Stimulating Macrophage Activity in Mice and Humans by Oral Administration of Quillaja Saponin

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Quillaja saponin (QS) was examined for its immunostimulating effect on mice and humans after oral administration. Mice fed QS for 24 h significantly increased in chemotactic and phagocytosis activities of peritoneal macrophages. This enhancing effect in both activities continued for 4-d after QS administration. Mice fed QS for 24 h prior to an interperitoneal challenge with Escherichia coli showed a higher survival rate than the control group. Peripheral blood analysis of volunteers showed significant increases in chemotactic and phagocytosis activities after oral administration of QS for 7 d. Furthermore, the volunteers did not show significant changes in immunoglobulin, transaminase, IL-1β, or TNF-α levels, or in serum albumin concentrations. Thus orally administered QS can effectively enhance the immune response through stimulation of macrophages without adverse effects.

Key words: quillaja saponin; immunostimulating; macrophage; Escherichia coli infection; toxicity

Saponins are a group of compounds characterized by structures containing steroid or triterpenoid aglycone and one or more sugar chains that are present widely in the plant kingdom.1) Quillaja saponin (QS) is extracted from the bark of Quillaja saponaria Molina. It has two normonoterpene ester moieties. Due to the presence of lipid-soluble aglycone and water-soluble sugar chains in its structure, QS is surface active compounds with detergent, wetting, emulsifying, and foaming properties. Hence, it is widely used in the food and beverage industry as an emulsifier or foaming agent. In addition, it has been credited with a number of beneficial activities, including expectorant, antimicrobial, anti-inflammatory, anticancer, and cholesterol-lowering ones.2) For many years QS has also been of interest due to its effect on the immune system and its application in vaccines.3–5) QS impacts the immune system through its adjuvant activity, that is, its ability to improve the effectiveness of vaccines. It has been reported that the aglycone portion of saponin binds the cholesterol molecule on the gut cell membrane to form a complex molecule. This new molecule disrupts the gut barrier, increases intestine permeability, and consequently facilitates the passage of large invading molecules from the intestine to the peripheral circulation.6–8) Oral administration of QS with antigens has been reported to enhance cytokine production to specific antigens without producing any reaginic antibody.5) It is widely used as a veterinary vaccine adjuvant, especially for rabies, and foot-and-mouth diseases.3,4) No studies have reported on the immunostimulative effects of oral administration of QS alone.

Macrophages are important players of the innate immune system that participate in eliminating pathogens. Contact of macrophages with pathogens provokes the expression of pro-inflammatory cytokines and mediators that orchestrate pathogen killing and further coordinate the immune response. During the past three decades, immunotherapy has become an important approach in treating human diseases through the use of regimens designed to modulate immune responses. The objectives of this study were (A) to determine whether dietary QS can influence the activity of macrophages in mice and humans, (B) to assess the immunomodulatory properties of QS on E. coli-challenged mice, and (C) to investigate the effects of oral administration of QS on liver function and the inflammatory response in human volunteers.

Materials and Methods

Materials. Quillaja saponin (QS) containing 45% saponin according to HPLC analysis was purchased from Maruzen Pharmaceuticals (Hiroshima, Japan). Dulbecco’s Modified Eagle Medium (DMEM), streptomycin, penicillin, and Gibmsa stain solution were from Nacalai Tesque (Kyoto, Japan). Latex beads (0.81 μm in diameter) were from Sigma-Aldrich (St. Louis, MO). A sport drink powder, Pocari Sweat™, was from Otsuka Pharmaceutical (Tokyo). Brain Heart Infusion (BHI) broth was from Becton, Dickinson (Spark, MD). Mono-poly resolving medium (MPRM) was from Nihon Pharmaceutical (Tokyo). Biotrak™ human interleukin (IL)-1β and human tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kits were from Amersham Pharmacia Biotech (Little Chalfont, UK). E. coli C11 (JE5665) was from the Japanese National Institute of Genetics (Shizuoka, Japan). Unless specified otherwise, all the other chemicals used were guaranteed to be of reagent grade.

Animals. Specific pathogen-free (SPF) 4-week-old male ICR mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed under regulated conditions (22 °C on a 12-h light-dark cycle with lights on at 7 A.M.), and had free access to standard food purchased from Oriental Yeast (Tokyo) and sterile water, unless otherwise indicated. All animal experiments were approved by the Kyoto Women’s University animal committee.

Mouse peritoneal macrophage stimulation, harvesting, and culturing. QS solutions were prepared at various concentrations in a sterile 20 mM sodium phosphate buffer pH 7.2 containing 0.15 M NaCl (PBS).

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One hundred microliters of QS solution was orally injected into each mouse with the dose at 0.05–50 mg/ QS/kg. All the control mice were administered the same volume of sterile PBS instead of the QS solution. At 24 h after QS administration, the mice were euthanized by cervical dislocation. The peritoneal fluid was collected after intra-peritoneal injection of 5 mL of DMEM containing 100 U/mL of penicillin and streptomycin (DMEM (+)+) with gentle massage of the abdomen. Peritoneal exudate cells were washed with DMEM (+) 3 times and suspended in DMEM (+) to a concentration of 5 × 10^6 cells/mL. The concentration of the cell suspension was estimated by using a 1:10 solution of trypan blue and counting the number of living cells on a hemacytometer. Then 200 μL of the cell suspension was distributed into petri dishes and incubated in a humidified atmosphere of 5% CO2 for 37 °C for 2 h to allow the cells to attach. Nonadherent cells were removed by rinsing 3 times with sterile PBS. Adherent cells were resuspended in DMEM (+) before determination of the activity of the macrophage cells.

To determine the activation period of the macrophages due to QS, 100 μL of QS solution at a dose of 0.5 mg QS/kg was fed to each mouse. After QS administration on days 0, 1, 2, 4, and 10, the peritoneal cells of the mice were harvested and cultured as described above.

Chemotaxis assay. The chemotactic activity of macrophages was determined by a modified Boyden chamber technique. Briefly, a 500-μL suspension of 0.5% formalin treated E. coli cells (1 × 10^6 cells/mL) in DMEM (+) was inserted into the lower compartments of the chemotaxis chambers. Then macrophage cells suspended to concentration of 5 × 10^6 cells/mL in 200 μL DMEM (+) were seeded into the upper compartment. The two compartments were separated with a polycarbonate filter (5μm pore size) (Nuclepore, Piasonton, CA). The chambers were incubated at 37 °C in humidified air with 5% CO2 for 18 h. Then the filter was removed. The macrophages in the pores of filter were fixed with 75% methanol for 3 min, and the cells were stained with Giemsa stain solution for 15 min. After rinsing with PBS and drying, the membranes were mounted on glass slides. The stained macrophages were counted in five fields at a magnification of ×400. Chemotactic activity was expressed as the number of macrophages that reached the lower side of the chamber per field of vision.

Phagocytosis assay. Macrophages were cultured at 37 °C in humidified air with 5% CO2 for 18 h on 18-mm coverslips in 8-well plates at 1 × 10^5 cells/mL/well in DMEM (+). Then 200 μL of latex beads (0.03%) was added to each well and this was further incubated under the same conditions, for 1 h. The cells on the coverslips were then rinsed twice with cold PBS, fixed with 75% methanol for 5 min, and stained with Giemsa stain solution at room temperature for 15 min. The stained macrophages were observed in five fields at a magnification of ×400. Cells that ingested more than five beads were counted as bead-positive cells. The phagocytic activity of macrophages was represented as the percentage of bead-positive cells among total cells.

Survival rate of mice infected by E. coli. E. coli cultured in BHI broth at 37 °C for 24 h was harvested by centrifugation at 5,000 × g at 4 °C for 10 min and washed 3 times with sterile PBS. Bacteria cells were suspended in sterile PBS and adjusted to a concentration of 1 × 10^6 cfu/mL in the same solution, as estimated using a standard curve of E. coli cfu/mL and absorbance at 600 nm. The mice were fasted for 1 night and then fed 100 μL of QS in sterile PBS at a dose of 0.05–5 mg of QS/kg. The control mice were fed 100 μL of sterile PBS. Twenty-four h later, each mouse was intraperitoneally injected with 0.5 mL of E. coli suspension. The animal survival rate was recorded every day until 5 d after E. coli injection.

Oral administration of quillaja saponin in humans. Eight healthy male volunteers (22–23 years of age) participated in the study. A QS drink was prepared by mixing 4 g of sport drink powder and 15 mg of QS in 100 mL water. The volunteers were served QS drink at dose of 0.5 mg of QS/kg/d after breakfast for 7 d. A blood sample was collected in a heparinized tube from each volunteer before intake of the QS drink and 24 h after last administration of the QS drink. The study was approved by the local research ethics committee, and it followed the Declaration of Helsinki. The volunteers gave written informed consent to the protocol.

Human plasma and macrophage cell preparation. The blood plasma and macrophage cells from each volunteer were isolated. Briefly, 14 mL of heparinized whole blood was layered onto 12 mL of MPRM, and this was centrifuged at 2,000 × g for 20 min at room temperature. The upper layer was collected as blood plasma and stored at −80 °C for cytokine determination. The Buffy coat under the layers of plasma, containing monocytes (hereafter referred to as macrophage cells), was collected and washed 3 times with 5 mL of DMEM. The macrophage cells were suspended to a concentration of 5 × 10^5 cell/mL in DMEM. Macrophages from each volunteer were tested for chemotaxis and phagocytosis activities as described above.

Biological analysis of human blood. Blood samples from the volunteers were measured for the concentrations of immunoglobulins E (IgE) and G (IgG), glutamic oxalo-acetic transaminase (GOT), glutamic pyruvic-transaminase (GPT), gamma-glutamyl transferase (γ-GOT), and C-reactive protein (CRP) by Japan Clinical Laboratory (Kyoto, Japan). Quantitative measurements of cytokine, IL-1α, and TNF-α in the blood plasma were done using commercial available ELISA kits for human IL-1α and TNF-α. ELISA assay was performed following the manufacturer’s instructions.

Statistical analysis. Survival curves were obtained by the Kaplan-Meier method, and mean survival times due to QS dietary treatment were statistically analyzed by the log-rank test. The results of the other experiments were expressed as mean ± SD. The data for macrophage activity and the biological qualities of the blood from the volunteers were analyzed by two-sided paired t-test. Dose and time-dependent responses of peritoneal macrophages from mice were evaluated by two-sided Student’s t-test. p-Values of less than 0.05 were considered to be statistically significant. All statistical analyses were performed using Excel Statistics 2008 (SSRI, Tokyo).

Results

Effects of dietary QS on mouse peritoneal macrophages

First, we examined the chemotactic and phagocytosis activities of the peritoneal macrophages harvested from the mice 24 h after oral QS administration. To measure the chemotactic activity of the peritoneal macrophages, we used formalin-treated E. coli as the chemotactic agent. The number of macrophages migrating through the membrane filter on the E. coli suspension was taken to represent the chemotactic activity. Six migrating peritoneal macrophages from the control mice were observed (Fig. 1A). The numbers of migrating peritoneal macrophages from the mice orally fed QS at doses of 0.05, 0.5, 5, and 50 mg/kg, were significantly higher than those from the control mice, 36, 45, 42, and 38 cells, respectively (p < 0.05).

The macrophages harvested at the same time were also evaluated for phagocytosis activity, expressed as the percentage of macrophages that phagocytosed latex beads. No promotion of phagocytosis activity was observed in the peritoneal macrophages from the mice fed QS at 0.05 mg/kg (Fig. 1B), as phagocytosis activity was only 17.0%, almost same as that of the control mice (13.8%). The phagocytosis response of macrophages was elevated by orally fed QS to 0.5, 5, and 50 mg/kg (p < 0.05). Feeding the mice at a dose of 0.5 mg/kg induced phagocytosis activity in the mouse macrophages to 35.5%. The phagocytosis activity of the mouse peritoneal macrophages at 22.5 and 24.8% was detected in the mice fed QS at doses of 5 and 50 mg/kg.
Next, we investigated the activation period for the chemotactic and phagocytosis responses of macrophages following oral administration of QS (Fig. 2). We found that the chemotactic activity of the macrophages rose from six to 45 cells at day 1. The numbers of migrating macrophages harvested at days 2 and 4 were 26 and 27. The chemotactic activity of the peritoneal macrophages returned to seven cells, almost the same level as before oral administration of QS, at day 10. The phagocytosis activity of the peritoneal macrophages increased remarkably, from 14.1 to 43.5%, at day 1, and this persisted until day 4 (41.7%). A weaker induction response by QS was observed at day 10. However, the phagocytosis activity of the mice macrophages was still higher (28.9%) on day 10 than day 0.

Effects of QS administration on mice challenged with E. coli

The protective effect of orally administered QS against pathogenic infection in mice was examined. QS at various doses was orally fed to the mice 24 h prior to challenge with E. coli, and they were monitored for survival rate for 5 d. The intraperitoneal infection caused by E. coli was acute in the control mice (Fig. 3). Only 30 and 20% of all the control mice survived after challenge with E. coli for 2 and 3 d, and all of the control mice were dead at day 4. Compared to the control mice, orally fed QS mice tolerated the E. coli challenge well. The mice fed 0.5 mg/kg of QS exhibited considerably elevated survival rates, of 60%, at day 5.

Statistical examination of average survival time by log-rank test showed 3.80 d for the 0.5 mg/kg QS treated group versus 2.50 d for the control group ($\chi^2 = 3.87$, $p < 0.05$). The survival rates of mice treated with 0.05 and 5 mg/kg (3.30 and 3.00 d, respectively), even though they did not significantly differ from that of the control mice, were apparently higher. Fifty and 40% of
the mice treated with QS at doses of 0.05 and 5 mg/kg survived at day 2. The mice from both groups showed survival rates of 30% at day 3 and this persisted for an extended period. Accordingly, dietary QS has a preventive effect in *E. coli* infection in mice.

**Effects of QS administration on human serum macrophages**

A QS drink at a QS concentration of 150 ppm, providing QS at a dose of 0.5 mg/kg/d, was served to volunteers for 7 d consecutively. The immunostimulatory effect of oral administration of QS in humans was determined by comparing the serum macrophage activity of the volunteers before and after intake of the QS drink. The macrophages from the volunteers after QS intake showed significantly higher chemotactic activity than before QS intake (43.9 ± 7.3 vs. 7.5 ± 1.3 cells/area, *p < 0.05*) (Fig. 4). In the same way, oral administration of QS induced considerably a higher level of phagocytosis activity in the macrophages than before QS administration (64.0 ± 7.2 vs. 24.5 ± 4.5%, *p < 0.05*).

**Effects of dietary QS on liver function and the inflammatory response of the human subjects**

After administration of the QS drink for 7 d, the levels of GOT, GPT, and γ-GPT activity in the blood plasma of the volunteers did not change significantly. Also there was no difference in the vitamin serum albumin levels before and after QS intake. On the other hand, significant elevations in α1-globulin and α2-globulin (*p < 0.05*) after 7 d of QS administration were observed. The IgG and IgE levels in the serum of the volunteers were not stimulated by oral administration of QS alone. The result for CPR levels of blood taken before and after administration were negative, indicating that the concentration of CRP was lower than 0.4 mg/dL. The concentrations of IL-1α and TNF-α were undetectable by means of the ELISA kit, indicating that the concentrations of these pro-inflammatory cytokines in the blood of the volunteers were lower than 0.1 pg/mL and 2.5 pg/mL, even after administration of QS.

**Discussion**

The ability to move toward antigens and phagocytize them is thought to be an essential function of macrophages and to be their role in the immune system. In this study, we determined the ability of macrophages to approaching a chemoattractant, lipopolysaccharide, expressing on the cell membrane of *E. coli*, and to ingest latex beads, which were presented as foreign particles. We found that oral administration of QS was very effective in inducing both activities of macrophages. QS feeding showed a preventive effect on pathogenic infection in mice. Oral administration of QS at a dose of 0.5 mg/kg, providing the most immunostimulating effect in mice, also enhanced the chemotactic and phagocytosis activities of macrophages in humans as well.

In the mice fed saponins alone, saponins are not absorbed in the alimentary canal, even though great enhancement of cell proliferation in the spleen and

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**Table 1. Effects of Orally Administered Quillaja Saponin on Biological Qualities of Blood of the Volunteers**

<table>
<thead>
<tr>
<th>Items</th>
<th>Before administration of QS</th>
<th>After administration of QS (0.5 mg/kg/d)</th>
<th>Significance of QS-induced response</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT (IU/L)</td>
<td>16.75 ± 2.57</td>
<td>17.5 ± 2.31</td>
<td>NS</td>
</tr>
<tr>
<td>GPT (IU/L)</td>
<td>13.88 ± 4.99</td>
<td>15.25 ± 8.39</td>
<td>NS</td>
</tr>
<tr>
<td>γ-GPT (IU/L)</td>
<td>22.00 ± 6.85</td>
<td>20.38 ± 6.49</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (%)</td>
<td>67.85 ± 3.52</td>
<td>66.05 ± 4.05</td>
<td>NS</td>
</tr>
<tr>
<td>α1-Globulin (%)</td>
<td>2.28 ± 0.20</td>
<td>2.61 ± 0.26</td>
<td><em>p &lt; 0.05</em></td>
</tr>
<tr>
<td>α2-Globulin (%)</td>
<td>7.66 ± 1.39</td>
<td>8.46 ± 1.10</td>
<td><em>p &lt; 0.05</em></td>
</tr>
<tr>
<td>IgG (mg/dL)</td>
<td>1347.38 ± 281.99</td>
<td>1323.88 ± 236.32</td>
<td>NS</td>
</tr>
<tr>
<td>IgE (mg/dL)</td>
<td>377.88 ± 140.68</td>
<td>399.88 ± 172.59</td>
<td>NS</td>
</tr>
</tbody>
</table>

Blood samples were collected before and after oral intake of QS (0.5 mg/kg/d) for 7 d. Values shown are means ± SD (n = 8). NS, Not significant. *Significant administration of QS (*p < 0.05*).
mesenteric lymph nodes has been reported. A study of the bioavailability of soyasaponin in humans indicates that ingested soyasaponin has low absorbability in human intestinal cells and appears to be metabolized to soyasapogenol B by human intestinal microorganisms in vivo and excreted in the feces. Similarly to other saponins, orally administered QS possibly does not enter the blood circulation and reach immune system at the cell level by blood circulation. One of the potential mechanisms by which orally administrated QS can exert its immunostimulatory effects is by activating the surfaces of intestinal epithelium cells, in view of the amphipathic property of QS. Studies other plant-origin saponins suggest that the aglycone moiety portion of saponins interacts with the cell membrane via specific receptors such as toll-like receptor-4, resulting in the production of cytokine via these receptors. Hence, besides facilitating the uptake of antigens into the endogenous pathway by its adjuvant activity, QS influences the secretion of immunomodulating cytokines such as ILs and interferons (IFNs) that are capable of activating macrophages, either directly or indirectly.

Nevertheless, in this study, no change in IL-1α or TNF-α level in the serum from the volunteers was observed after QS administration. To elucidate the mechanisms and cytokines underlying the induction of macrophage activity by oral administration of QS, further study is necessary. As macrophages are responsible for antigen processing and antigen presentation in antibody formation and the response to T-cell products in cell-mediated immunity, induction of macrophages by orally administered QS might be the main reason for the increase in the immunopotential effects in mice against invading pathogens. It has been reported that feeding saponin before rabies intracerebral challenge enhanced the production of serum rabies-neutralizing antibody in mice. Thus oral administration of QS alone not only stimulates macrophage activity, but also prompts adaptive immune responses to the invading substances.

Complications, such as liver damage or hemolysis of red blood cells at the site of attack, might be caused by many saponins. The toxicity of a saponin depends on its source, composition, concentration, and method of administration. While saponins show toxicity when on its source, composition, concentration, and method of administration. The concentration of QS permitted to be used in soft drinks by way of emulsifiers and stabilizers in Food Regulations 1975 in the UK (Statutory Instrument 1975. no. 1486) is <200 ppm. Despite the concentration of QS used in this study (150 ppm), lower than that stipulated, to assure the safety in QS utilization in humans, the current study investigated the effect of QS, orally administered, on liver function and inflammatory responses in humans. Oral administration of QS at 150 ppm, a dose of 0.5 g/kg/d, for 7 d did not influence indices of the liver function, GOT, GPT, or γ-GTP activities, the concentration of proinflammatory cytokine, IL-1α and TNF-α, or acute inflammatory index, CPR, in serum from the volunteers. Moreover, dietary QS showed no effect on IgG and IgE production. Even though the concentrations of α1-globulin and α2-globulin were significantly elevated (p < 0.05), they were in the normal concentration ranges of these proteins in the blood: 0.1–0.3 g/dL for α1-globulin and 0.6–1.0 g/dL for α2-globulin. Accordingly, the results suggest that oral administration of QS has no adverse effect on liver activity or inflammatory responses.

The data reported here suggest the prospect of applying QS as a potent macrophage-stimulating agent that might be used to prevent pathogenic infection, both in animals and in humans.

Acknowledgments

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