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The Extract of *Gynostemma pentaphyllum* Enhanced the Production of Antibodies and Cytokines in Mice

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Gynostemma pentaphyllum is a popular herbal tea in China and some Asian countries. The modulatory function of *G. pentaphyllum* total plant extracts on immune cells was evaluated in this study. The extract was intraperitoneally injected into mice for 5 consecutive days. The production of antibodies from B cells or cytokines from T cells was determined mainly with ELISA. After the treatment, serum IgM and IgG2a were significantly enhanced and showed dose-dependent effect. Moreover, serum IgA and IgG1 were also increased when received the extract at the doses of 0.05 or 0.50 g/kg/day. In addition to the serum levels, the injection of the extract enhanced the production of all antibodies from LPS-activated spleen cells. Furthermore, more cytokines were secreted from Con A-stimulated splenocytes of *G. pentaphyllum*-treated mice. Our results suggest that the extract of *G. pentaphyllum* might promote immune responses through the activation of T and B cells.

Key words——*Gynostemma pentaphyllum*; antibodies; cytokines; mice

INTRODUCTION

Gynostemma pentaphyllum (Cucurbitaceae) is one of the widely used herbal medicines in Southeast China. It is a perennial liana which grows in wild fields in Southern China, Japan, and Korea. In China, it is named as ''Jiao-Gu-Lan'' and usually used as an herbal tea. According to the principles of traditional Chinese medicine, the use and expecting clinical effects of *G. pentaphyllum* is very similar to *Panax ginseng*. Indeed, about 90 kinds of gypenosides have been also isolated from *G. pentaphyllum*.[1] In addition, the structures of more than six gypenosides from *G. pentaphyllum* were reported to be the same with the ginsenosides from *P. ginseng*.[2]

In recent years, several pharmacological effects of *G. pentaphyllum* have been reported, such as anticancer,[3] anti-gastric ulcer,[4] treatment of hepatitis,[5] and hyperlipidaemia.[6] Moreover, insulin releasing substance had also been isolated from this plant.[7] This substrate improved glucose tolerance and enhanced plasma insulin levels in rats with hyperglycemia. *G. pentaphyllum* was reported to activate immune response for cancer patients.[8]

The function of T and B lymphocytes are most important for host adaptive immunity. The antibodies that are secreted by B cells block infectious microbes or foreign antigens. Among the subtypes of antibodies, IgM appears first in primary response when the hosts encounter antigens.[9] Consequently, the production of specific IgG is able to neutralize the microbes or antigens. Nowadays, it is well accepted that the cytokines produced form $CD4^+$ T cells instruct the patterns of antibody subtypes.[10] Activated $CD4^+$ helper T (Th) cells are mainly divided into two subpopulations, including Th1 and Th2 cells,[11] based on the cytokine expression patterns. Th1 cells secret more interleukin (IL)-2, IL-12, and interferon (IFN)-γ;[12] however, Th2 cells produce more IL-4, IL-5, and IL-13.[13] IL-10 had been grouped as Th2-type cytokine, but it is now considered as the regulatory cytokine.[14]

Although *G. pentaphyllum* have been used widely in several countries and some clinical effects have

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been approved, its modulatory function on T and B cells has not been evaluated. With the introperitoneal injection of the *G. pentaphyllum* extract into mice, the aim of this study was to examine the effect of the extract on the serum immunoglobulin levels or the cytokine production from active spleen cells.

MATERIALS AND METHODS

Preparation of *G. pentaphyllum* Extracts *Gynostemma pentaphyllum* total plant extracts were prepared by the Department of Chinese Herbal Pharmacy, Chang Gung Memorial Hospital. Briefly, dried herb was soaked in water and boiled for 50 minutes. The decoction was then lyophilized and was reconstituted with phosphate buffered saline (PBS) before use. The solution was shaken overnight and steriled with 0.25 μm filters, aliquoted and stored at a -80°C freezer.

LPS Assay Owing to hope to know whether *G. pentaphyllum* was contaminated by sold gram-negative bacterium. We used Limulus Amebocyte Lysate-had kit to assay LPS content (Cape Cod, E. Falmouth, MA, USA). LPS was defined that was contaminated over 0.5 EU/ml in the test.[15,16]

Animals and the Administration of *G. pentaphyllum* Extracts Male BALB/c mice aged 6–8 weeks (average weight: 20–25 g) were purchased from Laboratory Animal Center, College of Medicine, National Taiwan University. Mice were accommodated one week before the experiments and maintained and handled according to the guidelines of Animal Care Committee of Chang Gung University and NIH Guides for the Care and Use of Laboratory Animals.

Mice were divided into four groups (six mice per each group) and injected intraperitoneally with normal saline (as normal control) or various doses of *G. pentaphyllum* extracts, including 0.05 g, 0.5 g, and 5.0 g per body weight daily (g/kg/day) for 5 consecutive days. The experiment was repeated twice.

Serum Collection and Splenocyte Cell Cultures Mice were sacrificed on day sixth. The blood was harvested and centrifuged at 6,000 rpm for 20 min. The serum was then collected and stocked at -80°C.

Single cell suspensions were prepared as previously described.[17] The spleen cells (5×10^5 cells/ml) were cultured in a medium containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 1 μg/ml lipopolysaccharide (LPS) for five days. The immunoglobulin concentrations in the culture supernatants were determined using the ELISA technique. To determine the effects of *G. pentaphyllum* on the cytokine production, the spleen cells (1×10^6 cells/ml) were cultured in the presence of 1 μg/ml concanavalin A (Con A) for two days. Culture supernatants of Con A-activated spleen cells were collected, and the cytokine concentrations were measured using the cytokine-specific ELISA technique.

ELISA Assay To determine the concentrations of IgA, IgG, IgM, IgG1, and IgG2a, we used sandwich enzyme-linked immunosorbent assay (ELISA) technique. Microtiter plates were coated with 100 μl of rabbit anti-mouse IgG+IgA+IgM, anti-IgG1, or anti-IgG2a antibodies (Zymed Laboratories, San Francisco, CA, USA) in PBS. After an overnight incubation at 4°C, the plates were washed and blocked with 1% gelatin for 1 h at 37°C. After washing, 100 μl of serial dilutions of standard IgG, IgG1, IgG2a, IgA, IgM and dilutions of serum were added, then incubated for 2 h at 37°C. Consequently, a horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed Laboratories) was added followed with the incubation of 100 μl of *o*-phenylenediamine (OPD) solution (0.4 mg/ml) for 20 min at room temperature. Finally, the reaction was stopped by 25 μl of 3N H_2SO_4. The optical density at 490 nm in each well was read using an ELISA reader.

The amounts of interleukin (IL)-2, interferon (IFN)-γ, IL-4 and IL-10 were measured with the use of ELISA kits specific to each cytokine (R & D Systems, Minneapolis, MN, USA). The minimum detectable concentration is 9.4 pg/ml for IFN-γ, 15.6 pg/ml for IL-2, 7.8 pg/ml for IL-4, and 15.6 pg/ml for IL-10.

Statistics Analysis The data were analyzed using One-way analysis of variance (ANOVA) with post-hoc Dunnett's test. Values were presented as the mean$\pm$the standard error (SE). Probability values (p) of less than 0.05 were considered to be significant.

RESULTS

***G. pentaphyllum* Induced Higher Levels of Immunoglobulin in Mice Serum** Each group of BALB/c mice received intraperitoneal injection of various doses (0, 0.05, 0.5, or 5 g/kg/day) of *G. pentaphyllum* for 5 consecutive days and then were sacrificed on day 6. The mice were designated as Nor-

mal, 0.05 G, 0.50 G, and 5.00 G groups, respectively. LPS assay of high dose 5.00 G group was about 0.192 EU/ml *in vitro*. The data would not influence immune response in mice.[16] The serum immunoglobulin levels were examined before and at the end of the experiments. The total IgG concentration in serum did not change with or without the *G. pentaphyllum* treatment (Fig. 1(A)). The administration of *G. pentaphyllum* at 0.05 or 0.5 g/kg/day enhanced the serum IgG1 levels (Fig. 1(B), 2.63 ± 0.41 mg/ml for 0.05 G or 2.53 ± 0.31 mg/ml for 0.50 G vs. 1.48 ± 0.10 mg/ml in Normal, $p=0.06$ or $p<0.01$, respectively). The highest dose of *G. pentaphyllum* (5.00 G), however, suppressed the serum IgG1 level (0.65 ± 0.15 mg/ml, $p<0.01$). On the other hand, higher doses of *G. pentaphyllum* significantly promoted more serum IgG2a (Fig. 1(C), 0.44 ± 0.03 mg/ml for 0.05 G, 0.59 ± 0.10 mg/ml for 0.50 G, or 0.65 ± 0.05 mg/ml for 5.00G *vs.* 0.35 ± 0.01 for Normal, $p<0.05$, $p=0.07$, or $p<0.01$, respectively).

The treatment of *G. pentaphyllum* changed the serum IgA levels similar to the effect of this herb to serum IgG1 levels (Fig. 1(D)). Nevertheless, *G. pentaphyllum* extract had dose-dependent enhancement

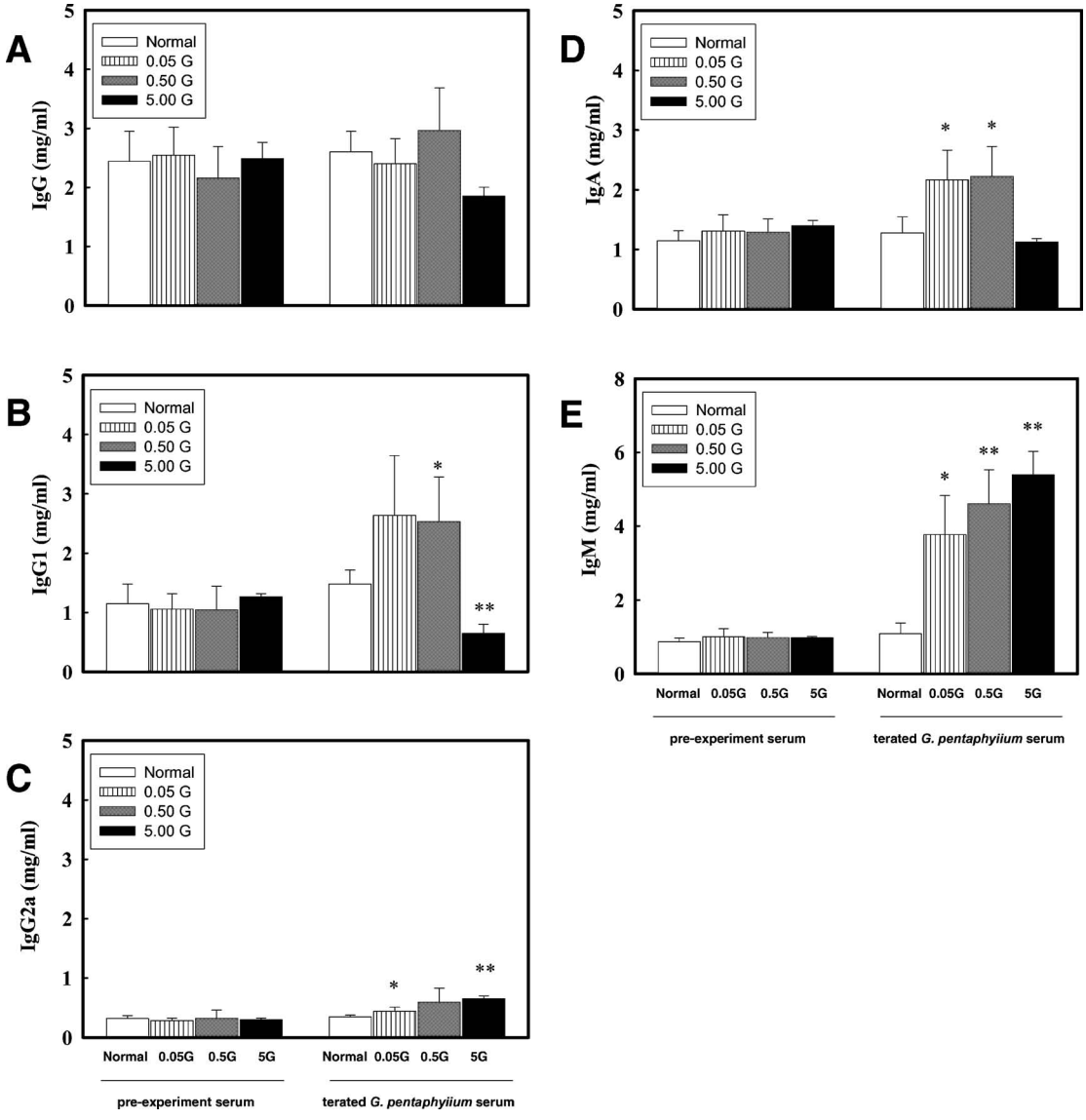

Fig. 1. *G. pentaphyllum* Extract Injected Intraperitoneally Induced Higher Serum Levels of Immunoglobulin

Serum was collected from mice pre-experiment or after the injection of *G. pentaphyllum* for 5 consecutive days ($n=6$). The mice were designated as Normal, 0.05 G, 0.50 G, and 5.00 G groups for various doses (0, 0.05, 0.50, or 5.00 g/kg/day) of *G. pentaphyllum* extract, respectively. The concentrations of serum IgG (A), IgG1 (B), IgG2a (C), IgA (D), and IgM (E) were determined with ELISA. Data were presented as mean±standard error (SE). *indicates $p<0.05$, and **indicates $p<0.01$, when compared to the serum level in Normal group.

on serum IgM concentrations (Fig. 1(E), 3.77±0.43 mg/ml for 0.05 G, 4.60±0.38 mg/ml for 0.50 G, and 5.39±0.64 mg/ml for 5.00 G *vs.* 1.08±0.12 for Normal, $p<0.05$, $p<0.01$, or $p<0.01$, respectively).

***G. pentaphyllum* Enhanced Immunoglobulin Production from Spleen Cells** The supernatants of splenocytes cultures were collected after the stimulation with 1 μg/ml of LPS for 3, 4, or 5 days. The immunoglobulin concentrations in the supernatants were determined with ELISA. The results in Fig. 2 (A) indicated that higher dose of *G. pentaphyllum* extract enhanced more prominent IgG secreted from activated spleen cells. The culture durations slightly affected the production of IgG. Significant increase was observed for the concentration of both IgG1 and IgG2a subtypes (Figs. 2(B) and 2(C)) with the highest dose of treatment, particularly with the IgG1 production from 4-day culture (0.055±0.006 μg/ml *vs.* 0.011±0.001 μg/ml, $p<0.01$) and IgG2a production from 5-day culture (0.081±0.005 μg/ml *vs.* 0.006±0.001 μg/ml in normal, $p<0.01$). In addition, longer culture durations enhanced the secretion of IgG1 and IgG2a. Compared to the cultures for 3 days, the IgG1 production of 5-day culture was increased about 2 folds from the 0.50 G and 5.00 G mice. Similar, but more prominent pattern was observed with the IgG2a production, 2.38 folds for 0.50 G group and 13.18 folds from the 5.00 G group. The dose- and culture duration-dependent production of IgA (Fig. 2(D)) and IgM (Fig. 2(E)) was also detected.

***G. pentaphyllum* Enhanced the Production of Cytokines from Activated Spleen Cells** Spleen cells at the concentration of 10^6 cells/ml were cultured with Con A for 2 days. The supernatants were collected and the cytokine concentrations were determined with ELISA. The results in Fig. 3(A) showed that administration of *G. pentaphyllum* extract enhanced the production of IFN-γ from Con A-activated spleen cells (compared to 48.3±6.3 pg/ml in Normal group, 93.5±13.5 pg/ml for 0.05 G, $p<0.01$; 112.6±8.2 pg/ml for 0.50 G, $p<0.01$ or 109.7±8.7 pg/ml for 5.00 G, $p<0.01$). The production of IL-2, another Th1-type cytokine, showed dose-dependent response of *G. pentaphyllum* administration (Fig. 3 (B), compared to 545.9±86.5 pg/ml in Normal group, 1068.1±67.2 pg/ml for 0.05 G, $p<0.01$; 1486.2±123.1 pg/ml for 0.50 G, $p<0.01$; and 1751.7 ±59.9 g/ml for 5.00 G, $p<0.01$).

For the production of Th2-type cytokines, all three doses enhanced IL-4 secretion from activated spleen cells. However, the highest IL-4 production was detected from 0.05G mice (Fig. 3(C), 202.5±27.4 pg/ml *vs.* 65.8±11.5 pg/ml in Normal group, $p<0.05$). Nevertheless, the production of IL-10 showed dose-dependent response with the injection of *G. pentaphyllum* extract (Fig. 3(D), compared to 27.5±4.0 pg/ml in Normal group, 48.7±4.3 pg/ml for 0.05 G, $p<0.05$; 71.7±8.2 pg/ml for 0.50 G, $p<0.01$; and 91.8±5.6 pg/ml for 5.00 G, $p<0.01$).

DISCUSSION

G. pentaphyllum is a common herbal tea in China and other Asian countries. Despite some anti-cancer and anti-infection activities, it has been described to have immune modulatory effects for cancer patients and serves as a potential treatment for respiratory inflammation.[3,8] However, the mechanism for this action remains not clear. The present study demonstrated that the extract of *G. pentaphyllum* enhanced serum immunoglobulin levels and the production of antibodies from LPS-activated spleen cells. In addition, the production of cytokines from Con A-stimulated spleen cells was also improved with *G. pentaphyllum* treatment.

The significant and dose-dependent increase of serum IgM levels indicates that the treatment of *G. pentaphyllum* might improve primary immune response.[18] IgA, the most dominant immunoglobulin in mucus[19] and also serves as one of important mediators in the first line defense, was also enhanced with the treatment of the doses of 0.05 or 0.5 g/kg/day. This provides the preliminary evidence for the immune stimulatory activity of *G. pentaphyllum*. On the other hand, the extract at the doses of 0.05 or 0.5 g/kg/day increased serum IgG1 and IgG2a concentration. Both subtypes are considered to function as adaptive or more specific immune responses. It has been well accepted that the cytokines of Th2-type, such as IL-4, regulate the immunoglobulin class-switch and result into more IgG1 production.[13] The ConA-stimulated spleen cells of *G. pentaphyllum*-treated mice produced more IL-4 in our study. In addition, the production of IgG2a is correlated with the activity of Th1 cells.[20] Therefore, the dose-dependent increase of serum IgG2a levels implicated that the extract of *G. pentaphyllum* might also have the activity to enhance the Th1 activity.

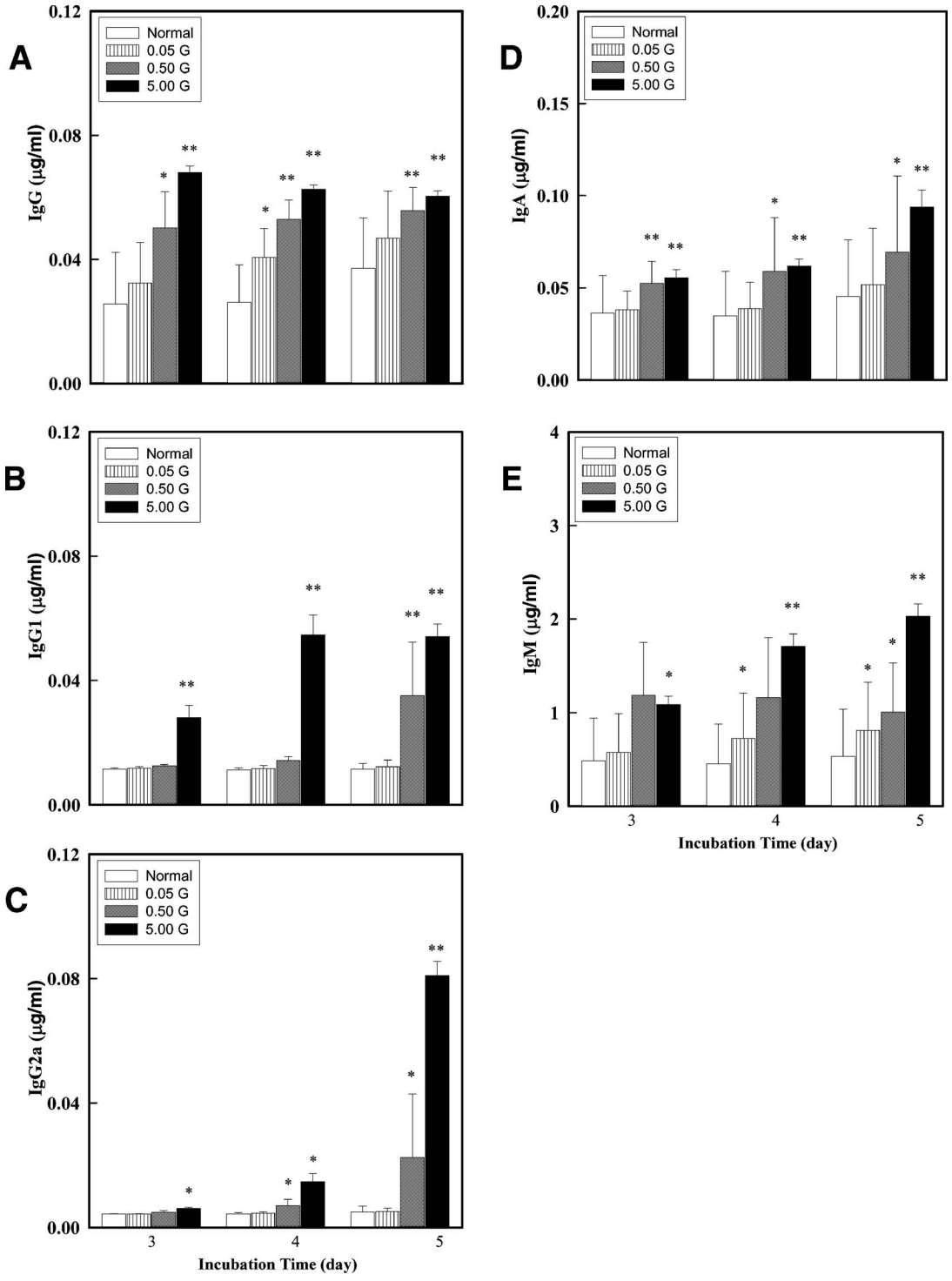

Fig. 2. *G. pentaphyllum* Enhanced Immunoglobulin Production from Spleen Cells

Mice were treated as described in the legend to Fig. 1. Spleen cells (5×10^5 cells/ml) were incubated with 1 μg/ml of LPS. The supernatants were collected after the cultures for 3, 4, and 5 days. The concentrations of IgG (A), IgG1 (B), IgG2a (C), IgA (D), and IgM (E) were determined with ELISA. Date was presented as mean$\pm$SE. *indicates $p<0.05$, and **indicates $p<0.01$, when compare to the Normal group.

With the stimulation of LPS to splenocytes, more significant amount of IgM was produced from mice treated with higher dose of *G. pentaphyllum*. Although LPS is a typical B cell-independent antigen,[21] the results indicated that the extract of *G. pentaphyllum* should be able to activate B cells *in vivo* and pre-deposit the capacity to respond to LPS stimulation. In addition to the similar pattern for the

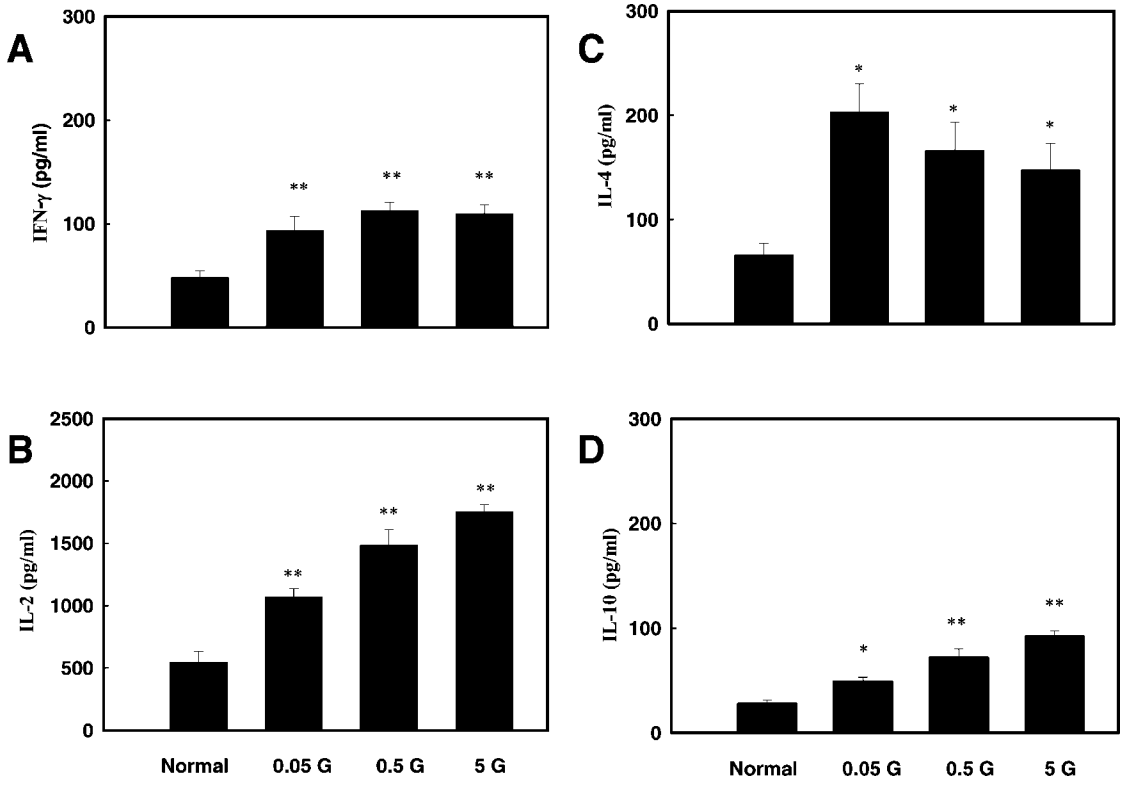

Fig. 3. *G. pentaphyllum* Enhanced the Production of Cytokines from Activated Spleen Cells

Mice were treated as described in the legend to Fig. 1. Spleen cells (1×10^6 cells/ml) were incubated with 1 μg/ml of Con A for 2 days. Culture supernatants were collected and the concentration of IFN-γ (A), IL-2 (B), IL-4 (C), and IL-10 (D) were measured by ELISA. Date was presented as mean$\pm$SE. *indicates $p<0.05$, and **indicates $p<0.01$, when compared to the Normal group.

production of IgA from LPS-stimulated spleen cells, more dramatic production of IgGs (including total IgG, IgG1, and IgG2a) from LPS-stimulated splenocytes was observed. Since LPS itself should not have the ability to drive immunoglobulin gene class switch from IgM to IgG1 or IgG2a, the enhancement of IgG1 or IgG2a production from activated cells might be from indirect effects of LPS stimulation.

In order to understand the effect of *G. pentaphyllum* on the responses of T cells, we first measured the cytokine levels in serum from the treated mice. The concentrations of all tested cytokines, including IL-2, IL-4, IL-10, and IFN-γ, were lower than the detectable levels (data no shown). Thus, we stimulated the spleen cells with Con A, a T-cell mitogen. We tested IL-2 and IFN-γ as the representative cytokines for Th1-response. IL-2 is a T cell growth factor that promotes T cell proliferation.[22] It can also enhance immunoglobulin synthesis and J-chain transcription in B cell.[23] IFN-γ, another important cytokine secreted from Th1 and NK cells, is very important for anti-virus infection and enhances anti-tumor activity.[24] The results in Fig. 3 indicated the dose-dependent enhancement of IL-2 and IFN-γ production from Con A-activated spleen cells. This might provide the mechanism for the significant production of IgG2a from spleen cultures. In addition to the effect of Th1 cells, *G. pentaphyllum* should have some effect on Th2 cells. The production of IL-4 from Con A spleen cells were also increased with the treatment of *G. pentaphyllum* at different doses. IL-4, secreted by Th2 and dendritic cells,[25,26] actives B cells and promotes isotype switching to IgG1 and IgE.[13] It is difficult to estimate the effect of *G. pentaphyllum* extract on IgE production, due to the undetectable IgE level in serum or culture supernatants in this study. The IgG1 levels in serum or activated splenocytes were increased followed by the treatment of herbal extract, although dose-dependent responses were only observed from the cultures of activated splenocytes. In recently years, IL-10 was reported as the important mediator of T regulatory cells that could regulate and suppress effector T cells.[27] T cells from mice receiving the highest dose of *G. pentaphyllum* secreted most IL-10 than other groups. It is possible that high dose of *G. pentaphyllum* induced some regulatory immune

responses.

The chemical structures of the compounds isolated from this plant had been identified to have high correlation with the ginseng saponins.[1] Recently, some reports showed that the ginseng or saponins could treat gastric ulcer and reduce bacterial load as well as promote ''chi''.[28] Higher serum levels of IgM, IgA, and IgG were detected from mice injected with ginseng extract intraperitoneally.[17] More IL-2, IL-4, and IL-10 were also produced from the spleen cells cultured with Con A. Moreover, the mice received saponin Rb1 promoted anti-inflammatory response when the mice were treated with LPS.[29] A previous study also demonstrated that Rg1, another saponin, enhanced IL-4 secretion and inhibited IFN-γ production.[30] Therefore, the extract of *G. pentaphyllum* might have the effects similar to ginseng saponins. Besides possible immune regulatory effects, *G. pentaphyllum* can be applied for treatment hyperlipidaemia to reduce serum cholesterol.[5] Recently, Norberg et al. isolated a insulin-releasing substance that evidenced *G. pentaphyllum* could reduce blood-sugar.[7] The antioxidant and hepatoprotective effects of *G. pentaphyllum* extract were reported[5] and this herb could cause apoptosis in human hepatoma cells.[31] Most importantly, no significant chronic toxicity was observed in rats treated with high doses of *G. pentaphyllum*. Normal liver function was demonstrated by normal ranges of alanine aminotransferase (ALT) and asparate aminotransferase (AST) in rats received 6-month of the extract.[32] The serum ALT and AST levels of our mice were also in normal range (data no shown).

In conclusion, our results demonstrated that the administration of *G. pentaphyllum* extract could promote the antibodies and cytokines production from B and T lymphocytes. Further fractionation of the extract and the identification of effective compound(s) might provide certain scientific substantiation at molecular level for how this herb modulates immune responses. Moreover, whether this herb affect specific immure responses is also an important point for the understanding of this popular herb.

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