Immunostimulating Effects of Polysaccharides of *Panax ginseng* by Oral Administration into BALB/c Mice

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Ginsan, polysaccharide isolated from the root of *Panax ginseng* C.A. Meyer, has been shown to be a potent immunomodulator, producing several cytokines and stimulating lymphoid cells to proliferate. In this study, ginsan was orally inoculated into BALB/c mice up to 39 days and the activity of immune cells containing macrophages and T cells was analyzed. Moreover, the production of cytokines, e.g., tumor necrotic factor-α (TNF-α), interferon-γ (IFN-γ), GM-CSF and IL-12 was also analyzed. In results, the phagocytosis of macrophages was increased. About 13% cytotoxicity of NK cells was observed in 22 days and 29 days of administration. But, oral administration did not highly affect the proliferation of T cells. In cytokine analysis, 150 mg/kg and 300 mg/kg at 22 days and 29 days showed three times more increase in TNF-α than the controls. IFN-γ showed 1.07 and 1.16 times more increase at 150 mg/kg and 300 mg/kg over 22 days, respectively more than the controls. 32 days of 150 mg/kg and 300 mg/kg induced GM-CSF of about 1.3 times more than the controls. IL-12 was not induced in samples more than the controls. Ginsan could be a potential immunostimulator. Therefore, our study suggests that it can be adapted as an immunostimulator that requires a relatively short oral administration.

Key Words: *Panax ginseng*, Ginsan, Macrophage, NK cell, Cytokine

INTRODUCTION

The root of *Panax ginseng* C.A. Meyer (Araliaceae) has been one of the most popular natural tonics used in Asian countries. Ginsan, polysaccharide isolated from the root of *Panax ginseng* C.A. Meyer, has been shown to be a potent

immunomodulator, producing several cytokines (TNF-α, IL-1β, IL-2, IL-6, IL-12, IFN-γ, GM-CSF) and stimulating lymphoid cells to proliferate (Shin et al., 2002; Song et al., 2002; Song et al., 2003). It was reported that ginsan was capable of stimulating immune effector cells such as T cells, cytotoxic T lymphocytes (CTL), and natural killer (NK) cells (Song et al., 2002). Pretreatment of mice with ginsan protected them from lethal effects of ionizing radiation (DMF for radiation-induced lethality = 1.45) (Ivanova et al., 2006). In addition, it has been proposed that cytokines produced from splenocytes could be involved in the generation of cytotoxic cells by ginsan (Yun et al., 1998). In addition, ginsan possesses marked radioprotective and antiseptic properties (Lim et al., 2002; Song et al., 2003). The water extracts of ginseng showed antitumor activity against

certain kinds of tumor cells in mice (Lee et al., 1971) and inhibited the incidence of lung tumors by carcinogens (Yun et al., 1983). Effects of the ginsan polysaccharides of *Panax ginseng* have been elucidated for the production of cytokines and stimulation of immune cells. However, it has not yet been reported that oral administration of ginsan into mice would have stimulating effects on BALB/c mice. In this study, ginsan was orally inoculated into BALB/c mice up to 39 days and the activity of immune cells containing macrophages and T cells were analyzed. Moreover, the production of cytokines, e.g., tumor necrotic factor-α (TNF-α), interferon-γ (IFN-γ), GM-CSF and IL-12 was also analyzed.

MATERIALS AND METHODS

Extraction of polysaccharides of *Panax ginseng* and their administrations into BALB/c mice

To extract polysaccharides of *Panax ginseng* C.A. Meyer, 4 to 8 volumes of water were added to its roots, and then the oncoming mixture was extracted at 75℃ to 80℃ for 36 to 72 hours. This process was repeated three times. The extracts were filtered with a 0.22 μm-sized filter and slowly cooled. The chilled extracts were filtered again with the filter above and concentrated at 60℃ in a vacuum. The concentrated extracts were mixed with absolute ethanol and were precipitated in a cold condition until polysaccharides were completely precipitated. A layer of saponin was then removed. The resulting polysaccharides were dissolved with water and were concentrated in a vacuum. They were maintained in a cold condition for over 2 weeks and the remaining enzymes were inactivated. The final stabilized polysaccharides were autoclaved and freeze-dried. This prepared powder of *Panax ginseng* C.A. Meyer was provided by healthbiomed Co. Ltd.. For their oral administration into 6-week female BALB/c mice (Nara biotech, Korea), 150 mg/kg and 300 mg/kg were prepared and the administration was performed daily up to 39 days. All experiments to use animals were approved by Namseoul Animal Committee (No. NSU-14-06).

Phagocytosis assay of macrophages using *Escherichia coli*

Macrophages were isolated from peritoneal exudate cells as described by Klimetzek and Remold (Klimetzek et al., 1980). Thioglycollate-elicited peritoneal exudate cells were obtained from BALB/c mice following intraperitoneal injection of 1 mL thioglycollate and lavage of the peritoneal cavity with 5 mL of medium after 4 days. Peritoneal exudate cells were seeded at densities of 1×10^5 cells/well into a 96-well cell culture plate. After 24 hour incubation, unattached cells were removed and adherent cells were used for the association assay. To analyze phagocytic ability of macrophage, association assays were applied because macrophages possess similar effects with *Acanthamoeba* (Jung et al., 2007). Briefly, macrophages were cultured in 96-well plates in RPMI 1640. Once confluent, the unbound macrophages were aspirated and bound macrophages were washed once with phosphate-buffered saline (PBS). Macrophages were incubated with *Escherichia coli* (2×10^6 cfu/well/0.5 mL of PBS) and plates incubated for up to 1 hr at room temperature. Following this incubation, macrophages were washed with PBS three times to remove non-associated bacteria and macrophage counted using a haemocytometer. Finally, macrophage were lysed by adding sodium dodecyl sulfate (SDS) (0.5% final concentration) to each well for 30 min and the numbers of macrophage were enumerated by plating on LB agar plated as previously described (Alsam et al., 2006). The percentage of macrophage association was calculated as follows: recovered *E. coli* (cfu)/total *E. coli* (cfu) $\times$ 100 = % *E. coli* associated with macrophage. In addition, the ratio of bacteria to macrophage was calculated as follows: recovered *E. coli* (cfu)/number of macrophage = *E. coli*/macrophage ratio.

Activity of natural killer (NK) cells using YAC-1 cell

Spleen was isolated from BALB/c mice; and the spleen cells, by grinding using slide glasses were washed with RPMI 1640 media containing fetal bovine serum, 2 mmol/l glutamine and 100 mg/l penicillin-streptomycin. They were inoculated with 1×10^5 cells/well into 96-well cell culture plates. YAC-1 as a target cell against natural killer (NK)

cell was added to the plates of spleen cells at a ratio of 1:5 (Yoo et al., 2014). The co-incubation was maintained at 37℃ for 5 hr and lactate dehydrogenase assay (LDH) were performed to analyze cytotoxicity of NK cells (Jeong SR et al., 2005). Briefly, 50 μL of reacted supernatant in each well was transferred to a 96-well assay plate. After addition of 50 μL of reconstituted assay buffer from a Cyto-Tox96 Non-radioactive Cytotoxicity Assay kit (Promega, Madison, WI), the plate was incubated for 30 min at RT, and then 50 μL of stop solution was added. The reactants were read at 490 nm using an enzyme-linked immunosorbent assay reader. The formula used to calculate the percent *in vitro* cytotoxicity was as follows: (sample value - control value)/(total LDH release - control value) × 100.

T cell proliferation assay

To analyze T cell proliferation by polysaccharides of *Panax ginseng*, T cells were isolated from mice spleens by columns (R&D systems). Briefly, single cell suspensions of murine leukocytes were depleted of erythrocytes by use of a hypotonic lysing reagent. Processed cells that were to be loaded onto the column were resuspended in 1 mL of 1

× column wash buffer. The top cap of the column was removed first to avoid drawing air into the bottom of the column. Next, the bottom cap was removed. After the column buffer had drained down to the level of the white filter, the 1 mL cell suspension was applied to the top of the column. The cells were incubated at room temperature for 10 min. After the incubation step, T cells were eluted from the column with 4 aliquots. The collected T cells are centrifuged at 250 × g for 5 min. T cells (1.5×10^5 cells/well) were inoculated into a 96-well cell culture plate. After 4 days, 10 μL of CCK-8 solutions were added to the plate. Next 5 hr incubation was done and absorbance was measured at 450 nm using a microplate reader.

Measurement of cytokines

To analyze whether polysaccharides of *Panax ginseng* stimulated immune cells, cytokines, e.g., tumor necrotic factor-α (TNF-α; R&D systems), interferon-γ (IFN-γ; Koma biotech), GM-CSF (Koma biotech), IL-12 (Koma biotech) were measured. Briefly, 100 μL of mice sera or standards were added to an assay plate and washed. 100 μL of the detection antibody was added and streptavidin-horseradish
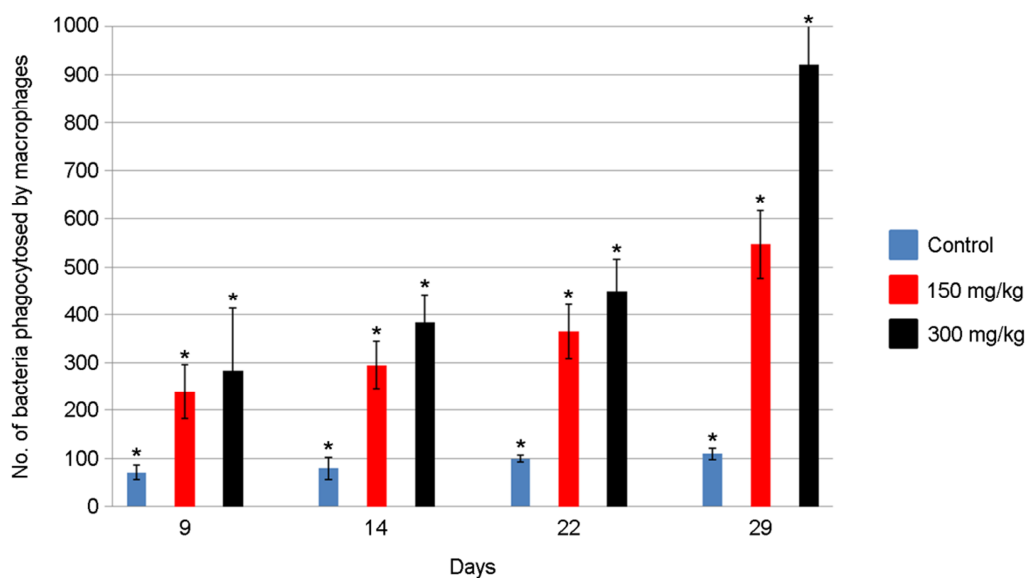
Fig. 1. Phagocytosis of macrophages in BALB/c mice by oral administration of ginsan. It was analyzed up to 29 days. Phagocytosis of the macrophages was analyzed using their bacteria uptake by counting colony forming unit (CFU). Results are presented as the mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P < 0.05$, using paired T-test, one-tail distribution.
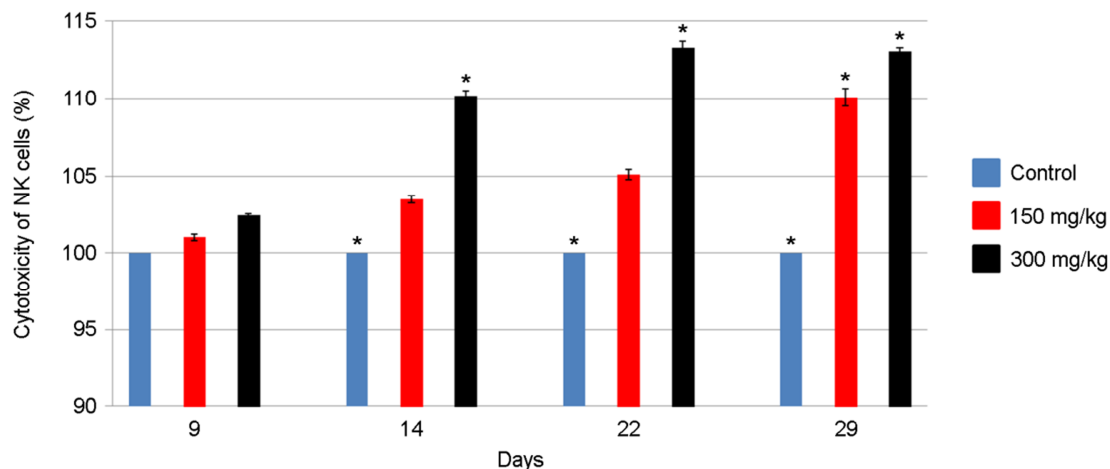
Fig. 2. Cytotoxicity of NK cells isolated from mice spleens. It was analyzed up to 29 days. 150 mg/kg and 300 mg/kg was compared with controls of saline-inoculated mice. Results are presented as the mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P < 0.05$, using paired T-test, one-tail distribution.

peroxidase (HRP) to each well. Finally, substrate and stop solution were applied and absorbance was measured at 450 nm.

RESULTS

Phagocytosis of macrophages to *E. coli*

To understand the immunostimulating effects of ginsan, the phagocytosis of macrophages was analyzed. Using a bacterium of non-pathogenic *E. coli*, the procedures were performed and they were modified as compared with a previous report (Jung et al., 2007). Overall, there was 2 to 3 times more increase in the phagocytosis of macrophages from 150 mg/kg and 300 mg/kg than the controls of only saline-administration (Fig. 1). For longer administration, phagocytosis seemed to be higher but ratios compared with controls and samples were similar. Asterisks indicated a significant difference showing $P < 0.05$. It suggested that ginsan would increase the phagocytosis of macrophages by oral administrations.

Cytotoxicity of NK cells against YAC-1

About 5% of the spleen is made up of NK cells. For the activity of NK cells, YAC-1 cell have been used as a target cell. Here, to understand the effect of ginsan, the cyto-toxicity of NK cells was analyzed using YAC-1 cell. When initial analysis of NK cell-cytotoxicity in 9-day administration was performed, there was a little increase in its cytotoxicity (Fig. 2). And about 13% cytotoxicity was observed in 22 days and 29 days of administration. Asterisks indicated a significant difference showing $P < 0.05$. Taken together, it suggested that ginsan would increase the cyto-toxicity of NK cells isolated from BALB/c spleen by oral administrations.

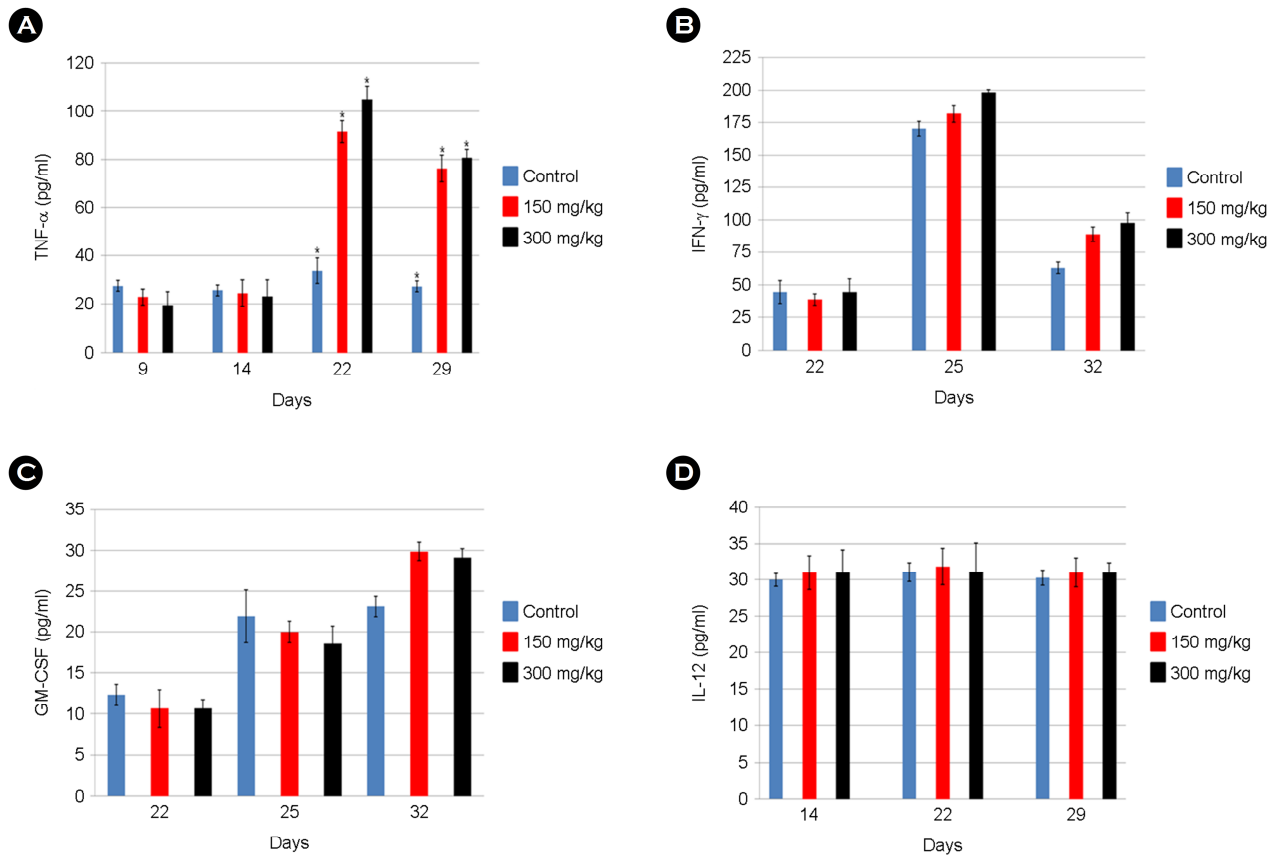**Proliferation of T cells by oral administration of ginsan**

As shown in figure 1 and 2, ginsan would stimulate immune cells such as NK cell and macrophage. To under-stand whether ginsan induced T cell proliferation, T cells were isolated from mice spleens and their numbers were analyzed by a biochemical assay (Fig. 3). As compared with the controls of only saline-inoculated mice, 150 mg/kg and 300 mg/kg did not show higher proliferations of T cells. Our results showed that oral administration did not have significant effect on the proliferation of T cells.

Production of cytokines

Previous reports showed that ginsan stimulated immune cells and induced the production of several cytokines. It was not well understood that oral administration of ginsan
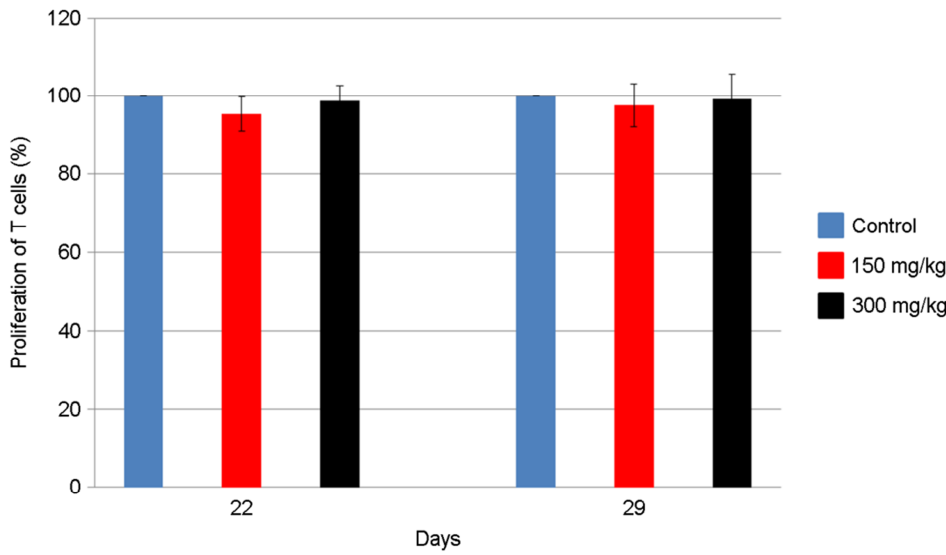
Fig. 3. Proliferation of T cell isolated from mice spleens. After T cells were isolated, they were incubated at 37℃ under 5% CO_2 for 4 days and analyzed by a biochemical assay using a CCK-8 kit.

Fig. 4. Production of cytokines by oral administration of ginsan. TNF-α, INF-γ, GM-CSF and IL-12 were analyzed up to 32 days. They are indicated with amount of pg/mL rather than absorbance. Results are presented as the mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P < 0.05$, using paired T-test, one-tail distribution.

induced the production of cytokines in BALB/c mice. Ginsan could stimulate immune cells such as NK cell and macrophage as shown in figures. It implied that ginsan induced the production of cytokines. TNF-α was measured until 29 days of oral administration in BALB/c. It was not increased at 9 and 14 day analysis but 150 mg/kg and 300 mg/kg at 22 and 29 days showed three times more increase than the controls (Fig. 4A). INF-γ showed the increase 1.07 and 1.16 times of 150 mg/kg and 300 mg/kg at 22 days, respectively more than the controls (Fig. 4B). Twenty nine days of 150 mg/kg and 300 mg/kg induced 1.4 and 1.56 times more than the controls (Fig. 4B). GM-CSF showed similar patterns with INF-γ. Interestingly, 32 days of 150 mg/kg and 300 mg/kg induced about 1.3 times more than the controls (Fig. 4C). IL-12 was not induced in samples more than the controls (Fig. 4D). Asterisks indicated a significant difference showing $P < 0.05$. Taken together, ginsan induced TNF-α, INF-γ and GM-CSF but did not induce IL-12 by oral administration into BALB/c mice.

DISCUSSION

P. ginseng has been widely used in oriental medicine. Ginsan, a polysaccharide isolated from the root of *Panax ginseng* C.A. Meyer, is a potent immunomodulator that produces cytokines and stimulates lymphoid cells to proliferate (Shin et al., 2002; Song et al., 2002; Song et al., 2003). In the present study, ginsan could increase the phagocytosis of macrophages and cytotoxicity of NK cells but was not enough to proliferate T cells by oral administrations. It induced TNF-α, INF-γ and GM-CSF but did not induce IL-12 by oral administration into BALB/c mice. It was reported that ginsan could modulate mucosal immune response in asthma model (Lim et al., 2009) and bacterial infection (Na et al., 2009). On hand, ginsan may serve as an efficient supplement for the oral vaccine because ginsan significantly increased both secretory and serum antibodies to orally administered antigens as ginsan enhanced antibody response to orally introduced antigens by modulating the COX expression in the Peyer's patches (Na et al., 2010). Ginsan is a component of a natural product and it should not have any side effect. For example, it was reported that ginsan in the dose of 100 mg/kg did not alter serum ALT, AST, ALP activities, and total bilirubin and albumin levels indicating no hepatotoxic action of ginsan itself in mice (Song et al., 2004). Ginsan is a group of polysaccharides, suggesting that it can use saccharides-signaling pathway. The cell activation pathway of ginsan was similar to that of lipopolysaccharide (LPS) (Shin et al., 2002). Moreover, since ginsan does not have lipid moiety, such as lipid A, which bind to LPS-binding protein (LBP), it is likely that ginsan can activate macrophages by directly binding CD14 via LBP-independent pathway (Shin et al., 2002).

Taken together, this study suggested that ginsan would be a potential immunostimulator. Therefore, our study suggests that it can be adapted as an immunostimulator that requires a relatively short oral administration.

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