Ganoderma lucidum polysaccharides enhance the function of immunological effector cells in immunosuppressed mice

Xiao-Ling Zhu a,∗, Alex-F. Chen b, Zhi-Bin Lin a,∗

a Department of Pharmacology, School of Basic Medical Science, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100083, PR China
b Departments of Pharmacology and Neurology and the Neuroscience Program, Michigan State University, East Lansing, MI 48824-1317, USA

Received 18 April 2006; received in revised form 23 October 2006; accepted 16 November 2006
Available online 21 November 2006

Abstract

The present study was designed to determine in vivo efficacy of Ganoderma lucidum polysaccharides (Gl-PS) for enhancing the activity of immunological effector cells in immunosuppressed mice. Mice were injected intraperitoneally (i.p.) once daily with low-dose (2.5 mg/kg), intermediate-dose (25 mg/kg), and high-dose (250 mg/kg) of Gl-PS, respectively, for 7 consecutive days 24 h after i.p. injection of an immunosuppressing anti-tumor agent cyclophosphamide (Cy, 300 mg/kg). In Cy-treated mice, compared to vehicle, low-dose Gl-PS accelerated recovery of bone marrow cells, red blood cells and white blood cells, as well as splenic natural killer cells and natural killer T cells, and enhanced T and B cell proliferation responses on day 8, cytotoxic T lymphocyte activity on day 5, as well as NK cell and lymphokine activated killer cell activity on days 7–9. Furthermore, it promoted phagocytosis and cytotoxicity of macrophages on day 12. The above beneficial effects induced by the low-dose Gl-PS treatment did not result in any side effects. These results demonstrate the efficacious effects of low-dose Gl-PS treatment for enhancing the activity of immunological effector cells in immunosuppressed mice, and may provide a basis for applying this herb as an efficacious adjacent immunopotentiating therapy against cancer chemotherapy-induced immunosuppression.

© 2006 Elsevier Ireland Ltd. All rights reserved.

Keywords: Ganoderma lucidum (Leyss. ex Fr.) Karst. (Ling Zhi); Polysaccharide; Cyclophosphamide; Cytotoxicity; Immunomodulation

1. Introduction

Ganoderma lucidum (Leyss. ex Fr.) Karst. (Ling Zhi) (Aphyllophoromycetideae) (the family Polyporaceae) was first indexed in the Shen Nong’s Materia Medica (206 BC–8 AD) as a longevity-promoting and tonic herb of the non-toxic superior class, and has been used in traditional Chinese medicine (TCM) for more than 2000 years to prevent and/or treat various human diseases such as hepatitis, chronic bronchitis, gastritis, tumor growth and immunological disorders. According to ‘Fuzheng Guben’, one of the major TCM therapeutic principles, Ganoderma lucidum (Gl) is capable of strengthening body resistance and improving constitutive homeostasis in patients (Lin, 2001). Gl polysaccharides (Gl-PS), a glycopeptide isolated from the water-soluble polysaccharides of Gl, is a major effective component of Gl (Lin, 2001). Our and some others previous studies have demonstrated that Gl-PS exhibit immunomodulatory and anti-tumor effects (Hou et al., 1995; Zhang et al., 2002; Bao et al., 2002; Lin and Zhang, 2004; Gao et al., 2005; Sung et al., 2005; Zhu and Lin, 2005; Zhu and Lin, 2006). Cyclophosphamide (Cy) is the most widely used alkylating agent in cancer chemotherapy to date. The anti-tumor effect of Cy is in proportion to the dose of Cy administered, often resulting in immunosuppressive and cytotoxic effects (Singh et al., 1993). Chemotherapy-induced leukopenia leads to significant morbidity and mortality, a major limiting factor in clinical chemotherapy without efficacious remedies. As a tumor grows progressively, the immune system of tumor-bearing hosts is frequently impaired (Bear, 1986; Hoover et al., 1990). Our previous studies have shown that Gl-PS significantly ameliorate the inhibitory effects in lymphocytes induced by immunosuppressing anti-tumor agents. Therefore, this study was designed to determine in vivo efficacy of Gl-PS for enhancing the activity of immunological effector cells in immunosuppressed mice.
by anti-tumor agents such as mitomycin and etoposide in vitro (Lei and Lin, 1993). However, the in vivo efficacy of GI-PS on immunological effector cells, which play a key role against tumor growth under immunosuppression, is poorly understood. The present study was thus designed to elucidate the effects of GI-PS on immunological effector cells in immunosuppressed mice induced by Cy treatment.

2. Materials and methods

2.1. Animals and drugs

Inbred strain male (6–8 weeks old) C57BL/6j (H-2b) mice were purchased from the Department of Experimental Animals, Health Science Centre, Peking University, Beijing, China. Ganoderma lucidum (Leyss. ex Fr.) Karst. (Ling Zhi) (Aphyllorhomycteidae) (Polyporaceae) cultivated with wood log was obtained from The Ganoderma lucidum Production Base in Taining County, Fujian Province in China. GI has special characteristics such as umbrella like fungi composed of pileu and stipe, pileu semicircular, circular or reniform, dorsal surface yellow-brown to red-brown, with annular ridges and radial veins, ventral surface nearly white to brownish, with many small pores in which there are numerous basidiospores, stipe mostly laterally located curved in zigzag way, yellow-brown, corky texture and light weight. The quality of Ganoderma lucidum fruit bodies was monitored by Dr. Xiaolan Mao, a senior researcher of the National Institute of Microbiology and Microbiological Institute, China Academy of Science. The selected suitable Ganoderma lucidum strain for cultivation with wood log is Ga0801 (No. of strain) and was preserved by Profs. Shuqian Lin and Saizhen Wang of Fuzhou Institute of Green Valley Bio-Pharm Technology in China. GI-PS were isolated from boiling water extract of the fruit bodies of Ganoderma lucidum, followed by ethanol precipitation, dialysis, and protein depletion using the Sevag method, as we previously described (Cao and Lin, 2002; Lin et al., 2003). The component sugar and molecular weight distribution of the glycopeptides were determined by gel permeation chromatography (GPC) and high performance liquid chromatography (HPLC). The structure of the glycopeptides was detected by IR, 1H NMR and 13C NMR. As a polysaccharide peptide, the isolated GI-PS has a molecular weight of 584,900, with a ratio of polysaccharides to peptides of 93.61–6.49%. The polysaccharides consist of d-rhamnose, d-xylene, d-fructose, d-galactose, d-mannose, d-glucose, and uronic acid with a molar ratio of 0.793:0.964:2.944:0.167:0.389:7.94:0.33. The glycoside linkage was major β- with minor α-bonding. The peptides contain 16 amino acids (Asp, Thr, Ser, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Phe, Lys, His, Arg, Pro) (Cao and Lin, 2002; Lin et al., 2003). As a water-soluble powder, GI-PS was dissolved in physiological saline, filtered through a 0.22 μm filter and stored at 4°C before use. Cy (Shanghai Hualian Pharmaceutical Co. Ltd., Shanghai, China) was dissolved in sterilized saline prior to its injection to mice. Levamisole hydrochloride (LH) was purchased from Hunan Dongting Pharmaceutical Co. Ltd. (Hunan, China).

2.2. Induction of immunosuppression and treatment protocols

Mice were injected intraperitoneally (i.p.) with a single sub-lethal dose of Cy (300 mg/kg) on day 0. Mice were divided into five groups 24 h after Cy injection and received i.p injections once daily for 7 consecutive days of (1) low-dose GI-PS (2.5 mg/kg), (2) intermediate-dose GI-PS (25 mg/kg), (3) high-dose GI-PS (250 mg/kg), (4) levamisole hydrochloride (10 mg/kg, an immunopotentiating agent) as positive controls, and (5) vehicle (i.e. sterile physiological saline) as negative controls.

2.3. Peripheral erythrocyte, leukocyte, splenocyte and bone marrow cell counts

Blood was collected on the day of sacrifice by retro-orbital bleed into heparin tubes. Single-cell spleen suspensions were pooled in serum-free RPMI-1640 medium (Gibco Laboratories, NY, USA) by filtering the suspension through sieve mesh with the aid of a glass homogenizer to exert gentle pressure on the spleen fragments. Erythrocytes were lysed with an ammonium chloride solution (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.2). Bone marrow cell (BMC) suspensions were prepared by flushing a femur with serum-free RPMI-1640 media through syringe needles several times. At the time of assay, total numbers of circulating erythrocytes, leukocytes, splenocytes and BMC were counted under light microscopy.

2.4. Phagocytosis of peritoneal macrophages

Peritoneal macrophages were prepared as described (Berrebi et al., 2003). Phagocytosis of macrophages was measured by neutral red uptake method as described (Weeks et al., 1987). Briefly, peritoneal exudates were induced by intraperitoneal injection of 2 ml 3% thioglycollate. Cells were harvested 96 h later from exudates after wash with cold phosphate buffered saline (PBS) containing 5 U/ml heparin. Cells were then cultured overnight in RPMI-1640 with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO2. The following day, all nonadherent cells were removed by washing with PBS. Adherent cells were detached using 10 mM EDTA in PBS and seeded at a density of 1 × 105 cells/well in the 96-well microplates with complete RPMI-1640 media. At 24 h after culture, the cells were washed and neutral red (50 ng/ml) was added. The plates were incubated for 3 h and cells were then washed to remove excess dye and blotted dry. The incorporated dye was resuspended in ethanol (50%) containing glacial acetic acid (1%) and the absorbance was measured at 540 nm in a microplate reader (Model 550 BIO-RAD). The absorbance (A) was translated into phagocytosis ratio for comparison: phagocytosis ratio = testA/normal controlA × 100%.

2.5. Assay of splenocyte proliferation induced by T cell and B cell mitogens concanavalin A and lipopolysaccharide, respectively

Splenocytes were placed into the 96-well flat-bottomed microplates in triplicate at 5 × 105 cells/well, then 2.5 μg/well
of concanavalin A (Con A) or 10 μg/well of lipopolysaccharide (LPS, both from Sigma, St. Louis, MO, USA) was added to the wells. The cells were then incubated in a total volume of 200 μl/well. Serum free RPMI-1640 medium was used as control. Cell proliferation was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay 72 h after culture (Wang et al., 2002). MTT (Sigma, St. Louis, MO, USA) solution of 20 μl (5 g/l) was added to each well. After 4 h incubation, the cells were lysed and the purple formazan crystals were solubilized for detection at 570 nm. The absorbance (A) was translated into lymphocyte proliferation ratio for comparison: lymphocyte proliferation ratio = testA/normal controlA × 100%.

2.6. Preparations of anti-tumor effector cells

Splenocytes were prepared as the effector cells for splenic natural killer (NK) activity assay as described previously (Li et al., 1998). Splenic cytotoxic T lymphocytes (CTL) were prepared as described (Li et al., 1998). Briefly, splenocytes (1 × 10^7 cells/well) isolated from C57BL/6j mice (H-2b) as responders were cultured with 25 mg/l mitomycin C-treated P815 sensitized cells (stimulator; H-2b) at a 50:1 ratio in 24-well tissue culture plates. On day 5 after incubation, the responder cells were harvested as CTL. We confirmed that the positive rate of T cells was greater than 94% and the positive ratio of NK cells was less than 1% after incubation for 120 h, as determined by flow cytometry, which corresponded with the previous report (Li et al., 2000). To generate lymphokine-activated killer (LAK) cells, splenocytes at a concentration of 1 × 10^9 cells/l were incubated with 300 IU/ml rIL-2 and IFN-γ, 100 U/ml final concentration (PeproTech EC Ltd. London, UK), and the adherent cells were washed twice and incubated overnight in the presence of LPS (2 μg/ml) prior to cytotoxicity assay.

2.7. Cytotoxicity assays

Tumoricidal activity of the effector cells was assayed by measuring lactate dehydrogenase (LDH) released from the target cells using a cytotoxicity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s protocol. Briefly, effector cells (2 × 10^5 per well) in the 96-well microplates were co-cultured with target cells at an E:T ratio of 20:1. After 24 h of culture, supernatants were evaluated for LDH activity released by the damaged cells. Spontaneous LDH release from effector cells, cell-free culture media, and target cells lysed with Triton X-100 were served as controls. The percentage of specific cell release was calculated as percentage-specific cytotoxicity (% C). All assays were performed in triplicate. YAC-1 cells were used as target for NK cells and CTL, and P815 cells were used as target for LAK cells and macrophages.

2.8. Cell staining for phenotype analysis

Effector cells were freshly harvested and washed twice with ice-cold FACSscan buffer (PBS containing 2% FCS and 0.1% sodium azide). Twenty percent of mixed mouse and rat sera were used to block non-specific antibody binding before cells were then stained with the monoclonal antibody (mAb) against CD3 coupled to fluorescein isothiocyanate (FITC) (Santa Cruz, CA, USA) or mAbs against NK1.1 coupled to phycoerythrin (PE) (Pharmingen, CA, USA) for 30 min at 4°C in the dark. The stained cells were washed twice, fixed with 1% paraformaldehyde in FACScan buffer, and then analyzed by a FACScan flow cytometer (Becton Dickinson, NJ, USA).

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett’s t-test. Results were presented as mean ± S.E.M. Values of P < 0.05 were considered to be a statistically significant finding.

### Table 1

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>Day 3</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC</td>
<td>WBC</td>
</tr>
<tr>
<td>Gl-PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250.0</td>
<td>1.8 ± 0.03^c</td>
<td>2.0 ± 0.04^b</td>
</tr>
<tr>
<td>25.0</td>
<td>1.9 ± 0.03^c</td>
<td>1.9 ± 0.04^b</td>
</tr>
<tr>
<td>2.5</td>
<td>2.5 ± 0.04^c</td>
<td>2.9 ± 0.06^b</td>
</tr>
<tr>
<td>LH</td>
<td>10.0</td>
<td>2.5 ± 0.04^c</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.5 ± 0.06^c</td>
<td>2.7 ± 0.06^b</td>
</tr>
<tr>
<td>Normal</td>
<td>7.5 ± 0.08</td>
<td>6.8 ± 1.5</td>
</tr>
</tbody>
</table>

The data are expressed as mean ± S.E.M. of six mice. Gl-PS: Ganoderma lucidum polysaccharide; LH: levamisol hydrochloride. ^P < 0.05 and ^P < 0.001 vs. normal mice.
3. Results

3.1. Effects of Gl-PS on peripheral red blood cells (RBC), white blood cells (WBC), splenocytes and bone marrow cells (BMC) in Cy-treated mice

The numbers of peripheral RBC and WBC, splenocytes and BMC in Cy-treated mice receiving various treatment protocols were examined at different time points, and the results were shown in Tables 1 and 2. Cy treatment markedly reduced the numbers of RBC and WBC, splenocytes and BMC on day 3 (Table 1). Treatments with Gl-PS of low- or intermediate-dose restored BMC and RBC counts to normal levels in Cy-treated mice on days 9 and 24, respectively. Low-dose Gl-PS treatment also resulted in partial but significant recovery of WBC counts compared with saline vehicle group on day 24 (Table 2). While levamisole hydrochloride (LH) treatment also increased RBC counts on day 24, it failed to augment other cell counts compared to Gl-PS treated mice. No evident toxic or side effects were observed in Gl-PS treated mice during these experiments.

3.2. Effects of Gl-PS on concanavalin A or lipopolysaccharide-induced lymphocyte proliferation

The normal murine lymphocyte proliferation ratio induced by Con A or LPS and treated with RPMI medium 1640 was regarded as 100%. The proliferative responses of lymphocytes to both T cell and B cell mitogens (Con A and LPS, respectively) were reduced markedly on day 3 in Cy-treated mice, when compared to the normal lymphocyte proliferation ratio (Fig. 1A). Treatments with low- and intermediate-dose of Gl-PS or levamisole hydrochloride (LH) promoted recovery of both T and B cell proliferation responses on day 8, whereas the lymphocytes proliferation ratio in high-dose of Gl-PS and the vehicle groups was still significantly lower than the normal (Fig. 1B).

3.3. Effects of Gl-PS on macrophage phagocytosis and anti-tumor activity in Cy-treated mice

The phagocytosis of macrophages from normal mice was regarded as 100% by neutral red uptake method. As shown in Table 2, compared to vehicle and normal, on day 12 in Cy-treated mice, injection with low-dose Gl-PS or levamisole hydrochloride (LH) significantly enhanced whereas intermediate-dose Gl-PS treatment restored impaired macrophage phagocytosis, and furthermore, treatment with low-dose Gl-PS significantly increased the cytotoxicity (an indicator of their anti-tumor activity) against P815 cells (NK-resistant) in activated macrophages.

Table 2

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>RBC, $\times 10^{12}$ l$^{-1}$</th>
<th>WBC, $\times 10^{9}$ l$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gl-PS (mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250.0</td>
<td>4.1 $\pm$ 0.3$^b$</td>
<td>3.6 $\pm$ 0.4$^b$</td>
</tr>
<tr>
<td>25.0</td>
<td>6.2 $\pm$ 0.4</td>
<td>4.3 $\pm$ 0.4$^b$</td>
</tr>
<tr>
<td>2.5</td>
<td>7.8 $\pm$ 0.3</td>
<td>5.0 $\pm$ 0.3$^c$</td>
</tr>
<tr>
<td>LH (mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>6.7 $\pm$ 0.7</td>
<td>3.9 $\pm$ 0.8$^b$</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.4 $\pm$ 0.6$^b$</td>
<td>3.7 $\pm$ 0.3$^b$</td>
</tr>
<tr>
<td>Normal</td>
<td>8.2 $\pm$ 0.9</td>
<td>6.1 $\pm$ 0.4</td>
</tr>
</tbody>
</table>

The data were expressed as mean $\pm$ S.E.M. of six mice. Gl-PS: Ganoderma lucidum polysaccharide; LH: levamisole hydrochloride. $^b$P < 0.05 and $^c$P < 0.001 vs. normal mice. $^b$P < 0.05 vs. Cy-treated mice administrated saline vehicle.
Fig. 3. Effects of GI-PS on macrophage cytotoxicity in Cy-treated mice on day 12. The cytotoxicity of activated macrophage was determined by LDH assay of the supernatants from cultured P815 target cells. Effectorstargets = 20:1. Data were expressed as mean ± S.E.M. of six mice. GI-PS: *P < 0.05 and **P < 0.01 vs. normal mice; ΔP < 0.001 vs. Cy-treated mice administrated saline vehicle.

from Cy-treated mice compared to the vehicle and normal, while intermediate-dose GI-PS or LH treatment restored impaired macrophage cytotoxicity, but cytotoxicities in high-dose GI-PS and vehicle groups were lower than the normal (Fig. 3).

3.4. Effects of GI-PS on splenic natural killer cell (NK), lymphokine-activated killer cell (LAK) and cytotoxic T lymphocytes (CTL) cytotoxicities in Cy-treated mice

A single sublethal dose of Cy inhibited NK cell, LAK cell and CTL cytotoxicity significantly by day 5 in vehicle group, compared with normal mice as shown in Fig. 4A. In Cy-treated mice, only low-dose GI-PS as well as levamisole hydrochloride (LH) restored the CTL cytotoxicity to normal on day 5 (Fig. 4A). LH, intermediate- and low-dose GI-PS had similar effect on NK cell and LAK cell cytotoxicity on day 7, but that cytotoxicities in high-dose GI-PS and vehicle groups still were lower than the normal (Fig. 4B). On day 9, above parameters in all groups restored normal, injection with low-dose GI-PS significantly augmented LAK cytotoxicity further compared to the vehicle and the normal, and NK cell cytotoxicities of LH, intermediate-and low-dose GI-PS group in Cy-treated mice were stronger than that of normal and vehicle (Fig. 4C).

3.5. Effects of GI-PS on immunophenotypes of splenocytes in Cy-treated mice

Immunophenotypes were evaluated by determining the absolute numbers of splenocytes in Cy-treated mice on day 9. Compared to normal mice, the numbers of both CD3+ T lymphocytes and CD3+ NK1.1+ (NKT) cells in Cy-treated mice were decreased to a similar extend in all experimental groups (Table 3). Treatment with low- and intermediate-dose of GI-PS rescued NK1.1+ cell numbers to the normal levels, an effect that was not mimicked in other treatment groups. In addition, treatment with low-dose GI-PS significantly increased the number of NKT cells as compared to the saline vehicle group (Table 3).

4. Discussion

In the present study, we determined, for the first time, the efficacy profiles of chronic, low-dose GI-PS treatment for enhancing the activity of immunological effector cells and haematopoiesis in immunosuppressed mice. The major new findings are that in cyclophosphamide-induced immunosuppressed mice, chronic treatment with low-dose GI-PS resulted in (1) accelerated recovery of bone marrow cells (BMC), red blood cells (RBC) and white blood cells (WBC), as well as splenic natural killer (NK) cells and natural killer T (NKT) cells, (2) enhanced T and
B cell proliferation responses, cytotoxic T lymphocyte (CTL) activity as well as NK cell and lymphokine activated killer (LAK) cell activity, (3) augmented macrophage phagocytosis and anti-tumor cytotoxicity, and (4) achieved the greatest effect on promoting granulocytopoiesis. None of mice treated with Gl-PS died, nor did their body weights change significantly (P > 0.05, data not shown) during the experiment period. The above beneficial effects induced by the low-dose Gl-PS treatment were not paralleled with any evident toxic or side effects. Thus, in vivo treatment with low-dose Gl-PS accelerates the recovery of immunosuppressed mice from leukopenia, myelosuppression and immunosuppression, the common conditions associated with cancer chemotherapy.

In sublethal dose (300 mg/kg, i.p.) of cyclophosphamide treated mice, the immunosuppression manifest in markedly reduced the numbers of peripheral RBC, WBC, splenocytes and bone marrow cells, inhibited lymphocyte proliferative responses to both T and B mitogens, and activity of NK cell, LAK cell and CTL. These data are consistent with previously published studies (Ballas, 1986; Gazit and Kedar, 1994; Shen et al., 1994; Yang, 1994). Although cyclophosphamide is the mainstay cancer chemotherapy agent, its immunosuppressing activity represents a major clinical challenge and is a main limiting factor for sustained clinical use. One of the few remedies is the use of levamisole, an immunopotentiating agent. Although levamisole has been shown to improve the function of immunological effector cells, it causes a number of severe side effects including serious neurological symptoms, gastric haemorrhage and vomiting of blood, colic, anaemia and vasculitis (Bagga and Hari, 2000; Grohn et al., 1984; Palcoux et al., 1994; Joly et al., 1998; Tenbrock et al., 1998). For the past several decades, attempts have been made to search for safer immunomodulating agents, and one of the special focuses has been on the biological response modifier (BRM) derived from natural products. The results of the present study demonstrate that low-dose Gl-PS, when injected intraperitoneally in mice, affords the greatest protection for immune effector cells and hematopoietic progenitors against the sublethal effects induced by cyclophosphamide.

Our findings showed that a single sublethal dose of cyclophosphamide reduced the counts of peripheral blood cells on day 3, which corresponded with other reports (Cox, 2000; Yeager et al., 1982), and low-dose Gl-PS restored the bone marrow cells to the normal levels on day 9, had the same effect on RBC on day 24, and markedly increased WBC counts compared with the saline vehicle group, despite that the WBC in all cyclophosphamide-treated groups were under normal levels by day 24.

The proliferation of T and B lymphocytes is known as a response to the stimulation induced by antigen or mitogens. While cellular multiplication induced by concanavalin A is commonly used to detect T lymphocyte immunity in vitro, the LPS-induced activation of B cells and subsequent immunoglobulin synthesis reflect B lymphocyte immunity (Quakyi et al., 1997). Previous reports have shown that cyclophosphamide suppresses both humoral and cellular immune responses (Balow et al., 1975; Rondinone et al., 1983; Morato et al., 1996; Masnaya and Ratner, 2000). Indeed, a single dose of cyclophosphamide (200 mg/kg) inhibited the proliferative response to both B and T cells (Quakyi et al., 1997). Although the suppressed antibody productions are reconstituted gradually after 3–6 weeks, inhibition of mitogen-stimulated lymphocyte proliferation is sustained even after 5 weeks (Moynhian and Cohen, 1989). In the present study, the proliferative responses to both T and B mitogens were reduced markedly on day 3 in all cyclophosphamide-treated groups due to the exposure of the mice to maximal tolerated dose of cyclophosphamide (i.e. 300 mg/kg). Treatment with low- or intermediate-dose of Gl-PS promoted the recovery of T and B cell proliferation responses on day 8, to a similar extent as to the effect induced by levamisole. Our results also showed that cyclophosphamide treatment impaired macrophage phagocytosis, consistent with the previously reported findings of such toxicity induced by cyclophosphamide in vivo (Marcinkiewicz et al., 1994). Treatment with low-dose Gl-PS resulted in enhanced phagocytosis and anti-tumor activities of macrophages. As shown previously, CTL, NK cell and LAK cell activities are significantly reduced following treatment with low dose (150–100 mg/kg) or sublethal dose (300 mg/kg) of cyclophosphamide, which are not recovered until day 9 (Ballas, 1986; Gazit and Kedar, 1994; Shen et al., 1994; Yang, 1994).

Our data showed that treatment with low-dose Gl-PS accelerated recovery of CTL on day 5, NK cell and LAK cell activity on day 7, and significantly enhanced NK cell and LAK cell activity on day 9. NKT cells are a novel subpopulation of T cells that express NK markers (e.g. NK1.1 in certain mouse strain) (Mauroka et al., 1998). They have been shown to play a role in immunoregulation and tumor surveillance as anti-tumor effector cells (Brutkiewicz and Sriram, 2002; Smyth et al., 2002). Treatment with low-dose Gl-PS also accelerated the recovery of splenic NK cell and NKT cell numbers in cyclophosphamide-treated mice. Recent clinical studies suggested that subgroups

---

**Table 3**

Effects of Gl-PS on immunophenotypes of splenocytes on day 9 after Cy treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD3+ × 10^6</th>
<th>NK1.1+ × 10^6</th>
<th>CD3+ NK1.1+ × 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gl-PS (mg/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250.0</td>
<td>4.3 ± 0.3c</td>
<td>0.5 ± 0.1s</td>
<td>1.7 ± 0.1c</td>
</tr>
<tr>
<td>25.0</td>
<td>4.8 ± 0.6c</td>
<td>1.2 ± 0.3</td>
<td>2.3 ± 0.2c</td>
</tr>
<tr>
<td>2.5</td>
<td>5.3 ± 0.6c</td>
<td>1.3 ± 0.3</td>
<td>2.8 ± 0.2c</td>
</tr>
<tr>
<td>LH (mg/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>4.4 ± 0.9s</td>
<td>0.8 ± 0.2c</td>
<td>2.1 ± 0.1c</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.8 ± 0.3c</td>
<td>0.7 ± 0.1b</td>
<td>1.3 ± 0.1c</td>
</tr>
<tr>
<td>Normal</td>
<td>18.1 ± 0.9</td>
<td>1.3 ± 0.1</td>
<td>4.4 ± 0.3</td>
</tr>
</tbody>
</table>

The data were expressed as mean ± S.E.M. of six mice. Values were expressed as the absolute number per spleen × 10^6. Gl-PS: Ganoderma lucidum polysaccharide; LH: levamisole hydrochloride. *P < 0.05 and **P < 0.001 vs. normal mice; $P < 0.05 vs. Cy-treated mice administrated saline vehicle.
of advanced-stage caner patients treated with Gl-PS 5.4 g/day orally for 12 weeks might be responsive, which resulted in tumor necrosis factor-α decreased. The results of the clinical studies were significantly variable (Gao et al., 2003; Huang et al., 2005), but our experiment results from mice were less fluctuant comparatively. So the number of patients enrolled need to be increased to evaluate clinical response and toxicity.

In summary, the present study demonstrate, for the first time, that chronic treatment with low-dose Gl-PS results in accelerated recovery of immuno-suppression in cyclophosphamide-treated mice, without evident side effects. Our findings may provide a mechanistic basis for using Gl-PS as an alternative means in lessening chemotherapy-induced immunosuppression in cancer patients via its actions of immunopotentiation. Taken the efficacious and safe profiles together, our experimental results also provide support for future clinical studies of applying this ancient Chinese herb medicine in cancer patients undergoing chemotherapy.

Acknowledgements

Gl-PS was kindly provided by Profs. Shuqian Lin and Saiizhen Wang of Fuzhou Institute of Green Valley Bio Pharm Technology. Research Fund of Shanghai Green Valley Holding Co. Ltd. supported this study.

References

Quayi, E.K., Carter, P.H., Tsai, C.M., Marti, G.E., 1997. Immunization with meningococal membrane-bound lipo-ooligosaccharide accelerates granulo-
cyte recovery and enhances lymphocyte proliferation in myelosuppressed mice. Pathobiology 65, 26–38.