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Effects of Ganopoly® (A *Ganoderma lucidum* Polysaccharide Extract) on the Immune Functions in Advanced-Stage Cancer Patients

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ABSTRACT

Preclinical studies have established that the *Ganoderma lucidum* polysaccharide (GLPS) fractions have potent anti-tumor activity, which has been associated with the immuno-stimulating effects of GLPS. However, it is unclear whether GLPS has immuno-modulating effects in humans in vivo. This study aimed to investigate the effects of Ganopoly®, the polysaccharides fractions extracted from *G. lucidum*, on the immune function of advanced-stage cancer patients. Thirty-four advance-stage cancer patients were entered onto this study, and treated with 1800 mg Ganopoly®, three

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times daily orally before meals for 12 weeks. Immune parameters (cytokines, T cell subsets, mitotic response to phytohemagglutinin (PHA) and natural killer (NK) activity) were compared between baseline and after 12-week treatment. Thirty patients are assessable for their immune functions. Treatment of Ganopoly® for 12 weeks resulted in a significant ($P < 0.05$) increase in the mean plasma concentrations of interleukin (IL-2), IL-6, and interferon (IFN)-γ, whereas the levels of IL-1 and tumor necrosis factor (TNF-α) were significantly ($P < 0.05$) decreased. A marked variability among patients with advanced-stage cancer was observed in the numbers of each lymphocyte subset at baseline. The mean absolute number of $CD56^+$ cells was significantly ($P < 0.05$) increased after 12-week treatment of Ganopoly®, whereas the numbers of $CD3^+$, $CD4^+$, and $CD8^+$ were just marginally increased compared to baseline levels, with the CD4:CD8 T cell ratios unchanged. PHA responses after 12-week treatment with Ganopoly® were enhanced in most patients, when compared to pretreatment baselines ($P < 0.05$). In addition, Ganopoly® treatment resulted in a significant increase ($P < 0.05$) in the mean NK activity compared to baselines (34.5 ± 11.8% vs 26.6 ± 8.3%). The present study indicates that Ganopoly® enhanced the immune responses in patients with advanced-stage cancer. Clinical evaluations of response and toxicity are ongoing.

Key Words: *Ganoderma lucidum*; Polysaccharide; Tumor; Efficacy; Safety.

INTRODUCTION

At present, the major forms of cancer treatment are surgery, radiation, chemotherapy and immunotherapy (Gibbs, 2000; Ratain and Relling, 2001). However, these therapies are only successful when the cancer is detected at an early stage, or limited to certain types of cancer (e.g. leukemia). Due to limited diagnostic means for detecting precarcinoma status and cancers at early stages, most patients present in the advanced stage of cancer or with extensive local infiltration. For advanced tumors, in particular those tumors developed from epithelial tissues such as lung, colon, breast, prostate and pancreas, these therapies are less successful. Drug resistance and dose-limiting toxicities are the major problems for the success of cancer chemotherapy (Ratain, 1997; Ratain and Relling, 2001). Therefore, novel therapeutic approaches are required to kill cancer cells more effectively and selectively.

The approach to treat advanced cancer using natural medicines has drawn much attention recently (Vickers, 2000). Indeed, some natural medicines have been investigated as anti-cancer agents in cancer patients and some encouraging findings have been observed, although objective responses have rarely been found. For example, the treatment with the herbal supplement, PC-SPES, composed of 7 highly concentrated Chinese herbs and 1 US herb, caused a significant decrease in the serum prostate-specific antigen levels for > 57 weeks in 33 androgen-dependent prostate cancer patients (Small et al., 2000). Further in vitro and animal studies have found that PC-SPES can induce tumor cell apoptosis; inhibit tumor cell proliferation; downregulate BCL-2, BCL-6, proliferating cell nuclear antigen, prostate-specific antigen, and androgen receptor; and upregulate P53, BAX, and P21 proteins (Chen, 2001). All these activities may contribute to the beneficial effects of PC-SPES in prostate cancer patients.

A number of natural medicines have been reported to be immune stimulants in in vitro and animal studies (Wasser and Weis, 1999; Werner and Jolles, 1996). They can enhance cell-mediated immunity and NK activity, facilitating the killing the tumor cells by the body. For example, *Ganoderma lucidum*, a medicinal mushroom, has been reported to have anti-tumor activity in mice (Hwang et al., 1989; Lee et al., 1995b; Miyazaki and Nishijima, 1981; Sone et al., 1985; Wang et al., 1997). Further studies suggest that the *G. lucidum* polysaccharide (GLPS) fractions are involved in this anti-tumor action (Chang, 1996; Czop and Austen, 1985). GLPS is able to activate macrophages, T lymphocytes, and NK cells, and induce the production of cytokines such as tumor necrosis factor (TNF-α), interleukins (ILs) and interferons (IFNs) in in vitro with human immune cells and in vivo in mice (Liu et al., 2002; Wang et al., 1997; Zhang et al., 2002; Zhou and Gao, 2002). However, it is unclear whether GLPS has immuno-modulating effects in humans in vivo. This study aimed to investigate the effects of Ganopoly®, the polysaccharide fractions extracted from *G. lucidum*, on the immune function of advanced-stage cancer patients.

MATERIALS AND METHODS

Cancer Patients

Patients were entered onto the study if they met the following eligibility criteria and did not meet any of the exclusion criteria: $\geq$ 18 years; histologically confirmed, advanced-stage cancers arising from various tissues; $\geq$ 12 weeks of interval between prior anticancer therapy and entry and an interval of 60 days from prior cranial radiation therapy before enrollment for patients with primary brain tumors; an Eastern Cooperative Oncology Group (ECOG) performances status of 0–2 and a projected life expectancy of $\geq$ 12 weeks; adequate bone marrow function, renal function and liver function; and informed consent for participation. Ethical approval was from the Institutional Research Ethics Committee. Exclusion criteria were as follow: severe concurrent conditions; pregnant or lactating women; no objective measurable disease by physical examination and appropriate medical imaging studies; and patients who had taken or were taking *Ganoderma* preparations or any immuno-modulating agents. As shown in Table 1, 34 patients were included in this study. The tumor was mainly from lung, breast, liver, colon, prostate, bladder, and brain. Thirty-three of 34 (97%) patients had $\geq$ 2 disease/organ sites affected and 32 (94%) patients were treated with various modules. Thirty patients were assessable for immune functions after 12 weeks. Four patients were not assessed due to non-compliance (n = 2), lost to follow up (n = 1) or death (n = 1). The death of one patient with advanced liver cancer at week 7 was due to disease complication (grade 4 encephalopathy caused by late-stage hepatic cirrhosis). This patient stopped taking Ganopoly® at week 4. There is no evidence indicating his death was due to the side effect of Ganopoly®.

Ganopoly® Treatment

Ganopoly® was the only anti-cancer agent administered during the 12-week study period. Patients were treated with 1800 mg, three times daily orally before meals for 12

Table 1. Baseline patient characteristics.

Characteristics	
Male	20
Female	14
Age (median & range)	57.5 (31 − 77 years)
Cancer origin	
Lung	7
Colon	6
Breast	5
Liver	5
Prostate	4
Bladder	2
Brain	2
Unknown	3
ECOG performance status	
0	2
1	21
2	11
Previous treatment	
None	2
Surgery	23
Chemotherapy	6
Radiotherapy	10
Immunotherapy	12
Endocrine treatment	5
Traditional Chinese Medicine	19
$\geq$ 2 of above treatments (excluding surgery)	18
*Sites of metastasis**	
Soft tissue/lymph node only	28
Lung ± soft tissue only	12
Viscera	8
Liver	13
Other (e.g. spleen, bone and brain)	16
Number of disease/organ sites affected	
1	1
2	14
$\geq$ 3	18

weeks. Each capsule contained 600 mg extract of *G. lucidum*, with 25% (w/w) crude polysaccharides. As the fruiting body of *G. lucidum* contains approximately 0.5% (w/w) polysaccharides, a capsule of Ganopoly® was equal to 30 g fruiting body of *G. lucidum,* or total dose of Ganopoly® per day (5400 mg) was equal to 270 g fruiting body. The common dose of *G. lucidum* for folk use in China is 50−300 g per day, depending on disease severity (Chang, 1994). As most herbs used for chronic diseases are administered for at least 1−4 months, we chose 12 weeks as the treatment regimen.

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Ganopoly® was manufactured to the GMP standards and provided by Encore International Co., Auckland, New Zealand.

Determination of Plasma Cytokine Concentrations

Plasma from patients before and after treatment was assayed for the determination of IL-1, IL-2, IL-6, IFN-γ, and TNF-α using commercially available ELISA kits (R & D Systems, Abingdon, UK). All determinations were performed in triplicate.

Phytohemagglutinin (PHA)-Stimulated Lymphocyte Proliferation Assay

PHA-stimulated lymphocyte proliferation assay was performed using [^{3}H]-thymidine incorporation as measure of lymphocyte proliferation as described (Santin et al., 2000). Mononuclear cells were separated from the heparinized blood of cancer patients and normal donors by Ficoll-Hypaque gradients. Peripheral blood lymphocytes (PBL) (5×10^4 cells/well) were seeded in a 96 well/plates in 0.2 ml of RPMI 1640/5% human AB serum and incubated with 10 mg/l of PHA (Sigma-Aldrich, Beijing, China). PBL were tested for specific proliferation after 96 h. Cultures were pulsed with 1 μCi/well of [^{3}H]-thymidine for the last 16 h, and incorporated radioactivity was measured. All assays were carried out in triplicate wells.

Analysis of Lymphocyte Subsets by Flow Cytometry

The lymphocyte subset analysis included $CD3^+$ (T lymphocyte), $CD4^+$ (T-helper cells), $CD8^+$ (T-suppressor cells), and HNK-1 and $CD56^+$ (NK cells). Peripheral blood samples (20 ml) were collected from each patient into heparinized tubes before the onset of treatment and after 12-week treatment. Lymphocytes were separated by Ficoll-Hypaque centrifugation. Standard direct-labeling techniques were used as recommended by the manufacturers. Immunophenotyping was done by flow cytometry using conjugated antibodies able to detect specific epitopes. The antibodies for the identification of lymphocyte subsets were OKT4 ($CD4^+$) and OKT8 ($CD8^+$) from Ortho (Raritan, NJ). Immunofluorescence was examined by a FACScan analyser (Becton Dickinson). The proportion of lymphocytes stained with each monoclonal antibody was converted to the absolute number per microliter by multiplying by the number of lymphocytes per microliter derived from the whole blood count.

Measurement of Natural Killer Activity

NK cell activity was tested in the total peripheral blood mononuclear cells (PBMC) population against the NK-sensitive K562 tumor cells by means of ^{51}Cr release assay as previously described (Tsavaris et al., 2002). Briefly, effector PBMC were freshly isolated and plated in 100 μl aliquots in 96-well microtiter plates. Tumor target cells (10^7) were incubated with 100 μCi of sodium [^{51}Cr] chromate (Amersham) for 90 min

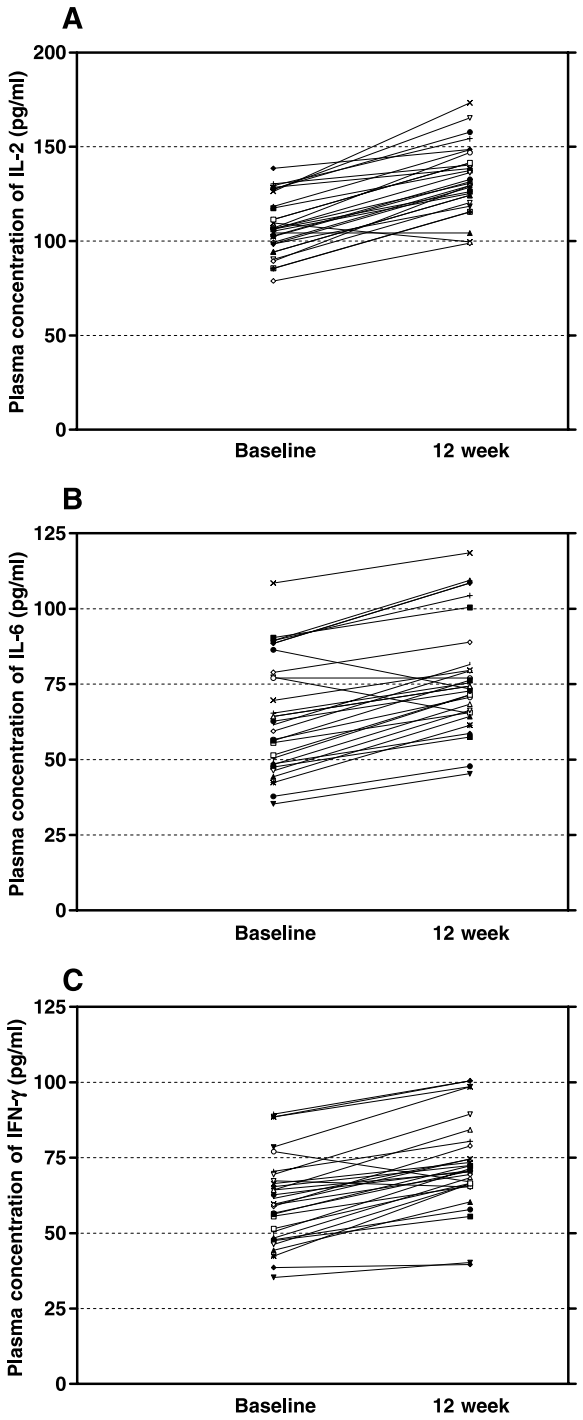

Figure 1. Changes in the plasma concentrations of IL-2 (A), IL-6 (B), and IFN-γ (C) at baseline and after 12-week treatment with Ganopoly® in advanced-stage cancer patients. Values are the mean ± SD.

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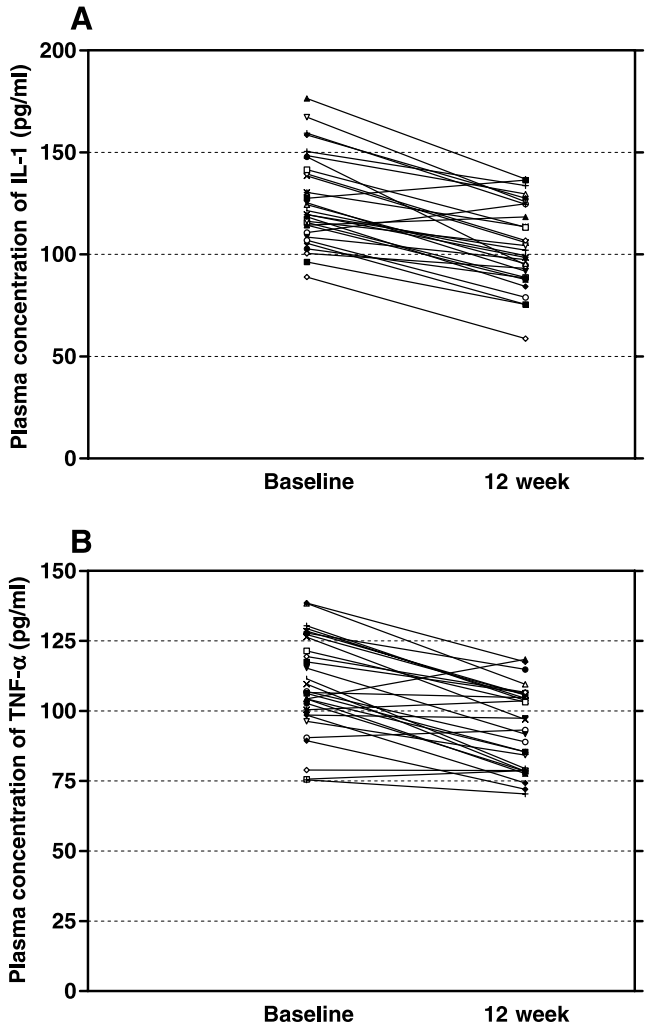

Figure 2. Changes in the plasma concentrations of IL-1 (A) and TNF-α (B) at baseline and after 12-week treatment with Ganopoly® in advanced-stage cancer patients. Values are the mean ± SD.

at 37°C, washed twice to remove excess isotope and a quantity amounting to 5×10^3 cells/well was added to the effector cells, to assess an effector to target (E/T) ratio of 40 to 1. After 18 h incubation at 37°C in a CO_2 incubator, 100 µl of supernatant were removed from each well for radioactivity counting using a γ-counter. Spontaneous and maximum release was established by incubating target cells in medium alone or with 5% Triton-X 100 respectively. Spontaneous release did not exceed 15% of the maximum release. In all cases, cultures were set up in triplicate and % specific target cell lysis was calculated as follows:

$$\%\ \text{specific target cell lysis} = \frac{\text{Mean cpm}_{\text{experimental}} - \text{Mean cpm}_{\text{spontaneous}}}{\text{Mean cpm}_{\text{maximum}} - \text{Mean cpm}_{\text{spontaneous}}}$$

STATISTICAL ANALYSIS

Data were analyzed using the analysis of variance, the two-tailed *t* test, and Student's *t* test for paired data. In all tests, the difference was considered significant when *P* values were less than 0.05.

RESULTS

Changes in Plasma Cytokine Concentrations

Treatment of Ganopoly® for 12 weeks resulted in a significant ($P < 0.05$) increase in the mean plasma concentrations of IL-2, IL-6, and IFN-γ (Figure 1); whereas the levels of IL-1 and TNF-α were significantly ($P < 0.05$) decreased (Figure 2).

Changes in the Number and Proportion of Lymphocyte Subsets

A marked variability among patients with advanced-stage cancer was observed in the numbers of each lymphocyte subset at baseline. The mean absolute number of $CD56^+$ cells was significantly ($P < 0.05$) increased after 12-week treatment of Ganopoly® (baseline vs 12 week: 238.3 ± 54.1 vs 277.4 ± 56.8) (Figure 3). However, the mean numbers of $CD3^+$, $CD4^+$, and $CD8^+$ were just marginally increased (Figure 4) compared to baseline levels. Treatment of Ganopoly® had no or little effect on total PBL counts and the CD4:CD8 T cell ratios.

Mitotic Response to PHA

As shown in Figure 5, PHA responses after 12-week treatment with Ganopoly® were enhanced in most patients (n = 24, 80%), when compared to pretreatment baseline

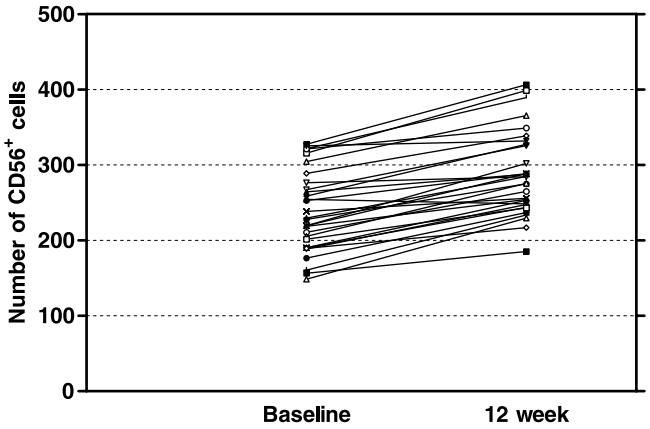

Figure 3. $CD56^+$ (NK cells) counts at baseline and after 12-week treatment with Ganopoly® in advanced-stage cancer patients. Values are the mean ± SD.

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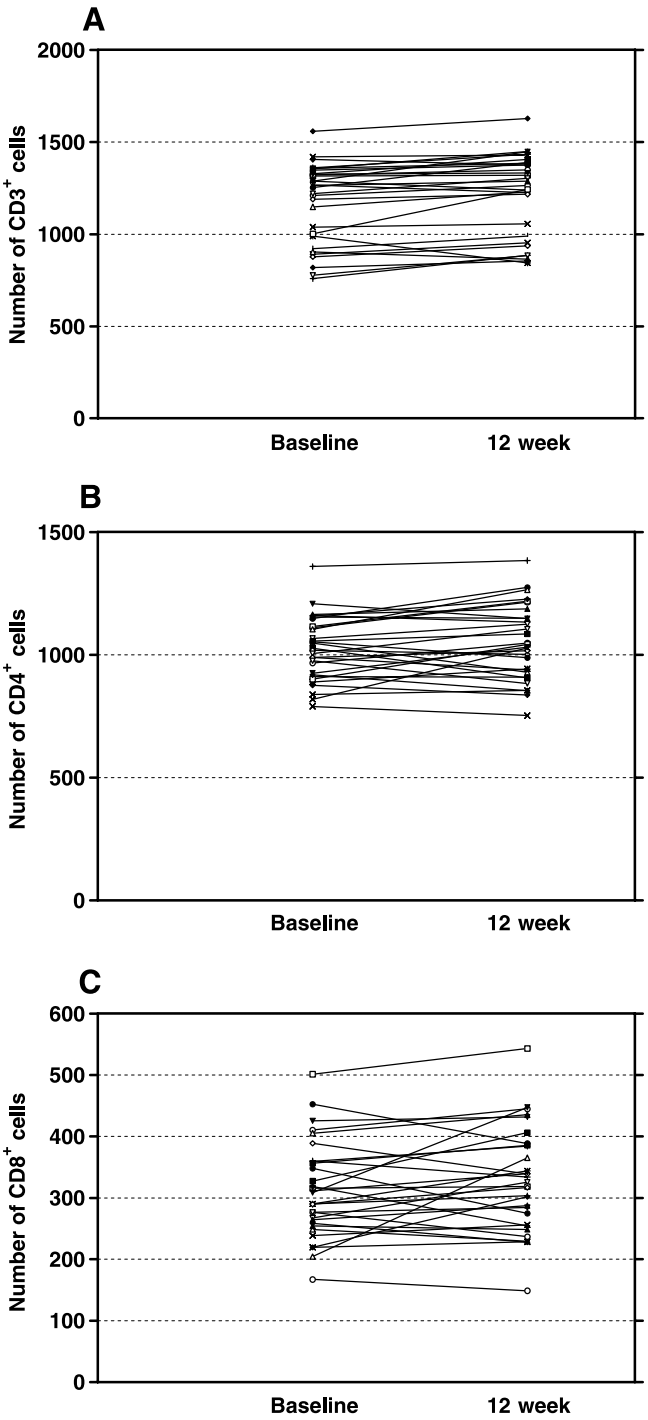

Figure 4. $CD3^+$ (T lymphocyte, A), $CD4^+$ (T-helper cells, B) and $CD8^+$ (T-suppressor cells, C) counts at baseline and after 12-week treatment with Ganopoly® in advanced-stage cancer patients. Values are the mean ± SD.

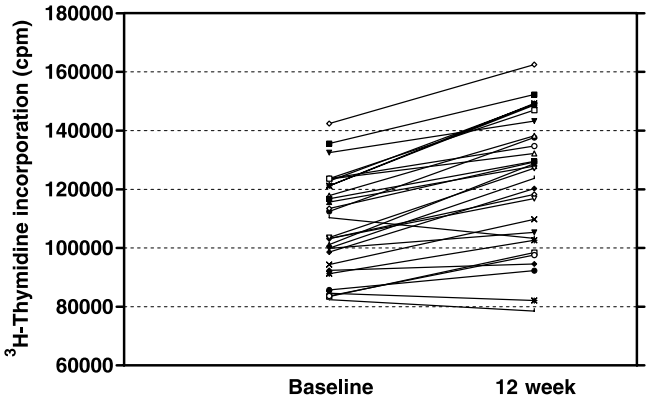

Figure 5. Mitotic response of peripheral blood lymphocytes to phytohemagglutinin (PHA) at baseline and after 12-week treatment with Ganopoly® in advanced-stage cancer patients. The data point is from at least three determinations.

levels (baseline vs 12 week: 108001.9 ± 16676.8 vs 121470.6 ± 21647.7 cpm, $P < 0.01$). However, the PHA response remained unchanged in 2 patients (6.7%) or was decreased in 4 patients (13.3%).

NK Activity

Baseline NK activity against K562 was evaluated in all patients and compared to the cytotoxic activity after treatments. A marked interindividual variability among patients in NK cytotoxic activity against K562 target cells was observed as demonstrated by a range of baseline killing varying from 14% to 55% at a 40:1 effector: target ratio. Treatment of Ganopoly® for 12 weeks resulted in a significant increase ($P < 0.05$) in the mean NK activity compared to baselines (34.5 ± 11.8% vs 26.6 ± 8.3%). In addition, there was a significant correlation ($r^2 = 0.3155$; $P = 0.0012$)

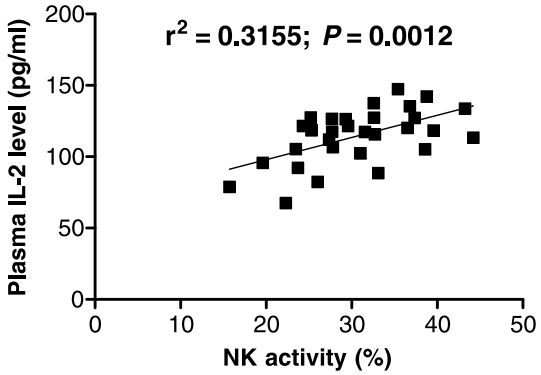

Figure 6. Correlation between plasma IL-2 levels and natural killer activity in advanced-stage cancer patients.

when the data for IL-2 after 12-week Ganopoly® treatment were plotted against the corresponding values of NK activity in 30 cancer patients (Figure 6).

DISCUSSION

The fate of the host-tumor interactions is considered to depend on the balance between the intrinsic aggressiveness (i.e. inherent metastatic potential) of the tumor and the strength of the host immune response. Cytotoxic T lymphocyte and NK activity play an important role in immunological surveillance in neoplasia and metastasis (Brittenden et al., 1996; Burnet, 1971; Cooper et al., 2001; Robertson and Ritz, 1990). Cytotoxic T lymphocytes have been shown to recognize specifically antigenic peptides in the context of major histocompatibility complex Class I molecules, while the ligands for triggering NK receptors involved in natural cytotoxicity are still largely unclear (Moretta et al., 2002). Human NK cells, comprising approximate to 15% of all circulating lymphocytes, can cause early production of cytokines and chemokines (e.g. ILs), and lyse tumor cells without prior sensitization (Cooper et al., 2001). In the late stage of cancer patients, NK activity is significantly decreased and associated with an impairment of cytokine production (Goto et al., 1999; Jovic et al., 2001; Lauerova et al., 2002). Low NK activity has been associated with poor prognosis in advanced cancer patients (Villegas et al., 2002). Therefore, a number of immunotherapeutic approaches aiming to enhance NK activity and production of cytokines such as IL-2 have been investigated in advanced-stage cancer patients, and beneficial effects have been observed in some patients (Agarwala et al., 2002; Brittenden et al., 1996). In addition to active and passive transfer of functional NK cells and cytokines, natural medicines may be an important complement to these approaches. This study indicates that Ganopoly® increased NK cell number and activity in those patients with advanced-stage cancer, providing evidence that Ganopoly® may act as a host defense potentiator. Similarly, a recent study shows that various natural medicines such as Transfer Factor Plus and Agaricus Blazeii Murill teas significantly increase NK activity, plasma TNF-α and PBL response to PHA (See et al., 2002).

Evidence has indicated that the immune function can be reduced or damaged in cancer patients receiving chemotherapy and radiotherapy, resulting in an decrease in cytotoxic T lymphocyte and NK activity (Kempf and Mitchell, 1985; Santin et al., 2000; Tsavaris et al., 2002). Therefore, standard chemotherapy and radiotherapy might negate or reduce the therapeutic benefits obtained by the increased tumor killing of the treatment. These negative effects may be particularly important in the treatment of immunogenic tumors where immunological function of the host is a determining factor for the clinical outcome of treatment. Thus, combination of chemotherapy or radiotherapy with immuno-modulating agents may provide a strategy for overcoming the immunosuppressive effects of chemotherapy/radiotherapy. Recently, many biological response modifiers have been combined with cytotoxic chemotherapeutic agents/radiation, in attempt to enhance anti-cancer activity and reduce toxicity. As many herbal medicines appear to work as biological response modifiers, they may become an important complementary approach to cancer treatment. These are agents or approaches modifying the relationship between the host and tumor by activating, increasing, and/or restoring the reactivity of immunological effector mechanisms that are involved in resistance to tumor growth and metastasis, leading to therapeutic effects

(Mihich and Fefer, 1983; Werner and Jolles, 1996; Zhou et al., 2002). It would be expected that Ganopoly® would negate the immunosuppressive effects of traditional chemotherapy/radiotherapy in cancer patients. Additionally, the inhibitory effects of Ganopoly® on TNF-α production may result in beneficial effects (e.g. improved life quality) in advanced-stage cancer patients. Increased TNF-α has been thought to contribute to cancer cachexia that is manifested by bodyweight loss, chronic nausea, fatigue, insomnia and profuse sweating (Argiles and Lopez-Soriano, 1999). Drugs that downregulate TNF-α can result in improvements of cachexia (Haslett, 1998). Thus, Ganopoly® may represent a useful approach to improve cancer cachexia.

This study indicates that Ganopoly has stimulated host defense response, as demonstrated by enhanced NK activity, PBL mitotic response to PHA and production of IL-2 and IFN-γ. The mechanism for these effects of Ganopoly® is unclear. It is well-known that many polysaccharides from natural sources have been identified to have significant enhanced effects on the immune system (Paulsen, 2001; Wasser and Weis, 1999; Werner and Jolles, 1996). Accumulating evidence indicates that the active β-D-glucans, consisting of a linear backbone of β-(1→3)-linked D-glucopyranosyl groups with varying degrees of branching from the C6 position, are the major immuno-modulating component of natural polysaccharides, although other forms of poly-saccharides such as heteropolysaccharides and glycoproteins may also play a major role (Kishida et al., 1988; Miyazaki and Nishijima, 1981; Sonet et al., 1985; Usui et al., 1981, 1983). They are potent stimulators of murine and human effector cells such as macrophages in vitro and in vivo by binding to membrane complement receptor type three (CR3, $\alpha_M\beta_2$ integrin, or CD11b/CD18) on immune effector cells (Battle et al., 1998; Konopski et al., 1994; Mueller et al., 2000; Muller et al., 1996). The β-D-glucan binding site (lectin site) of CR3 has been mapped to a region of CD11b located C-terminal to the I-domain and its distinct metal ion-dependent adhesion site for the many protein ligands of CR3 such as iC3b, ICAM-1 and fibrinogen (Diamond et al., 1993; Lee et al., 1995a; Thornton et al., 1996). CR3 receptors on macrophages are bound by β-D-glucan molecules and internalized, priming a series of molecular events. Further studies are needed to investigate whether it is the β-D-glucans in Ganopoly® that have the potent immune-stimulating effects and the mechanisms involved.

Although natural-occurring polysaccharides have been widely studied in in vitro and animals, human data are still sparse. To the best of our knowledge, this study is the first one to demonstrate that Ganopoly®, the polysaccharide fractions extracted from *G. lucidum* by a patented technique, enhanced the immune responses in patients with advanced-stage cancer. Clinical evaluations of response and toxicity are ongoing.

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