Long-Term Oral Administration of Ginseng Extract Modulates Humoral Immune Response and Spleen Cell Functions

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Abstract: Ginseng radix (Panax ginseng C.A. Meyer) is a popular herbal medicine in Oriental countries. We investigated the effect of long-term oral administration of ginseng extract on the antigen-specific antibody response. Male BALB/c mice were treated orally for 30 consecutive days with 2 g/kg of a 50% ethanol extract of ginseng root. Mice treated with ginseng and immunized with ovalbumin (OVA), resulting in an eight-fold increase in titers of anti-OVA immunoglobulin (Ig)G in the serum compared to the group receiving OVA immunization without ginseng treatment; the level of IgG was also significantly elevated in the mice treated with ginseng and immunized with OVA. Mice treated with ginseng without OVA immunization exhibited significantly reduced IgG and IgA production by spleen cells. However, IgG production was not affected in mice treated with ginseng and OVA immunization in spleen cells. Interleukin (IL)-2, interferon (IFN)-γ and IL-4 secretion by spleen cells from either ginseng-treated mice or OVA-immunized mice were down-regulated compared to that in the control group; while the production of IL-10 was unchanged. The percentage of CD8+ cells was significantly reduced in spleen cells from ginseng-treated, OVA-immunized mice. Thus, long-term oral administration of ginseng extract appears to potentiate humoral immune response but suppress spleen cell functions.

Keywords: Ginseng; Antibody Response; Cytokines; T-Cell Populations.

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Introduction

Ginseng (Panax ginseng, C.A. Meyer) is a widely used Chinese medicine and is a major constituent of tonic remedies in eastern Asian countries. Reports suggest that ginseng is an immunological modulator. Mice injected intraperitoneally (ip) with an ethanol extract of ginseng for 2 consecutive weeks had significantly increased natural killer (NK) cell activity, but did not change in either anti-sheep red blood cell (RBC) antibody response or spleen cell proliferation (Kim et al., 1990). Subcutaneous administration of ginseng extracts in an athymic rat model reduced serum anti-Pseudomonas aeruginosa immunoglobulin (Ig)M and IgA antibody levels after challenge with P. aeruginosa (Song et al., 1997a and b). In contrast, mice injected ip with a 50% ethanol ginseng extract for 3 consecutive days possessed elevated serum levels of IgM, IgG and IgA, and showed a dose-dependent increase in IgM, IgG and IgA secretion by lipopolysaccharide (LPS)-stimulated spleen cells. In addition, serum levels of interleukin (IL)-2, interferon (IFN)-γ, IL-4 and IL-10 were significantly elevated by the extract (Liou et al., 2004).

Chinese herbal medicine is normally taken orally on a long-term basis. Oral administration of an aqueous ginseng extract for 5 to 6 days significantly increased the sheep RBC-induced primary IgM response and secondary IgG and IgM responses. NK cell activity also increased after the treatment (Jie et al., 1984). Long-term oral administration (52 days) of a 50% ethanol ginseng extract decreased serum levels of γ-globulin and IgG1 (Kim et al., 1997a and b). A recent study revealed that male BALB/c mice treated orally with ginseng root extract for 5 consecutive days had reduced serum level of IgG, but elevated IgA level. Production of IgG by spleen cells also significantly decreased, but IgA production was increased. IL-2, IFN-γ (Th1-type cytokine) and IL-10 (Th1-type cytokine) production by concanavalin (Con) A-stimulated spleen cells was up-regulated in ginseng-treated mice compared to the control group. However, production of IL-4 (Th2-type cytokine) showed no significant change. The activity of NK cells was increased in the ginseng group, while percentages of T-lymphocytes (CD3+) and CD4+8−, CD4−8+ subset were reduced (Liou et al., submitted). The aim of this study was to extend the scope of previous studies and investigate the effect of long-term oral administration of a 50% ethanol ginseng extract on immune responses.

Materials and Methods

Preparation of Herbal Drug Extract

White ginseng (Panax ginseng, C.A. Meyer), four to five years old, was purchased from the Lao-Chen-Ge Chinese herbal drugstore, De-Hua Street, Taipei, and grounded into powder. The powder (5 g) was suspended in 100 ml of 50% ethanol and boiled until the volume was reduced to half. The remaining suspension was centrifuged at 10,000 g for 30 minutes and the supernatant collected and dried with a Speed Vac. The dried extracts were reconstituted using sterile distilled water to make a stock of 100 mg ginseng/ml.
Animal Treatment and Cell Culture

BALB/c mice were divided into four groups, each containing six male mice (National Laboratory Animal Center, Taipei, Taiwan). One group of mice was given 2 g ginseng extract per kg body weight for 30 days by gastric gavage. On the first day of ginseng treatment, the mice were injected ip with a mixture of 100 μg ovalbumin (OVA) and 2 μl of completed Freund’s adjuvant. The mice were boosted with the same amount of OVA on day 14. The second group of mice was given ginseng extract without OVA immunization. The third group received OVA immunization without ginseng treatment. The control group received sterile water orally and a saline injection. All mice were sacrificed on day 31, sera were collected, and spleen cells were isolated. The spleen cells (5 × 10⁵ cells/ml) were cultured in media containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, antibiotics and LPS (1 μg/10⁵ cells) for 5 days. Cultured supernatants were retained for further studies. To investigate the effects of ginseng on cytokine production, spleen cells (1 × 10⁶ cells/ml) were cultured in the presence of 1 μg/ml Con A for 48 hours. Cultured supernatants of spleen cells were collected, and cytokine concentrations were measured using an ELISA technique.

Titration of Anti-OVA Antibody

Anti-OVA antibody activities in mouse sera were estimated using an ELISA technique. Briefly, a 96-well microtiter plate (Nunc-Immuno Plate, MaxiSorp, Nunc, Denmark) was precoated with 1 μg OVA /well (10 μg/ml, 100 μl/well) and incubated at 4°C overnight. The plate was washed with phosphate buffered solution (PBS) and 0.05% Tween 20 solution and blocked with PBS and 1% gelatin. After blocking, a two-fold series dilution of the serum samples were added (100 μl/well) and the plate incubated at 37°C for 2 hours. After incubation, the plates were washed and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Zymed, CA, USA) was added (100 μl/well) and incubated at 37°C for 1 hour. The color was developed using a substrate solution containing 0.1 M citrate buffer (pH 4.5), 0.03% H₂O₂ and 0.1% o-phenylenediamine. The absorbance in each well was read at 490 nm using an ELISA reader (EL311, BioTek, VT), and the data analyzed using a log-logit model.

ELISA for Immunoglobulins

Immunoglobulin concentrations were measured using a sandwich ELISA technique as previously described (Liou et al., 2004). Briefly, a 96-well microtiter plate was precoated with 100 ng of rabbit anti-mouse IgG + IgA + IgM antibody (Zymed Laboratory, CA) per well and incubated at 4°C overnight. The plate was blocked with PBS and 1% gelatin. Then, the diluted samples and standards were added (100 μl/well). The plates were incubated at 37°C for 2 hours and then HRP-conjugated secondary antibody at 100 μl/well were added. After 1-hour incubation at 37°C, the color development, absorbance determination and the data analysis were performed as described above.
ELISA for Cytokines

A cytokine ELISA system (R&D Systems, MN, USA) was used to quantify cytokines as previously described (Liou et al., 2004). The capture antibody was a rat monoclonal antibody against mouse IL-2, IFN-γ, IL-4, or IL-10, respectively. The detection antibody was a biotinylated goat anti-IL-2, -IFN-γ, -IL-4, or IL-10 polyclonal antibody, respectively. After the antigen-antibody reaction was completed, a HRP-conjugated streptavidin (Zymed, CA, USA) was added and the plate incubated at 37°C for 1 hour. The color was finally developed by a substrate solution containing hydrogen peroxide (0.02%) and 0.4 mg/ml tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories, Inc., MD, USA). Plates were incubated in the dark for 30 minutes at room temperature and the reaction stopped by adding 100 μl of 2N sulfuric acid per well. The absorbance at 450 nm was read with an ELISA reader (EL311, BioTek, VT, USA). The data was analyzed using a log-logit model.

Flow Cytometric Assay for T-Cell Population Analysis

The effects of ginseng on CD3 (pan-T marker), CD4 (helper T-cell marker) and CD8 (cytotoxic T-cell marker) expression in T-lymphocytes were estimated by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). CD3, CD4 and CD8 expression were monitored using a Cy-Chrome conjugated hamster anti-mouse CD3 antibody (Becton Dickinson, San Jose, CA), phycoerythrin (PE)-conjugated hamster anti-mouse CD4 antibody (Becton Dickinson, San Jose, CA), or fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD8 antibody (Becton Dickinson, San Jose, CA), respectively. Briefly, the non-adherent spleen cells were washed three times with sterile cold PBS and the concentration of cells adjusted to 1 × 10⁶ cells/ml. Each sample was mixed with 1 μl of indicated fluorescence-conjugated antibody and incubated at room temperature for 15 minutes. After incubation, the cells were washed with cold PBS and centrifuged at 200×g for 10 minutes. The pellet was dispersed and mixed with 500 μl of 1.0% paraformaldehyde. The percentage of CD3+ cells in the spleen lymphocytes and the percentages of CD4+8− and CD4−8+ cells among the CD3+ cell population were analyzed by flow cytometry. The fluorescence-conjugated isotype controls were used to determine the quadrant line setting, and any that exhibited non-specific staining were subtracted from the percentage for each individual surface marker.

Statistical Analysis

Data from the control and drug treatment groups were analyzed by one-way ANOVA. The difference between the two means was assessed using Student’s t-test, where p < 0.05 was considered significant.
Results

Ginseng Regulated Antibody Response to OVA

The effect of ginseng on the OVA-induced antibody response was investigated. For most of the serum dilutions, the values of OD_{490} (the value of absorbance at 490 nm) from mice treated with ginseng extract during OVA immunization were significantly higher than those from mice not treated with ginseng extract (p < 0.05) (Fig. 1). The titer (reciprocal of the dilution factor at the end point of titration) for OVA with ginseng group was 3072, approximately eight-fold greater than that obtained for the OVA group (384). Mice treated with ginseng without immunization and the blank control group showed a baseline level of anti-OVA antibody activity.

Ginseng Elevated Serum Level of IgG

Sera samples from treated mice were collected and assayed for IgG, IgA and IgM concentrations using ELISA. Ginseng extract or OVA alone did not elevate IgG concentration in sera. However, oral administration of ginseng extract during the period of OVA immunization significantly elevated the serum level of IgG (p < 0.01) (Fig. 2). The serum levels of IgM and IgA in three experimental groups were unchanged in comparison with those of the control group (Fig. 2).

Ginseng Down-Regulated IgG and IgA Secretion by Spleen Cells

Ginseng extract treatment had a distinct effect on spleen cell functions. Mice treated with ginseng extract for 30 days exhibited significantly down-regulated IgG and IgA secretion.
Mice that received ginseng extract during OVA immunization showed no change in IgG secretion, but had a significantly reduced IgA secretion (Fig. 3). IgM secretion was unaffected by either ginseng treatment or OVA immunization.

**Ginseng Reduced Production of Cytokines**

The significant reductions in IgG and IgA production in spleen cells suggest that long-term oral administration of ginseng extract might down-regulate the Th2-type cytokine...
production. To study the modulatory effect of ginseng on cytokine production, spleen cells were isolated and stimulated with Con A, and the concentrations of cytokines in the cultured supernatant were measured. Results indicated that the concentration of IL-4 in cultured supernatants was decreased significantly in the three experimental groups (ginseng treatment alone, immunization with OVA without ginseng treatment, and treatment with ginseng extract during OVA immunization) as compared to the control (Fig. 4). The secretion of Th1-type cytokines, including IL-2 and IFN-γ, was also reduced significantly in these three experimental groups (Fig. 5). However, IL-10 (Tr1-type cytokine) secretion remained unchanged (Fig. 4).

Figure 4. Th2-type cytokine secretion by spleen cells in vitro. Data was mean ± SEM of six similar experiments. *p < 0.05 from the control group.

Figure 5. Th1-type cytokine secretion by spleen cells in vitro. Spleen cells were isolated and in vitro-cultured for 48 hours in the presence of 1 μg/ml of Con A. Supernatants were harvested and assayed for cytokines by ELISA. Data was mean ± SEM of six similar experiments. *p < 0.05 from the control group.
The amount of IgG1, IgG2a and IgG2b secretion by spleen cells in vitro was determined. Results indicated that the concentrations of IgG1 and IgG2b in spleen cell cultured supernatants were down-regulated by long-term ginseng treatment (Fig. 6), perhaps resulting from a significant decrease in IL-4 production. IgG2a secretion increased significantly in mice treated with ginseng extract alone or simultaneously immunized with OVA. Since IFN-γ is a potential inducer for class-switching from IgM to IgG2a, this result did not agree with the data presented in Fig. 5, which showed a decrease in IFN-γ production after ginseng treatment.

**Ginseng Regulated Secretion of IgG Subtypes in Spleen Cells**

The amount of IgG1, IgG2a and IgG2b secretion by spleen cells in vitro was determined. Results indicated that the concentrations of IgG1 and IgG2b in spleen cell cultured supernatants were down-regulated by long-term ginseng treatment (Fig. 6), perhaps resulting from a significant decrease in IL-4 production. IgG2a secretion increased significantly in mice treated with ginseng extract alone or simultaneously immunized with OVA. Since IFN-γ is a potential inducer for class-switching from IgM to IgG2a, this result did not agree with the data presented in Fig. 5, which showed a decrease in IFN-γ production after ginseng treatment.

**Effect of Ginseng Extract on T-Lymphocyte Population**

A series of immunofluorescence assays were performed to monitor the variation in the T-cell subpopulation after treating the mice with ginseng extract and OVA. Mice treated with ginseng extract alone or immunized with OVA alone had no change in the percentage of T-lymphocyte subpopulations. Mice treated with ginseng extract and immunized with OVA exhibited a significant reduction in the percentage of CD8+ T-lymphocytes. CD8+ T-lymphocytes consist mainly of cytotoxic T-lymphocytes, and are the major effector cells of the cellular immune response. The percentages of CD3+ and CD4+ cells also were reduced, but not significantly. Therefore, the ratio between CD4+ and CD8+ cells remained unchanged (Table 1).
Ginseng is a widely used Chinese herbal medicine. As previously reported, mice injected intraperitoneally with a 50% ethanol extract of ginseng for 3 consecutive days exhibited significant increases in IgM, IgG and IgA production. The same treatment also increased IL-2, IFN-γ, IL-4 and IL-10 (Th2-type cytokine) production (Liou et al., 2004). However, a recent study indicated that mice treated orally with ginseng root extract for 5 consecutive days had reduced serum level of IgG, but elevated level of IgA (Liou et al., submitted). LPS-stimulated spleen cells from ginseng-treated mice showed a significant decrease in IgG production, but an increase in IgA production. IL-2, IFN-γ and IL-10 production by Con A-stimulated spleen cells from ginseng-treated mice were up-regulated compared to the control group. However, no significant change in IL-4 production occurred. In addition, IgG2a secretion by spleen cells from ginseng group increased in a dose-dependent fashion, while IgG1 secretion was significantly decreased. The activity of NK cells increased in the ginseng group also; however, the percentages of CD3+ and CD4+8− and CD4−8+ subset lymphocytes were reduced (Liou et al., submitted). Thus, the short-term oral administration of ginseng appears to enhance a Th1-type immune response. The discrepancy in the results from these two studies might be related to the route of administration.

The present study extended our previous research and indicated that the duration of ginseng treatment affects the immunomodulatory activity of ginseng. The dosage chosen in this study is based on a dose-dependent experiment in our previous study (Liou et al., submitted). Data from the dose-dependent experiment suggested that 2 g of ginseng extract per kg body weight (2 g/ginseng/kg) was the optimal dose for short-term oral administration of ginseng extract. In the present study, mice treated orally with 2 g/ginseng/kg for 30 days (a long-term treatment) showed an eight-fold increase in the titer of anti-OVA IgG compared to titers in mice immunized with OVA alone. Mice treated with ginseng and OVA immunization had significantly elevated serum IgG levels. However, IgA and IgM levels were unchanged; suggesting that long-term treatment with ginseng extract augmented the OVA-induced humoral immune response.

Results from spleen cell function studies did not correlate well with those from antibody studies. Mice treated orally with ginseng for 30 days and immunized with OVA during the same period did not change IgG levels, but reduced IgA production in spleen cells. However, mice treated with ginseng alone exhibited significantly reduced IgG and IgA production.

### Table 1. Effect of Ginseng Extract on the T-Lymphocyte Subpopulation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Immunogen</th>
<th>CD3 (%)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>CD4/CD8</th>
</tr>
</thead>
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<tr>
<td>H2O</td>
<td>Saline</td>
<td>44.09 ± 2.69</td>
<td>30.88 ± 2.28</td>
<td>13.99 ± 1.02</td>
<td>2.21</td>
</tr>
<tr>
<td>Ginseng</td>
<td>Saline</td>
<td>39.80 ± 2.61</td>
<td>27.23 ± 2.40</td>
<td>11.92 ± 0.47</td>
<td>2.28</td>
</tr>
<tr>
<td>H2O</td>
<td>OVA</td>
<td>40.90 ± 2.49</td>
<td>28.62 ± 2.21</td>
<td>12.20 ± 1.10</td>
<td>2.34</td>
</tr>
<tr>
<td>Ginseng</td>
<td>OVA</td>
<td>36.82 ± 2.99</td>
<td>25.93 ± 2.65</td>
<td>10.65 ± 0.57</td>
<td>2.43</td>
</tr>
</tbody>
</table>

*Data were mean ± SEM of six experiments. *p < 0.05 from the control group.
in spleen cells. IL-2, IFN-γ and IL-4 secretion by spleen cells were down-regulated in the mice treated with ginseng alone, OVA immunization alone, and ginseng plus OVA immunization compared to the control group; however, secretion of IL-10 was unchanged. The results from IgG isotype studies indicated that IgG1 and IgG2b concentrations in spleen cell cultured supernatants were down-regulated by long-term ginseng treatment; however, IgG2a secretion was increased significantly. These results are consistent with those from a rat model showing that subcutaneous injection of 0.025 g/kg/day of an aqueous ginseng extract for two weeks increased IgG2a production but decreased IgG1 secretion. The authors suggest that the change from IgG1 to IgG2a subclasses indicates a shift from a Th2- to Th1-type immune response (Song et al., 1997a and b). IFN-γ is a potential inducer of this class-switch from IgM to IgG2a. However, we found a significant decrease in IFN-γ production upon ginseng treatment, suggesting that cytokines other than IFN-γ induced production of IgG2a. Thus, long-term oral administration of ginseng might negatively affect the function of spleen cells by down-regulating IgG and IgA production, decreasing type-1 cytokine production and reducing the percentages of CD8+ subsets. This data suggests that lymphoid tissues other than the spleen might be involved in promoting the OVA-induced humoral immune response through ginseng extracts.

Significant differences exist in the regulation of antibody and cytokine production induced after 5 days and 30 days of ginseng treatment. This is consistent with a previous report stating that oral administration of an aqueous ginseng extract for 5 to 6 days in mice increased the primary IgM response and secondary IgG and IgM responses to sheep RBC by 50%–100% at the highest dose (Jie et al., 1984). However, another study reported that oral administration of a 50% ethanol ginseng extract for 52 days decreased serum levels of γ-globulin and IgG1 by 56% and 68%, respectively (Kim et al., 1997).

Data from animal models and human trials indicate that ginseng radix possesses pharmacological activity including sedative, demulcent, aphrodisiac, anti-depressant and diuretic effects (Ernst, 2002). Data also support the role of ginseng radix as an immunomodulator. Currently, 16 double-blind, randomized, placebo-controlled human trials of ginseng root extract have been conducted. However, most of the well-designed clinical trials do not support many of the claims for the benefits of ginseng (Vogler et al., 1999). The pharmacological effects of ginseng extract are profoundly affected by the procedure used for extract preparation, dosage of treatment, route of administration and treatment duration. Our study on the immunomodulatory activity of a 50% ethanol-soluble ginseng extract yielded significantly different results, when the experimental animals were treated with different ginseng doses, different route of administration and different protocols for drug treatment. Therefore, the protocols utilized in clinical trials should be standardized to confirm any pharmacological effects of ginseng radix.

Acknowledgments

This work was presented by Chian-Jiun Liou to the Department of Biology, National Taiwan Normal University in partial fulfillment of the requirements for a PhD degree.
References


