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Antitumor effects of the partially purified polysaccharides from *Antrodia camphorata* and the mechanism of its action

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Abstract

Antrodia camphorata is a popular folk medicine that has attracted great attention due to its fame for antitumor activity against cancer. However, there is little information available about its action. In the present study, we purified a unique polysaccharide component from *A. camphorata* mycelia (*AC-PS*) and found that it has pronounced anti-tumor effects on both in vitro and in vivo model. Our results showed that *AC-PS* alone did not show any direct cytotoxic effect to human leukemic U937 cells, even at high concentration (200 μg/ml). However, it could inhibit the proliferation of U937 cells via activation of mononuclear cells (MNCs). Treatment of U937 cells with *AC-PS*-stimulated-MNC-CM could significantly inhibit its proliferation with 55.3% growth inhibition rate. The in vitro antitumor activity was substantiated by the in vivo therapeutical study of *AC-PS* in sarcoma 180-bearing mice. Intraperitoneal and oral administration of *AC-PS*, 100 and 200 mg/kg significantly suppressed the tumor growth with the inhibition rate of 69.1% and 58.8%, respectively. In vivo studies also showed that several immunoparameters, such as the spontaneous proliferation of spleen cells, after *AC-PS* administration, were two-fold higher than in control mice. Furthermore, the cytolytic activity of spleen cells also increased from 9.8 ± 1.1% in control mice to 34.2 ± 5.5% and 48.2 ± 2.5%, after oral and intraperitoneal treatment, respectively. Besides, the mice serum interleukin-12 levels increased significantly by *AC-PS* treatment. Considering all these results, it is suggested that *AC-PS* elicit its anti-tumor effect by promoting a Th1-dominant state and killer activities.

Keywords: *Antrodia camphorata*; Polysaccharides; Antitumor; Cytokine; Spleen cells; Sarcoma 180

Introduction

Edible mushrooms have been used as flavorful foods and as health nutritional supplements for several centuries. For the Chinese, some mushrooms are especially regarded as medical substances to increase health and longevity. Nevertheless, systemic studies of its bio-function were not performed until the last third of this past century when biochemical technology for dissecting these traditional medicinal mushrooms and isolating their most active anticancer constituents became available. A number of bioactive molecules have been identified in numerous

Abbreviations: *AC-PS*, *Antrodia camphorata* polysaccharides; MNCs, human blood mononuclear cells; MNC-CM, mononuclear cells conditioned medium.

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mushroom species (Mizuno et al., 1995), but their polysaccharide components have been established as the most promising pharmacologically active antitumor compounds (Jong and Birmingham, 1993; Mizuno et al., 1992). In the last few decades, the biological activities of these polysaccharides have attracted increasing attention because of their immunomodulatory and antitumor effects. For example, the in vitro study of Wang showed that polysaccharides-rich fraction from *Ganoderma (G.) Lucidum* had strong stimulatory effects on both macrophages and T-lymphocytes for various cytokines releasing activity (Wang et al., 1997). Several investigators have demonstrated that partially purified polysaccharides of *G. Lucidum* could significantly inhibit the growth of implanted Sarcoma 180 in animals (Miyazaki and Nishijima, 1981).

Antrodia (A.) camphorata (*AC*), a parasitical microorganism on the inner cavity wall of local evergreen *Cinnamomum kanchirai* Hay (Lauraceae), is a species known to be available only in Taiwan. It was initially identified by Zang and Su as a new *Ganoderma* species in 1990 (Zang and Su, 1990), but was recently identified as a new basidiomycete *Antrodia camphorata* in the Polyporaceae (Wu et al., 1997). This species is well known in Taiwan under the name of "niu-chan-ku"or "chang-chih", and is also popular there. However, because its host wood is a local species that is getting scarce, *A. camphorata* is difficult to find in the forest and is also very expensive. Because of the similarity of *A. camphorata* and *Ganoderma* species in several characteristics, *A. camphorata* is said to have many medicinal uses, such as remedy for food, alcohol, and drug intoxication, itching illness and especially cancer. In the past, phytochemical investigations have resulted in the isolation of a series of new steroid acids, triterpene acids and polysaccharides (Cherng and Chiang, 1995; Cherng et al., 1996; Chiang et al., 1995; Huang, 2000; Shen et al., 1997; Yang et al., 1996). However, there are few reports about the biological activity of *A. camphorata*. Regarding its antitumor activity, only the study of Chen and Yang demonstrated that the crude extract of *A. camphorata* possessed in vitro cytotoxicity against P388 murine leukemia cells (Chen and Yang, 1995). In this study, we extracted the polysaccharides enriched fraction from the wild air-dried *A. camphorata* mycelia, harvested from submerged cultures, and analyzed its antitumor effect in vivo in the sarcoma 180 tumor-bearing mice and on the growth of leukemic cells in vitro. We also analyzed the immunomodulatory effects, such as splenocytes proliferation, cytotoxicity, and cytokines production in healthy mice.

Materials and methods

Preparation of AC-PS. The polysaccharide-enriched fraction of *A. camphorata* was isolated based on a previous method with little modification (Huang, 2000). Briefly, the mycelia of *A. camphorata* (Biotechnology Center, Grape King Inc., Chungli, Taiwan) were air-dried, disintegrated, and extracted with boiling water (at the ratio of 1:25, w/v) for 8 to 12 h. The suspension was filtered or centrifuged to remove the insoluble matter, and the water-soluble polysaccharide-enriched fraction was isolated by ethanol precipitation in two steps from the concentrated extract. The crude polysaccharides obtained were then passed through a Sephadex G50 (Pharmacia Biotech., Sweden) gel filtration column, and were further purified by an anion exchange column of DEAE-cellulose.

Cell lines. Human myeloid leukemia cell line, U937 (provided by the American Type Culture Collection, Rockville, MA), was used in the in vitro inhibition study. The cell was maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), 1 mM sodium pyruvate (Sigma, St. Louis, MO), 100 IU/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma) and the culture was passaged every 2 or 3 days. YAC-1, a murine NK-sensitive T-lymphoma cell line (provided by the American Type Culture Collection), was used in the in vitro NK/LAK cytotoxicity assay. The cell was maintained in RPMI 1640 medium supplemented with 10% FCS. Murine Sarcoma 180 cell (provided by the American Type Culture Collection) was used in the tumor therapeutical study. It was maintained in DMEM medium supplemented with 10% FCS and the culture was passaged every 2 or 3 days.

Animal models for therapeutical study. ICR mice (6–8 weeks old, weighing 18–23 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. At day 0, the ICR mice were injected subcutaneously with 1×10^6 of Sarcoma 180 tumor cells. Mice in groups of eight or ten were randomly divided into five groups. Twenty-four hours after tumor implantation, the tumor bearing mice were orally or intraperitoneally (*IP*) treated with *AC-PS* at a dose of 25, 50, 100, or 200 mg/kg everyday for 4 weeks. Mice treated with equal amounts of normal saline served as control. The tumor size of each mice was measured by a caliper and calculated by the formula: $a \times b^2/2$, where a is the length and b is the width in millimeters. The inhibition ratio was calculated by the following formula: Inhibition rate = [1 − (mean tumor volume of experiment mice / mean tumor volume of control mice)] × 100%. This study was repeated three times, and the results were consistent.

Separation of blood mononuclear cells and conditioned media preparation. Human peripheral blood was obtained from normal adult volunteers after obtaining informed consents and leukocytes were separated by density centrifugation in a Ficoll-Hypaque solution (1.077 g/ml) at 400 × *g* for 30 min. Mononuclear cells (MNCs) recovered at the interface were resuspended in RPMI 1640 medium containing 10% FCS. To investigate the effect of *AC-PS* on stimulating, the immune cells, total MNCs (1×10^6/ml)

were incubated in RPMI 1640 medium containing 10% FCS with or without various concentrations of *AC-PS* at 37 °C for 3 days. After that, all culture media (also called conditioned media, CM) were collected, filtered, and stored at −70 °C until use. They were named *AC-PS*-stimulated-MNC-CM in the presence of *AC-PS* or non-stimulated-MNC-CM in absence of *AC-PS*, respectively. In parallel, lipopolysaccharide (LPS, 10 μg/ml; Difco Lab., Detroit, MI) was also used to prepare MNC-CM (LPS-stimulated-MNC-CM) as a positive control.

Treatment of leukemia cells. Human leukemic cell line, U937, maintained in RPMI 1640 medium containing 10% FCS was kept in an exponential status. The cells were cultured at an initial concentration of 1×10^5/ml in Petri dishes in the presence of 30% (vol/vol) *AC-PS*-stimulated-MNC-CM or non-stimulated-MNC-CM. To identify the growth inhibitory effect of *AC-PS* on leukemia cells, some cells were treated with *AC-PS* alone at the dosages of 100 and 200 μg/ml. Under these conditions, the amount of *AC-PS* added to the cultures was about three times the amount in *AC-PS*-MNC-CM. Cultures were then incubated at 37 °C for 5 days, and viable cells were counted by the trypan blue exclusion test.

Separation of murine splenocytes. Healthy ICR mice (in group of 10) were treated with oral or intraperitoneal (*IP*) administration of 100 mg/kg/day *AC-PS* for 15 or 30 consecutive days. Mice were sacrificed by cervical dislocation on the next day. The spleens were removed aseptically, stripped of fat and placed in a culture medium. The culture medium was RPMI 1640 supplemented with 4 mM L-glutamine (Gibco BRL) and 10% FCS. Single-cell suspensions were obtained by gentle homogenization of mouse spleen in a tissue homogenizer. The spleen lymphocytes were separated from the debris and then washed twice (5 min at 800 × *g* at 4 °C). Isolated cell suspensions were washed three times with the culture medium. Lymphocyte viability was assessed by trypan blue exclusion. The control groups of mice were similarly treated with normal saline. The viability of the cells used throughout was >95%.

Proliferation assay of mouse splenocytes. Bromodeoxyuridine (BrdU) incorporation method was used to measure cell proliferation. Splenocytes, 2×10^6/ml, exposed or not exposed to T cell mitogen (20 μg/ml *Phaseolus vulgaris* agglutinin, PHA, Sigma), were seeded in 24-well tissue culture microplates. At 18 h before the end of the incubation period (2 days in 5% CO_2 in a humidified 37 °C incubator), 10 μM of BrdU (Sigma) was added per well. The cells were then washed twice in 1 × PBS and centrifuged at 500 × *g* for 15 min at room temperature. One ml of 0.1 M HCl (0.1 M HCl, 0.5% Triton X-100) was added slowly to the cells and the mixture was incubated at room temperature for 30 min, so that DNA was denatured to form single-stranded molecules. After neutralization with sodium borate (0.1 M, pH 8.5), two wash-step centrifugations of the nuclei in PBS were made before treatment with anti-BrdU antibody (5 μl of anti-BrdU monoclonal antibody [ICN Pharmaceuticals, Costa Mesa, CA] in 50 μl of PBS, 0–4 °C overnight). Following antibody incubation, another two wash steps were performed. A secondary antibody was then added (5 μl of goat anti-mouse Fab2 fragments-FITC conjugated [Dako Diagnostics, Copenhagen, Denmark], in 50 μl of PBS at room temperature for 1 h), following a wash step in PBS. Finally, the cells were resuspended in 1 ml PBS. Samples were analyzed by flow cytometry at 488 nm excitation and fluorescence was filtered with a 525-nm band pass filter. Green fluorescence from the fluorescein FITC-antibody conjugate was considered a measure of BrdU incorporation. The results were expressed as the percentage of BrdU positive. The stimulation index (SI) was calculated by the following formula: SI = % BrdU in experimental cultures / % BrdU in control cultures.

Target cell labeling. The YAC-1 cell line was used as target cell and was cultured for 24 h before use in the splenocytes cytotoxicity assay. The target cells were labeled with DiOC18, a lipophilic carbocyanine membrane dye (Molecular Probes, Eugene, USA), at 3 mM in dimethyl sulfoxide (DMSO) as described previously (Piriou et al., 2000). Briefly, 1 ml of complete medium containing 5×10^5 cells was mixed with 4 μl of 3 mM DiOC18. The cells were incubated at 37 °C for 30 min and rinsed three times with PBS. Cell labeling and cell viability were confirmed by flow cytometry before use in the test. Cell viability was assessed by propidium iodide (PI, 10 μg/ml, Sigma), which permeates only through the membrane of dead cells and emits a red fluorescence. The test was performed when cells were labeled homogeneously and cell viability was higher than 90%.

Splenocytes cytotoxicity assay. The susceptibility of target cells (*T*) to effector cells (*E*) was measured in the flow cytometric assay as previously described with little modification (Piriou et al., 2000). Briefly, 2×10^6/ml of effector cells were incubated with DiOC18-labeled target cells in a total volume of 1 ml using an *E*/*T* ratio of 20:1. Replicates of the effector cells without target cells and target cells without effector cells were set up in parallel. The plates were held at 37 °C for 24 h in a humidified incubator under 5% CO_2. Fifteen minutes before the end of the incubation time, samples were vigorously resuspended and 10 μl PI solution (50 μg/ml in PBS, Sigma) was added to stain dead cells. For each group, the activity of cytotoxic T cells was measured by analyzing 1×10^4 target cells/sample using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Live target cells were identified only by green fluorescence, whereas dead target cells (Td) were indicated by both green and red fluorescence. The percentage of dead target cells (%Td) was calculated as follows: %Td = (Td/T) × 100. Specific lysis was calculated as: $\%Td_{\text{with effector cells}} - \%Td_{\text{without effector cells.}}$

Assay for cytokines. The serum levels of IL-4, IL-6, IL-12, TNF-α and IFN-γ were determined by commercially available solid-phase enzyme-linked immunoabsorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN).

Statistical analysis. Statistical comparison between control and *AC-PS* were carried out using Student's *t* test. A level of $P < 0.05$ was considered to be statistically significant.

Results

Effects of AC-PS and AC-PS-MNC-CM on leukemic cell proliferation

To clarify that *AC-PS* exhibited direct or indirect cytotoxic activity against tumor cells, the effects of *AC-PS* or *AC-PS*-MNC-CM on leukemia cells were examined. Table 1 shows the growth of U937 cells in the presence or absence of various additives. After 5 days of incubation, the number of U937 cells in the untreated culture increased from 1×10^5/ml up to 30.9×10^5/ml with viability ≥98%. There was no significant difference in the proliferation upon treating the cells with 100 and 200 μg/ml of *AC-PS* alone. However, the proliferation of U937 cells was remarkably inhibited by treatment with *AC-PS*-stimulated-MNC-CM, resulting in an inhibition rate of 55.3% ($P < 0.001$). Similar effects were also observed in U937 cells treating with LPS-stimulated-MNC-CM. As a vehicle control, non-stimulated-MNC-CM had no significant suppressive effect on leukemic cell growth at comparable concentrations (Table 1).

Antitumor activity and side effects of AC-PS in sarcoma 180–bearing mice

The antitumor activity of *AC-PS* against sarcoma-180 tumor in the ICR mice model was significant. As shown in Fig. 1A, the mean tumor volume reached 2367.9 ± 395.4 mm^3 in the saline-treated control 4 weeks after tumor implantation. In contrast, a significant inhibition of tumor growth was observed in the mice upon oral administration of 50 mg/kg *AC-PS* with the inhibition ratio of 21.6% (mean tumor volume was 1857.5 ± 300.2 mm^3). The antitumor potency of *AC-PS* was dose-dependent with the inhibition rate of 39.7% and 58.8% (mean tumor volumes were 1429.0 ± 263.1 and 976.1 ± 119.8 mm^3) was noted for 100 and 200 mg/kg, respectively. Intraperitoneal injection of

Table 1
Comparison of growth inhibition in leukemic U937 cells induced by *AC-PS* alone, non-stimulated-, LPS-stimulated, and *AC-PS*-stimulated MNC-CM

Treatment[a]	Cell number ($\times 10^5$/ml)	Percent of inhibition (%)[b]
Untreated control	30.9 ± 0.6	–
AC-PS alone		
100 μg/ml	30.2 ± 0.7	1.8
200 μg/ml	29.4 ± 0.5	5.0
MNC-CM (30%; vol/vol)		
Non-stimulated	28.4 ± 1.2	8.4
LPS-stimulated (10 μg/ml)	11.8 ± 0.5[c]	61.8
AC-PS-stimulated (100 μg/ml)	13.8 ± 0.6[c]	55.3

[a] Leukemic U937 cells (1×10^5/ml initially) were incubated at 37 °C for 5 days in the presence or absence of AC-PS, non-stimulated- or *AC-PS*-stimulated-MNC-CM. Data are expressed as mean ± SEM of five separate experiments.
[b] Percent of inhibition = (1–treated / untreated control) × 100%.
[c] $P < 0.001$ compared with non-stimulated MNC-CM.

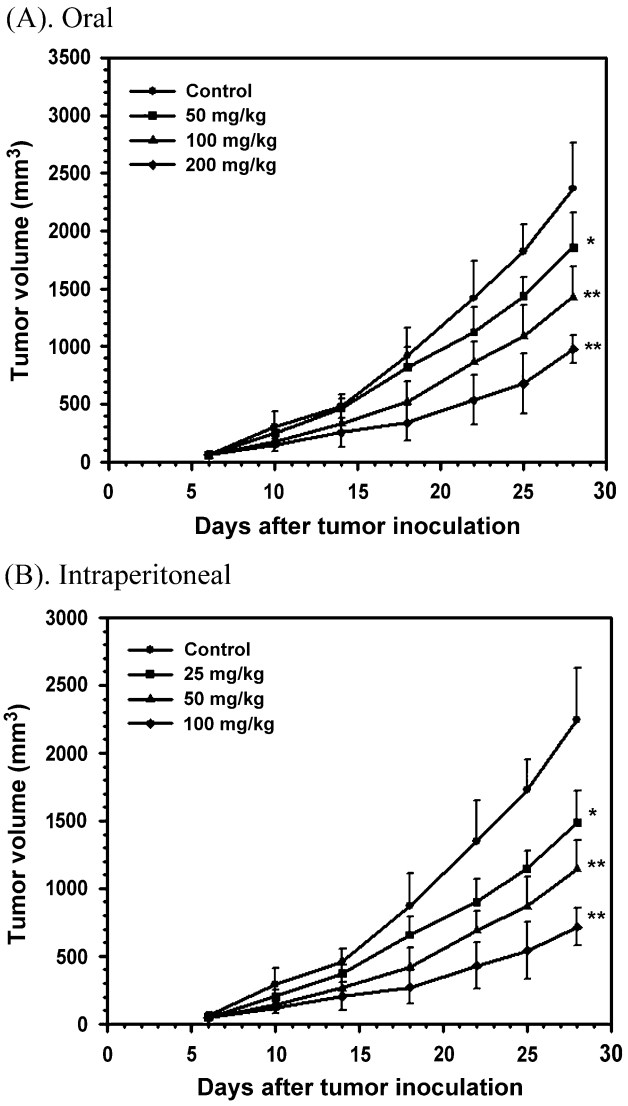

Fig. 1. Dose effects of (A) Oral or (B) Intraperitoneal administration *AC-PS* on tumor volume in sarcoma 180-bearing mice. Solid-type sarcoma 180 was prepared by subcutaneous injection of 1×10^6 cells into the right flank of mice on day 0. The indicated amounts of *AC-PS* were administered orally or intraperitoneally for 28 consecutive days, starting 24 h after the implantation of tumor cells. Control mice were treated with equal amounts of normal saline alone on the same schedule. The tumor volume was measured by a caliper and calculated by the formula: $a \times b^2/2$, where *a* is the length and *b* is the width in millimeters every 3–4 days. Results are expressed as means ± SEM, $n = 10$. *$P < 0.01$ and **$P < 0.001$ compared to the control group.

AC-PS inhibited tumor growth was even more effective than oral administration, thus intraperitoneal injection of 25 mg/kg had equal therapeutic efficacy (inhibition rate of 33.9%) as 100 mg/kg by oral administration (Fig. 1B). The inhibition rate for intraperitoneal administration of 50 and 100 mg/kg were 49.2% and 69.1%, respectively. Neither oral nor intraperitoneal administration of *AC-PS* had significant side effects such as reduced food intake or change of body weight gain in tumor-bearing mice.

Effects of AC-PS on spontaneous and mitogen-induced proliferation of splenocytes

To evaluate the in vivo immunostimulation effect of *AC-PS*, the proliferation of splenocytes from *AC-PS*-treated mice or controls were compared. As data showed in Table 2, the splenocytes from control mice showed a little spontaneous proliferation (with 10.4 ± 2.6% and 14.9 ± 0.8 % of BrdU incorporation for day 15 and day 30) and a moderate increase of cell number after PHA stimulation (with 22.3 ± 30% and 21.5 ± 1.8% of BrdU incorporation for day 15 and day 30, respectively). However, splenocytes from *AC-PS*-treated mice, whether oral or intraperitoneal administration, exhibited a significantly higher spontaneous proliferation (with 25.7 ± 3.0% and 26.8 ± 3.3% of BrdU incorporation after 15 days' administration and with 35.1 ± 3.6% and 35.2 ± 2.9% of BrdU incorporation after 30 days' administration for oral and intraperitoneal treated splenocytes, respectively). The proliferative response of splenocytes from *AC-PS*-treated mice was further enhanced by PHA treatment (with 38.0 ± 3.1% and 38.2 ± 2.7% after 15 days' administration and with 48.4 ± 3.5% and 49.2 ± 2.1% of BrdU incorporation after 30 days' administration for oral and intraperitoneal treated splenocytes, respectively).

Table 2
Effects of *AC-PS* on Splenocytes proliferation[a]

Treatment[b]	Splenocytes only		PHA activated-Splenocytes	
Day 15		SI[c]		SI[c]
Control	10.4 ± 2.6	–	22.3 ± 3.0	–
Oral-treated	25.7 ± 3.0[d]	2.5	38.0 ± 3.1	1.7
IP-treated	26.8 ± 3.3[d]	2.6	38.2 ± 2.7	1.7
Day 30				
Control	14.9 ± 0.8	–	21.5 ± 1.8	–
Oral-treated	35.1 ± 3.6[d]	2.4	48.4 ± 3.5[d]	2.3
IP-treated	35.2 ± 2.9[d]	2.4	49.2 ± 2.1[d]	2.3

[a] Bromodeoxyuridine (BrdU) incorporation method was used to measure cell proliferation. Data are expressed as mean ± SEM of 10 mice.
[b] Healthy ICR mice (in group of 10) were treated oral or intraperitoneal (*IP*) administration of 100 mg/kg/day *AC-PS* for 15 or 30 consecutive days. Mice were sacrificed by cervical dislocation on the next day after last administration. The spleens were removed aseptically, stripped of fat and placed in a culture medium.
[c] The stimulation index (SI) was calculated by the following formula: SI = % BrdU in experimental cultures / % BrdU in control cultures.
[d] $P < 0.001$ compared with control.

AC-PS enhances spontaneous and mitogen-induced cytolytic activity of splenocytes

To determine whether *AC-PS* enhances NK/LAK activity in vivo, the cytolytic activity of splenocytes against YAC-1 cells was examined in mice treated with *AC-PS*. As data shown in Fig. 2, the spontaneous cytolytic activity of splenocytes from the control mice was 9.8 ± 1.1%, and it increased to 14.5 ± 2.0% by PHA treatment. However, splenocytes from mice treated with *AC-PS* had significantly higher spontaneous cytolytic activity of 34.2 ± 5.5% and 48.2 ± 2.5% for oral and intraperitoneal *AC-PS* treatment ($P < 0.001$, $n = 10$), respectively. The cytolytic activities of splenocytes from *AC-PS*-treated mice were further enhanced by PHA-stimulation, that is, 57.0 ± 3.1% and 70.2 ± 4.4% for oral and intraperitoneal treatment, respectively.

Effects of AC-PS on the cytokines level in mice

We also examined the effects of *AC-PS* administration on the cytokines level in mice serum. As shown in Table 3, IL-12 production in *AC-PS*-treated mice were increased significantly. The serum level of IL-12 was increased from 6.1 ± 1.7 pg/ml for control mice to 29.3 ± 3.4 and 25.7 ± 4.4 pg/ml for oral and intraperitoneal *AC-PS* treated mice after 15 days' administration. The IL-12 production was further increased after 4 weeks of *AC-PS* treatment, from 4.6 ± 0.9 pg/ml for control to 83.7 ± 10.1 and 91.3 ± 15.8 pg/ml for oral and intraperitoneal *AC-PS*-treated mice, respectively. The other cytokines (IL-6, TNF-α and IFN-γ) were moderately increased by *AC-PS* treatment. Nevertheless, the IL-4 could not be detected in both control and the experimental mice serum.

Discussion

A. camphorata, a new basidiomycete of the polyporaceae identified in 1990, is well known in Taiwan as a traditional Chinese medicine. This medicinal fungus has attracted great attention due to its role in the antitumor activity against several different cancers, including ovary, gastric, breast, and liver cancers. However, because its host wood, *Cinnamomum kanchirai* Hay, is a local species and is getting scarce, *A. camphorata* is becoming difficult to find in the forest and is very expensive for medical use. So far, there is little information available about the anti-tumor activity of *A. camphorata*. Only Chen et al. had performed in vitro tests to demonstrate its antitumor activity (Chen and Yang, 1995). In the present study, we describe, for the first time, its in vivo antitumor activity against sarcoma 180 in ICR mice model. We also demonstrate that this effect was mediated by the enhancement of host immune defense, instead of direct toxicity to tumor cells, since the in vitro cytotoxicity was retained in the *AC-PS*-stimulated MNC conditioned medium.

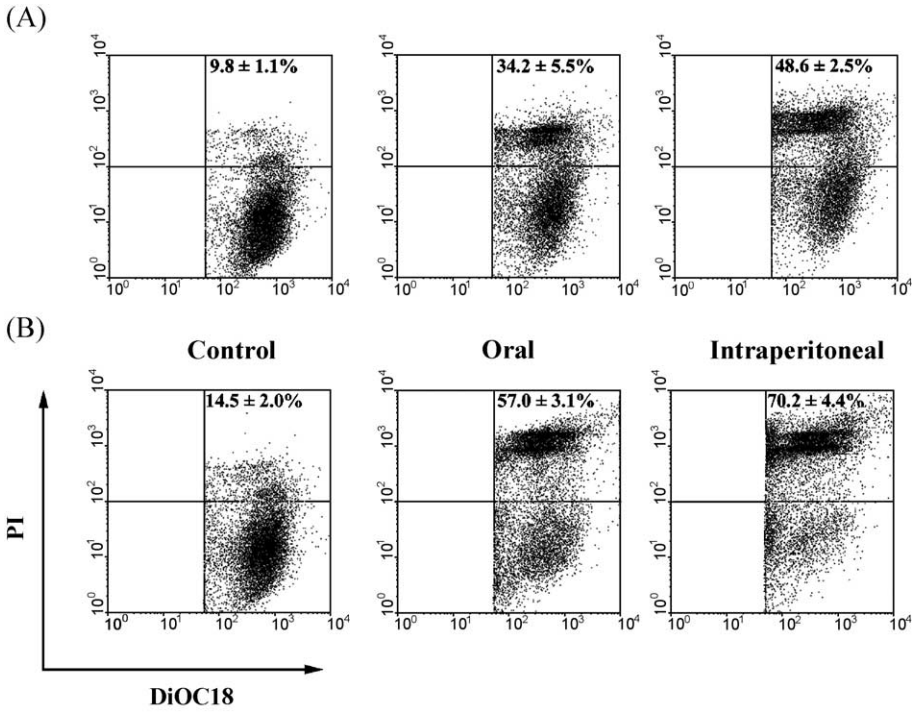

Fig. 2. *AC-PS* enhances (A) spontaneous and (B) mitogen-induced cytolytic activity of splenocytes. Freshly prepared YAC-1 as target cells (T) were labeled with DiOC18 and subsequently incubated with unstimulated or stimulated effector spleen cells (E) using *E*/*T* ratio of 20:1. Spleen cells, which are separated from either treated or untreated mice, were used as the effector cell. Fifteen minutes before the end of the incubation time, samples were vigorously resuspended and 10 μl PI solution (50 μg/ml in PBS, Sigma) were added to stain dead cells. After double staining, dead target cell population could be distinguished by two-parameter flow-cytometry. Results are expressed as means ± SEM, $n = 10$. $P < 0.001$ compared to the control group.

The antitumor activity of polysaccharides isolated from medicinal fungi were most attractive due to their low toxicity to normal cells and the apparent lack of side effects in clinical patients (Fukushima, 1989). Previously, many polysaccharides isolated from different medicinal fungi have been shown to have antitumor activity. Among them, Lentinan from *Lentinus edodes (Berk.) Sing*. (Suga et al., 1984), *PS-K/PS-P* from *Coriolus versicolor* (Mizushima et al., 1982; Yang et al., 1993) and *PS-G* from *Ganoderma lucidum* (Furusawa et al., 1992; Sone et al., 1985) are the three important fungal polysaccharides that have been widely used in antitumor investigations. Many investigators have suggested that the polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. This has been verified experimentally in the loss of antitumor effect of polysaccharides in neonatal thymectomized mice or after administration of anti-lymphocyte serum (Ooi and Liu, 2000); the antitumor activity of lentinan and other polysaccharides is inhibited by pretreatment with anti-macrophage agents (such as carrageenan). Thus, various effects of polysaccharides are thought to be due to the potentiation of precursor T cells and macrophages after specific recognition of tumor cells (Borchers et al.,

Table 3
Effects of *AC-PS* on cytokines production in mice serum

Treatment[a]	IL-4	IL-6	IL-12	TNF-α	IFN-γ
Day 15					
Control	ND[b]	2.4 ± 0.3	6.1 ± 1.7	ND[b]	ND[b]
Oral-treated	ND[b]	3.8 ± 0.3	29.3 ± 3.4[c]	0.8 ± 0.3	0.2 ± 0.1
IP-treated	ND[b]	6.1 ± 0.6	25.7 ± 4.4[c]	0.5 ± 0.3	0.2 ± 0.1
Day 30					
Control	ND[b]	2.4 ± 0.2	4.6 ± 0.9	ND[b]	ND[b]
Oral-treated	ND[b]	3.8 ± 0.3	83.7 ± 10.1[c]	4.8 ± 0.4	1.3 ± 0.4
IP-treated	ND[b]	13.4 ± 0.9	91.3 ± 15.8[c]	3.2 ± 0.3	1.5 ± 0.4

[a] Healthy ICR mice (in group of 10) were treated oral or intraperitoneal (*IP*) administration of 100 mg/kg/day *AC-PS* for 15 or 30 consecutive days. Serum was collected on the next day after last administration.
[b] ND: not detected.
[c] $P < 0.001$ compared with control.

1999). In the present study, the *AC-PS* we used could inhibit the in vivo growth of sarcoma 180 either through oral or intraperitoneal administration (Fig. 1). Moreover, in vitro study showed that the growth inhibition of U937 cells could only be induced by *AC-PS*-MNC-CM, but not by *AC-PS* alone even at doses of up to 200 μg/ml (Table 1). These are consistent with the previous reports that antitumor effects of polysaccharides may be through the indirect pathway by activating the host immune response. However, the immune-mediated prevention of tumor growth was through diverse mechanisms, which required the involvement of humoral and cell-mediated immune functions. All kinds of cells involved in the complex immune network, such as CTL, help T lymphocyte, B lymphocyte, activated macrophage, NK cell, and LAK cell were reported to be involved in the protection of host from tumor. For example, *PS-K* has been demonstrated to increase NK/LAK activation (Fisher and Yang, 2002); polysaccharides from *Ganoderma Lucidium* and *Coycydeps Sinensis* have been shown to inhibit the proliferation of leukemic cells via stimulating the production of cytokines by activated MNCs (Chen et al., 1997; Wang et al., 1997). For these reasons, we also examined the potentiality of *AC-PS* on the host immune defense system after oral and intraperitoneal administration. As shown in Table 2, splenocytes from *AC-PS*-treated mice displayed a significantly higher spontaneous proliferation rate after 30 days of administration as compared with those from untreated mice. Although we did not further characterize which population is the main responder to *AC-PS* treatment, the mixed population of immune cells contained in splenocytes indicated that *AC-PS* could exert its effects through complex immune network.

Besides the action on immune cells, augmentations of the cytokines production are known as another important function of polysaccharides. Cytokines are the major mediators of host defense, and they regulate communication between antigen-presenting cells (APCs), lymphocytes and other host cells during an immune response. Selective cytokine production, in particular, can determine the outcome of a response by stimulating protective or exacerbative immune mechanisms. Therefore, the cytokine repertoire present after the administration of immunomodulatory agents determine the type of host response directed against the tumor. In the present study, we have analyzed both Th1-type (TNF-α, IFN-γ, and IL-12) and Th2-type (IL-4 and IL-6) cytokines inducing pattern of *AC-PS* (Table 3). The findings of the present study clearly indicate that *AC-PS* is a potent inducer of Th1-type cytokines. Previous reports indicated that most polysaccharides induce Th1-dominant state mediated by IL-2 or IFN-γ induction (Kidd, 2000), but IL-12 induction by polysaccharides was not analyzed in the literature. Present study clearly indicated that *AC-PS* strongly induced IL-12 production. IL-12 is a heterodimeric 70 kDa glycoprotein that has been demonstrated in many studies to have potent antitumor and antimetastatic activities in murine model. Furthermore, it was reported that IL-12 could induce IFN-γ production, NK/LAK cell cytotoxicity, and activation of CD8+ T cells that is important for its antitumor immune response (Trinchieri and Scott, 1999). It might also explain the enhancement of cytotoxicity of splenocytes after *AC-PS* administration. Based on these results, it strongly suggested that *AC-PS* might elicit its antitumor effect by promoting a Th1-dominant state and killer activities.

Polysaccharides with antitumor action differ greatly in their chemical composition and configuration, as well as in their physical properties. Although it is difficult to correlate the structure and antitumor activity of complex polysaccharides, some relationship can be inferred. It has been suggested that the structural features such as β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points are needed for antitumor action (Chihara, 1992; Miyazaki and Nishijima, 1981). High molecular weight glucans appear to be more effective than the low molecular weight species (Mizuno, 1996; Mizuno, 1999a; Mizuno, 1999b). The polysaccharides used in the present study were a partially purified polysaccharide fraction. Its exact composition for the activation of host immune system remains to be further identified. However, Huang has shown that glucan fraction of *A. camphorata* is composed of a backbone of (1→3)-linked β-D-glucopyranosyl residues with an average molecular weight over 100 kDa (Huang, 2000).

Although *A. camphorata* was a popular folk medicine and had attracted great attention due to its role in the antitumor activity against several types of cancer, there is little information available about its action. This is the first report providing both in vitro and in vivo evidences of the therapeutical antitumor potential of this medicinal fungus. Our results suggested the antitumor activity of *A. camphorata* might act through the activation of host immune response. However, further investigation about the relationship between immunomodulation and antitumor activity is needed.

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