaction with CD40 ligands on the Con A-activated T cells, resulting in the augmentation of IL-12 production, which in turn promoted IFN-γ production by the T cells. Further studies are needed to examine these

possibilities.

In conclusion, our results suggest that the oral administration of RR promoted a Th1-type immune response. This further suggests that RR would be an effective dietary supplement for the prophylaxis of cancer and infectious diseases.

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