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Gandoderma lucidum Extract Promotes Immune Responses in Normal BALB/c Mice *In Vivo*

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Abstract. *Enhanced fruit and vegetable consumption is closely related to reduced cancer incidence as shown in epidemiological studies. Ganoderma lucidum, one of the most well-known traditional Chinese medicines, has been demonstrated to have pharmacological activities and antitumor effects, in Asian populations. However, the promotion of immune responses in normal BALB/c mice is unclear. In the present study, we investigated the immune responses of BALB/c mice after treatment with G. lucidum extract in vivo. The results demonstrated that G. lucidum extract was able to promote the proliferation of splenocytes under Concanavalin A or lipopolysaccharide stimulation. Compared with the control group, phagocytosis of macrophage was significantly enhanced by intraperitoneal administration of G. lucidum extract at both 3 and 6 mg/kg. Compared with the control group, natural killer cell activity was significantly enhanced by intraperitoneal administration of G. lucidum extract (6 mg/kg). Results of cytometric bead array and flow cytometry indicated that the expressions of interleukin-6 and interferon-γ also increased ($p<0.001$) by treatment with G. lucidum extract (3 and 6 mg/kg). In conclusion, the findings of this study implied that G. lucidum extract was able to effectively promote immune responses in BALB/c mice.*

It was reported that increased consumption of a plant-based diet may lead to reduced colon cancer risk (1, 2). Some of the phytochemicals in herbal-based dietary supplements may mediate physiological functions, including the immune responses related to cancer suppression *in vivo*. It is well known that several leukocytes are involved in immune responses. In particular, macrophages phagocytose antigens to present them to helper T-cells which then help B-cells to form plasma cells to release specific immunoglobulin to connect other associated antigens which will be phagocytosed by other macrophages *in vivo*.

Ganoderma lucidum has long been used in traditional Chinese medicine and it is popularly used as a dietary supplement in the Chinese population (3). Biologically active components of *G. lucidum* have been reported as having anticancer activity, such as the triterpenes, which induced cytotoxicity against mouse cancer cells *in vitro* (4) and inhibited growth and cancer metastasis in mice (5, 6); and polysaccharides, which increased the levels of inflammatory cytokines (7, 8) and inhibited the growth of tumors in mice (9, 10). *G. lucidum* induced cell cycle arrest *via* down-regulating cyclin D1 and induced apoptosis through up-regulating proapoptotic protein (BAX) (11), and suppressed cell invasion and anchorage-independent growth of breast cancer cells (12). *G. lucidum* inhibited proliferation and induced apoptosis in prostate cancer cells (13), and inhibited the growth of hepatoma cells through inhibiting protein kinase C and activating c-Jun *N*-terminal kinases (JNK) and p38 mitogen-activated protein (MAP) kinases (MAPKs) (14). Although various bioactivity studies of *G. lucidum* have been carried out, the effect on immune responses have not yet been completely clarified. Therefore, we investigated the effects of *G. lucidum* on immune responses *in vivo*.

Materials and Methods

Materials and reagents. Crude extracts of *G. lucidum* were kindly offered by Dr. Chang (China Medical University, Taichung, Taiwan). Olive oil was obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS),

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penicillin-streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

BALB/c mice. Sixty male BALB/c mice at 8 weeks of age (approximately 22-28 g) were obtained from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan).

Drug treatment. Forty BALB/c mice were randomly divided into 4 groups (10 mice per group) and were kept on a 12-h light/dark cycle at 25°C. *G. lucidum* (3 and 6 mg/kg) was administered by gavage in 1 ml of saline administered. Group I received saline only (control) and group II only olive oil. Group III was treated with *G. lucidum* (3 mg/kg) in olive oil by gavage. Group IV was treated with *G. lucidum* (6 mg/kg) in olive oil by gavage. Mice were treated once daily for 2 weeks before being weighed and sacrificed (15, 16).

Proliferation examination. Approximately 1×10^5 splenocytes/well which were isolated from each spleen of each mouse (control and experimental groups) in 1 ml of RPMI-1640 medium in 24-well plates were stimulated with Concanavalin A (Con A, 5 μM) or lipopolysaccharide (LPS, 5 μM) for 72 hours followed by centrifugation. The cells were collected for determination of cell proliferation by CellTiter 96 assay kit (Promega, Madison, WI, USA) as described elsewhere (15, 16).

Interleukin-6 (IL-6) and interferon-γ (IFN-γ) determination by cytometric bead array (CBA) method and flow cytometric analysis. Approximately 2.5×10^5 splenocytes which were isolated from each spleen of each mouse (control and experimental groups) in RPMI-1640 medium were cultured on 24-well culture plates. Cells were centrifuged and supernatants were subjected to assays for levels of cytokines as described elsewhere (17, 18). Analysis of IL-6 and IFN-γ which was conducted using a human Th1/Th2 CBA kit (BD Biosciences, San Diego, CA, USA) and was assayed by flow cytometry.

Phagocytosis experiments. Total macrophage cells were isolated from peripheral blood mononuclear cells (PBMCs) of each mouse (control and experiment groups) and were placed in 12-well plates. Approximately 50 μl of 1×10^5 cells/well then placed in 50 μl of RPMI-1640 medium with FBS and 50 μl of yellow fluorescent particles (BD Pharmingen, San Diego, CA, USA) were added and shaken in a shaker bath for 30 min at 37˚C. One millilitre of 4% paraformaldehyde was added and centrifuged at 1,500 rpm for 5 min. The supernatant was discarded and 1 ml of phosphate-buffered saline (PBS) was added which was then mixed well with 2 ml FBS followed by centrifugation at 1,500 rpm for 10 min. Discarded supernatant then mixed well and centrifuged again. The pellet was washed twice with PBS then 0.5 ml PBS was added then for flow cytometric analysis, as described elsewhere (17, 18).

Natural killer cell activity. Approximately 2.5×10^7 splenocytes in 1 ml of medium which were isolated from each spleen of each mouse (control and experimental groups) in RPMI-1640 medium were cultured on 24-well culture plates. The target cells YAC-1, ~2.5×10^5 cells) in 15-ml tubes were washed twice with serum-free RPMI-1640 medium then PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) was added to the cells which were mixed thoroughly for 2 min at 25˚C then 2 ml PBS were added for 1 min. Four milliliters of RPMI-1640 medium were added then cells were incubated for 10 min at 1,200 rpm and 25˚C. Finally, about 1×10^5 YAC-1 cells were placed onto 96-well plates in 100 μl then 5×10^6 splenocytes from each group were added to the individual wells and cells incubated for 12 h. NK cell activation was determined by flow cytometry as described elsewhere (18).

Statistical analysis. Data are expressed as mean±SD and differences between control and *G. lucidum*-treated groups were analyzed by Student's *t*-test with $*p<0.05$, $**p<0.01$, $***p<0.001$ used as the levels of significance.

Results

G. lucidum extract promoted the proliferation of splenocytes from BALB/c mice. The results are shown in Figure 1A and B. With Con A stimulation, both doses of *G. lucidum* extract promoted proliferation of splenocytes and this effect occurred in a dose-dependent manner. Compared to cells without Con A stimulation, only 6 mg/kg/day (high dose) increased the proliferation of splenocytes. Similarly, with or without LPS stimulation, only the high dose of *G. lucidum* extract significantly promoted proliferation (Figure 1B).

G. lucidum extract promoted the levels of IL-6 and IFN-γ in normal BALB/c mice. The results are presented in Figure 2A and B and indicate that *G. lucidum* extract increased the levels of IL-6 and IFN-γ at 3 and 6 mg/kg/day for 14 days.

G. lucidum extract promotes macrophage activity. The results of macrophage phagocytosis determination are presented in Figure 3. The percentage of macrophages with phagocytosed yellow fluorescent particles on *G. lucidum* extract treatment was significantly increased at 14 days with 3 and 6 mg/kg/day treatment as compared to day 0.

G. lucidum extract promotes NK cells activity. The results presented in Figure 4 show that the YAC-1 target cells were killed by NK cells from the mice after treatment with *G. lucidum* extract at 3 and 6 mg/kg/day at a target cell ratio of 50:1.

Discussion

More attention has been focused on increasing the human body's immunity against tumors (19, 20). Many reports have shown *G. lucidum* extract to have anticancer activities (4, 11-13) and a mixture of ganoderic acids (purified from *G. lucidum* mycelia) to have an inhibitive effect on proliferation of human and mouse carcinoma cell lines (21, 22). However, the immune functions of mice exposed to *G. lucidum* have not been clarified. The present results showed that *G. lucidum* extract can promote the proliferion of splenocytes from normal BALB/c mice after expose to both low and high doses of *G. lucidum* extract by oral treatment.

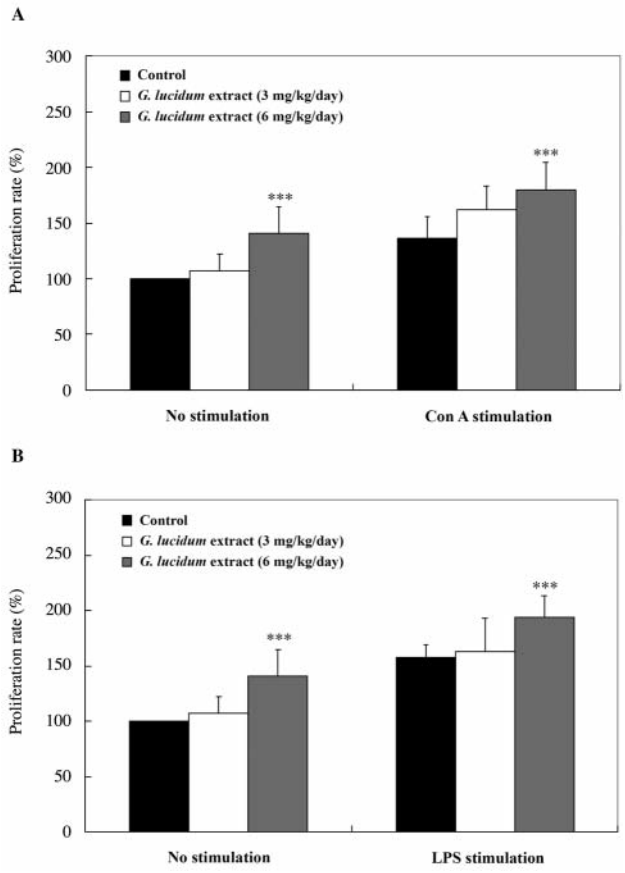

Figure 1. *G. lucidum extract promoted the proliferation of splenocytes from BALB/c mice. The splenocytes were collected from spleen of normal BALB/c mice which were pretreated with G. lucidum extract (0, 3 or 6 mg/kg/day) for 14 days then were stimulated with Con A (A) or LPS (B) for 72 hours before analysis for proliferation as described in Materials and Methods. Each data point is the mean±S.D. ***p<0.001 (n=10) compared to control.*

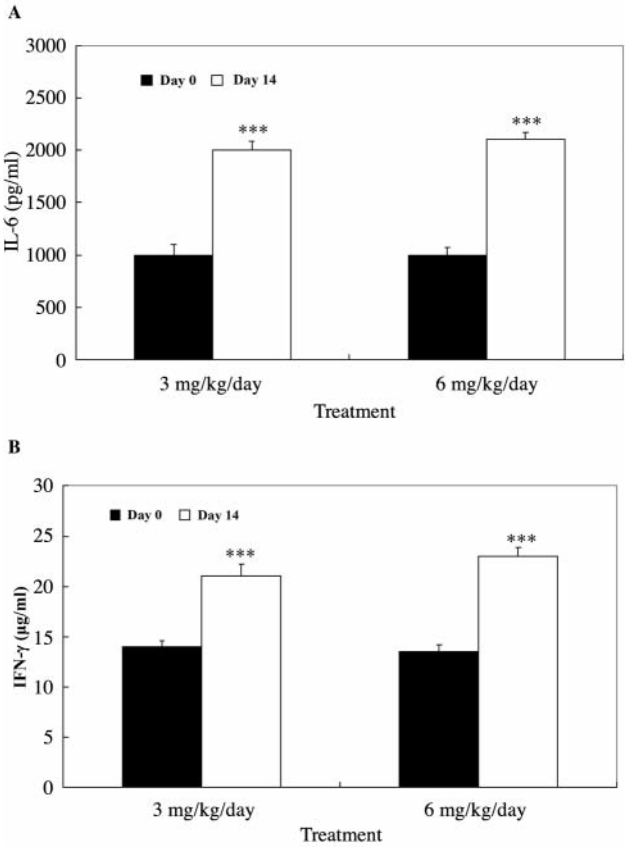

Figure 2. *G. lucidum extract affected the levels of IL-6 (A) and IFN-γ (B) from splenocytes of BALB/c mice. Approximately 2.5×10^5 splenocytes which were isolated from each spleen of each mouse (control and experimental groups) in RPMI-1640 medium were cultured on 24-well culture plates. Cells were centrifuged and supernatants were subjected to assays for levels of cytokines as described in Materials and Methods. Each data point is the mean±S.D. ***p<0.001 (n=10) compared to day 0.*

The administration of *G. lucidum* extract enhanced the expression of T-helper type 1 (TH1) and macrophage cytokines, including IFN-γ and IL-6 ($p<0.001$), and augmented phagocytosis and NK cell activities. Both there cytokines play an important role in specific immunological reactions to tumor cell growth and in promoting innate and adaptive immune responses (23). Cytokines play critical roles in regulating the outcome of antigen-specific T-cell responses and inflammatory response of macrophage, and thus have been a major focus in the study of the pathogenesis of autoimmunity.

In this study, as shown in Figure 2, the levels of IL-6 and IFN-γ were enhanced in BALB/c mice, which suggested that *G. lucidum* extract could be a potent inducer of TH1-type cytokines. It is well known that NK cells are involved in the nonspecific antiviral and antitumor defense in humans and it was reported that NK cells contributed to the elimination of transformed tumor cells (24). Our results also showed that *G. lucidum* extract promoted NK cell activity ($p<0.001$) in BALB/c mice (Figure 4). This adds to a better understanding of the effectiveness of *G. lucidum* extract for increasing immune responses. Cytokines regulate the innate immune system and increase NK cell activities, however, NK cells also regulate the adaptive immune system and responses to produce cytokines based on the observations of *G. lucidum* extract promoting the levels of and IFN-γ (Figure 2B). This result was consistent with other reported data that demonstrated the ability of NK cells to respond to IL-2 and IFN-γ (25, 26).

In conclusion, results from the present study demonstrated that *G. lucidum* extract elicits pronounced immunostimulatory activities in BALB/c mice. However, the associated possible pathway for causing the promotion of immune responses has not been completely verified. Further work needs to be carried out to investigate the signal pathway involved in

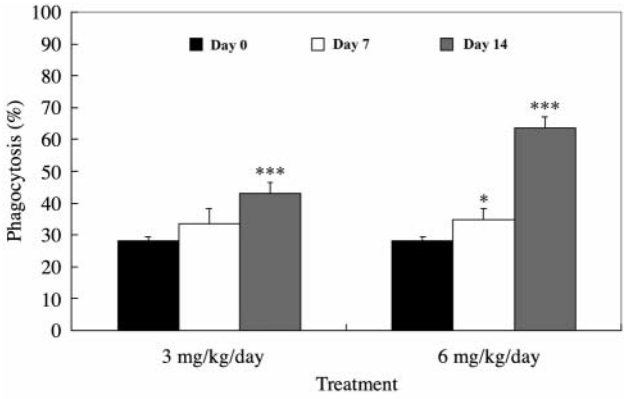

Figure 3. *G. lucidum extract promoted macrophage activity. After spleens were isolated from normal BALB/c mice exposed to 0, 3 and 6 mg/kg/day of G. lucidum extract for 14 days then macrophages were used for phagocytosis determination as described in Materials and Methods. Each data point is the mean±S.D.; *p<0.05, ***p<0.001 (n=10) compared to day 0.*

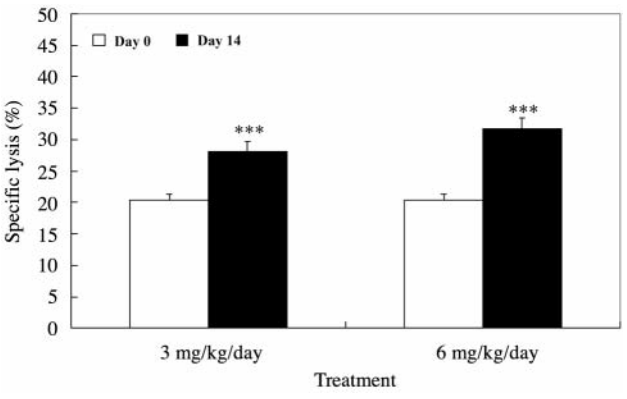

Figure 4. *G. lucidum extract promoted NK cell activity. After spleens were isolated from normal BALB/c mice exposed to 0, 3 and 6 mg/kg/day of G. lucidum extract for 14 days, NK cells were analyzed for their activity by flow cytometry as described in Materials and Method. Each data point is the mean±S.D.; ***p<0.001 (n=10) compared to day 0.*

T lymphocyte activation and macrophage and NK cell activities that were induced by *G. lucidum* extract. The results suggest that *G. lucidum* extract is a promising candidate for promoting immune response and a potentially valuable substance for pharmacological use.

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