Advance Access Publication 11 April 2007

eCAM 2008;5(1)85–90
doi:10.1093/ecam/nem001

Original Article

Phellinus linteus Extract Augments the Immune Response in Mitomycin C-Induced Immunodeficient Mice

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Phellinus linteus is a fungus distributed throughout Japan, Korea and China. Boiled water-soluble extracts from *P. linteus* (PLW) have shown anti-tumor and immunomodulatory properties in experiments done by intraperitoneal treatment, or in *in vitro* cell cultures. This is the first investigation on how oral administration of PLW influences immune responses. Here, we established immunodeficient mice by mitomycin C (MMC) and then researched how PLW influenced plaque-forming cell (PFC) production and populations of cytokine [interferon-(IFNγ-) and interleukin-4 (IL-4)]-producing T lymphocytes. PLW samples were administered orally for 19 days (1, 2 or 4 g/kg/day). PFC assay was followed using Jerne's method. IFN- and IL-4-producing T lymphocyte populations were measured by flow-activated cell sorter (FACS). These assays were conducted the day after the last oral administration. MMC groups were given MMC (1 mg/kg/day) intraperitoneally for 6 days with PLW administration. The number of PFC per 10^6 spleen cells increased significantly in the PLW (2 g/kg/day) group when compared with the MMC-control ($P<0.05$) while populations of IFNγ- and IL-4-producing T lymphocytes decreased by MMC treatment. However, the PLW group tended to increase more than the MMC-control. Our results indicated that PLW augments the immune response of the spleen in MMC-induced immunodeficient mice.

Keywords: immune response – mitomycin C – *Phellinus linteus*

Introduction

In recent years, natural foods have become a vital part of complementary and alternative medicine. Natural products that show anti-tumor activity have been gaining popularity among those faced with the deleterious side effects of treatment using chemotherapeutic drugs or synthetic compounds. In particular, a variety of mushrooms are immune potentiators, and polysaccharides and peptides with anti-tumor activity have been isolated from mushrooms (1) such as black mushroom shiitake, and *Agaricus blazei* Murill (2–5). Among these, *Phellinus linteus* has often been reported for its anti-tumor activity (6–13). Distributed in Japan, Korea and China as an aqueous extract, *P. linteus* is a traditional Kampo medicine for diarrhea. Its major compounds are polysaccharides, aminoacids, γ-aminobutyric acid, vitamins and sugar. Polysaccharides and proteoglycan isolated from *P. linteus* exhibited cytotoxic action on tumor cells (9) and induced functional maturation of murine dendritic cells (10,11). The aqueous extract of *P. linteus* (PLW) inhibited IgE-dependent mouse triphasic cutaneous reactions (12), and stimulated antibody production (13). These studies, however, were done by intraperitoneal treatment, or in *in vitro* cell culture system, and did not investigate the antibody response to oral administration, which is a much more convenient mode of treatment.

It is unclear how PLW activates the immune system. Controlled by cytokine-producing T lymphocytes, B lymphocytes appear to play an important role in the

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immune response when it is inhibited by overwork, stress, chemotherapeutic drugs and radiotherapy (14). Immunodeficiency caused by anti-tumor drugs such as mitomycin C (MMC) poses a serious problem for cancer patients. MMC has strong anti-tumor activity, but also restricts bone marrow activity (15–17). Therefore, patients treated with MMC often develop a serious immunodeficiency and it is for this condition that we considered the potential of PLW. Since PLW has modulated the immune response of Th1/Th2 cytokine secretion in murine splenocytes (18,19) and Mossman proposed that Th1/Th2 balance is associated with the immune system (20-22), PLW treatment offered promise as a treatment for this immunodeficient condition caused by MMC. We have already established the mechanism of oral administration of the mushroom by investigating how oral intake of Kampo medicine and fungus augmented various immune activities (23–27). We became convinced that PLW could be used for this condition and established an immunodeficient mouse model using MMC to investigate PLW's influence on the immune system.

Methods

Mice

Female C57BL/6J strain mice 8 weeks of age obtained from Japan SLC (Hamamatsu, Japan), were bred and treated in conformity with the guidelines for animal experiments at Kanazawa Medical University. They were housed for 1 week with a 12 h light–dark cycle in a temperature and humidity controlled room, and were given free access to food and water. After adaptation to the lighting conditions for 1 week, healthy mice were chosen for the investigation.

Preparation of *P. linteus*

Mycelium of *P. linteus* was a gift from Iwata Chemical Co., LTD. (Iwata, Japan). The boiled water extract (abbreviated as PLW) was prepared as follows: 1 kg of the dried Mycelium was refluxed with 10 l of water for 10 h, and the aqueous extract was freeze-dried (375 g, 37.5%). It was found to contain 6.4% protein, 83.2% polysaccharide (4.4% of β-glucan) and under 0.1% fat.

Preparation of Mitomycin C-Induced Immunodeficient Mice

Female mice 8 weeks of age were used. Mice in MMC-treated groups received intraperitoneal injections of MMC 1 mg/kg/day of their body weight for 6 days (28,29). According to a fixed rule, MMC medicine (obtained from Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan), was dissolved in distilled water. The control group was injected with an equivalent volume of saline. Weighing of the spleen and assay of its plaque-forming cells (PFC) were done 1, 2, 3 and 4 weeks after MMC treatment. The population of CD19-positive cells in the spleen was measured 1 week after treatment by flow cytometry.

Immunopotentiating Activity of PLW in Immunodeficient Mice

The schedule is shown in Fig. 1. Mice were divided into five groups (Control, MMC-control, MMC-PLW1, MMC-PLW2 and MMC-PLW3). Control and MMC-control groups were given distilled water, and MMC-PLW groups were administered orally 1, 2 or 4 g/kg/day of PLW for 19 days. MMC-groups were given MMC (1 mg/kg/day) intraperitoneally for 6 days with PLW administration (28–30). The day after the last administration, we measured weight change in the spleen, researched PFC production and the population of cytokine [interferon-γ (IFNγ) and interleukin-4 (IL-4)]-producing cells in the spleen.

PFC Production in Normal Mice Measured after 19 days of PLW

Female C57BL/6J mice were divided into four groups (Control, PLW1, PLW2 and PLW3). The control group was given distilled water and PLW groups were orally administered 1, 2 or 4 g/kg/day of PLW for 19 days. The day after PLW treatments, we investigated change in spleen weight and PFC production of spleen cells.

Assay of Plaque-Forming Cells

Sheep red blood cells (SRBC), used as antigens and obtained from Japan Biomaterial Center (Tokyo, Japan), were washed three times with saline. Mice received i.v. injections of 1×10^8 SRBC. After 4 days, spleen cells from these mice were centrifuged twice at 490*g* for 5 min and suspended in 10 ml of 10% fetal calf serum, Roswell Park Memorial Institute (FCS-RPMI) 1640 medium (sigma, USA). Spleen cells were counted by Tulk test, a standard method. The number of PFC against SRBC antigen was determined by the method of Jerne *et al.* (31). Briefly, 100 µl aliquots of the spleen cell suspension

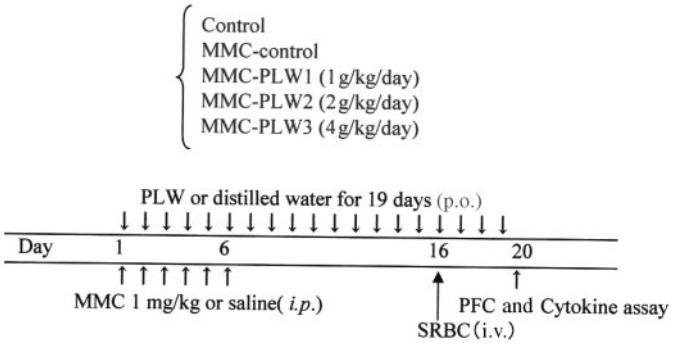

Figure 1. The experimental schedule of PLW on immune response.

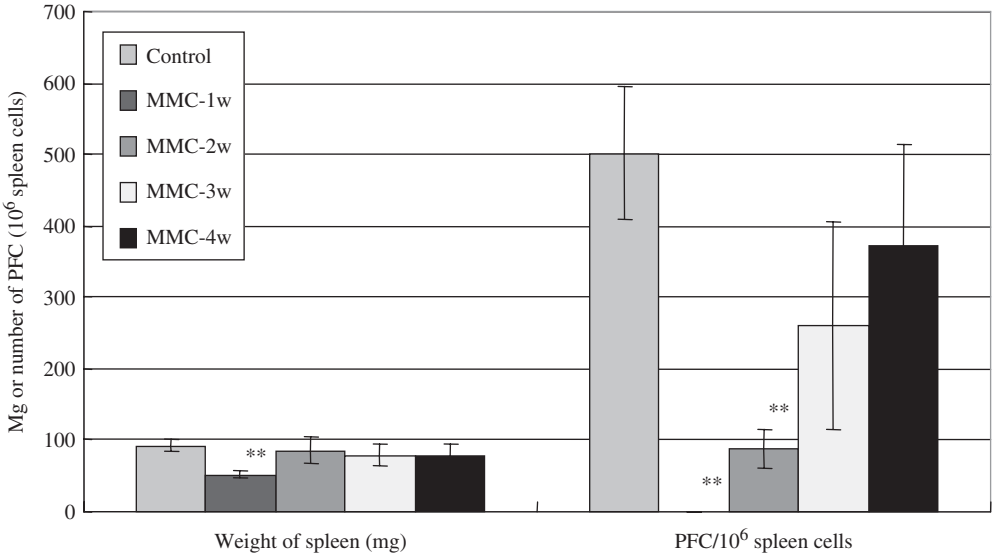

Figure 2. Recovered period from immunodeficient condition by Mitomycin C treatments (i.p., 1 mg/kg/day, 6 days) on the weight of spleen (mg) and the number of PFC per 10^6 spleen cells in normal mice. Each value represents the mean ± SD ($n = 7$); $^{**}P < 0.005$ versus control.

were mixed with 800 µl of Eagle's MEM agar, 100 µl of 8% SRBC and 0.5% DEAE-Dextran (Pharmacia, Sweden) in the final volume of 1.1 ml. These samples were incubated (37°C, 5% CO_2, 2 h) on the bottom layer (Eagle's minimum essential medium MEM agar). Guinea pig serum, isolated from the blood of the Hartley guinea pig, was added to the samples and incubated for 1 h. Hemolytic plaque of SRBC was counted by the naked eye.

Analysis of Surface Antigens

The expression of surface antigens and cytokines in spleen cells was determined by direct immunofluorescence (32,33). Spleen cells (1×10^6 cells/100 µl) were stained with final 0.5 µg of fluorescein-isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibody (mAb) at room temperature for 0.5 h. The FITC-conjugated anti-CD3 mAb, the PE-conjugated anti-IFNγ and -IL-4 mAb were obtained from Becton Dickinson. After incubation, these cells were centrifuged twice at 490*g* for 5 min with 1 ml of phosphate-based solution (PBS) and suspended in 0.5 ml of PBS containing 100 µg/ml $CaCl_2/MgCl_2$, 0.01% sodium azide and 1% FCS, and then analyzed using a FACScan flow cytometer (Becton Dickinson, USA).

Statistical Analysis

All values expressed as means ± S.D. Data were statistically analyzed by one-way analysis of variance (ANOVA) with the level of significance set at $P < 0.05$. Critical differences between means were evaluated by Dunnett's multiple comparison test set at $P < 0.05$.

Table 1. The population of CD19-positive cell 1 week after MMC treatment in spleen cells

	CD19-positive cells (%)
Control	50.5 ± 2.5
MMC-1w	47.1 ± 1.5

Each value represents the mean ± SD ($n = 7$).

Results

Antibody Production after Mitomycin C Treatment

In researching augmentation of the immune response to PLW administration in immunodeficient mice, we investigated the recovery period from MMC-initiated immunodeficiency. Results showed that PFC were not produced 1 week after the last treatment. However, production gradually recovered after 2 weeks (Fig. 2). The numbers of PFC per 10^6 spleen cells in each period were 88 ± 27 (2 weeks), 261 ± 145 (3 weeks), 371 ± 144 (4 weeks), showing significant differences at 1 and 2 weeks against the control ($P < 0.005$). Spleen weight at 1 week was lower than that of the control ($P < 0.005$). There was no significant difference between the populations of CD19-positive cells in the spleen (Table 1), suggesting that MMC treatment only slightly altered the CD19-positive cell population. Since recovery from immunodeficiency began 2 weeks after the last MMC treatment, we decided to assay PFC and flow-activated cell sorter (FACS) at that time.

Immunomodulating Activity of PLW in Immunodeficient Mice

We examined how PLW influenced splenic weight and PFC numbers per 10^6 spleen cells in immunodeficient

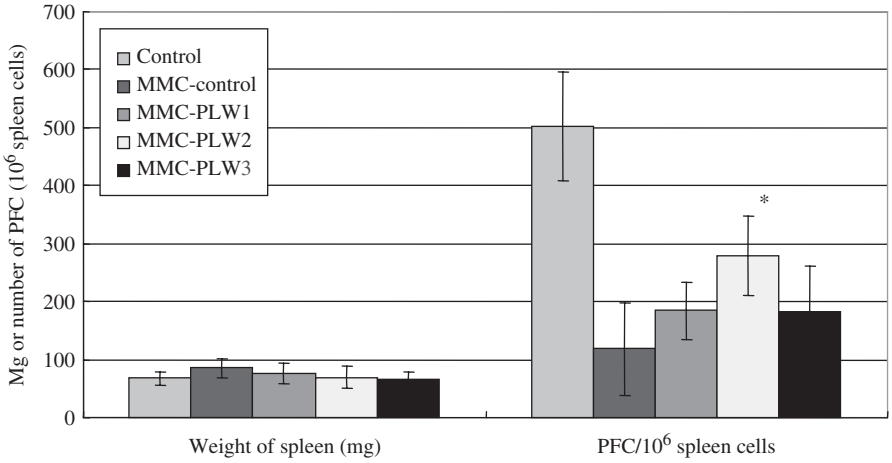

Figure 3. Effects of PLW treatments for 19 days on the weight of spleen (mg) and the number of PFC in mitomycin C-induced immunodeficient mice. Each value represents the mean ± SD ($n = 7$); *$P < 0.05$ versus MMC-control.

mice. There was no significant difference among the spleen weights of each group, as shown in Fig. 3. The number of PFC per 10^6 spleen cells was 502 ± 93 in the control, 118 ± 80 (MMC-control), 184 ± 49 (MMC-PLW1), 279 ± 69 (MMC-PLW2) and 183 ± 79 (MMC-PLW3), showing a significant difference between the MMC-control and MMC-PLW2 ($P < 0.05$) and suggesting that PLW augments antibody production in immunodeficient mice.

Cytokine (IFNγ and IL-4)-Producing T Lymphocytes in Spleen Cells

FACS data is shown in Table 2. The population of IFNγ- and IL-4- producing T lymphocytes decreased by MMC treatment while IFNγ- and IL-4-positive cells of PLW administration groups tended to increase (without significance) compared with the MMC-control. The population of CD3-positive cells was unchanged.

PLW Administration Did Not Increase Antibody Production in Normal Mice

Although PLW administration has shown immunopotentiating activity for immunodeficient conditions, its influence on normal conditions is unclear. Our results on normal mice showed that the number of PFC per 10^6 spleen cells were 350 ± 53 in the control, 448 ± 78 in PLW1, 446 ± 131 in PLW2 and 383 ± 101 in PLW3. No significant differences were observed among any of the groups, as shown in Fig. 4. PLW treatments for 19 days did not increase either antibody production or spleen weight.

Table 2. Effect of PLW oral administrarion for 19 days on T lymphocyte subsets ratio in Mitomycin C-induced immunodeficient mice

	CD3	IFNγ	IL-4
Control	46.39 ± 1.94	4.50 ± 0.28	0.76 ± 0.04
MMC-control	44.59 ± 1.45	1.75 ± 0.35	0.47 ± 0.15
MMC-PLW1	47.24 ± 6.93	2.11 ± 0.47	0.78 ± 0.29
MMC-PLW2	44.90 ± 1.84	2.74 ± 1.42	1.38 ± 0.75
MMC-PLW3	47.81 ± 0.60	2.22 ± 0.11	2.69 ± 0.75

Each value represents the mean ± SD ($n = 4$).

Discussion

As mentioned above, immunodeficiency is a serious side effect of chemotherapeutic drugs. We considered that this condition would be more receptive to PLW treatments than normal conditions and normal mice appear to be only slightly affected by PLW. Firstly, we made an immunodeficient mouse model by MMC treatment. We selected PFC assay in splenocytes as an index of immune activity. This is a typical method to directly observe antibody secreting cells. PFC production was completely inhibited by MMC treatments (1 mg/kg/day) for 6 days. The condition continued for 1 week, and gradually recovered without treatment. The spleen weights of the MMC-1w group were considerably lower than the control, and recovered completely after 2 weeks. However, there was no significant difference in CD19 antigen positive cells between MMC-1w and the control. This antigen exists on the cell surface membrane of non-activated B lymphocytes (34) and the treatment did not alter production suggesting that the maturation of lymphocytes was being inhibited and would not change during the treatment period.

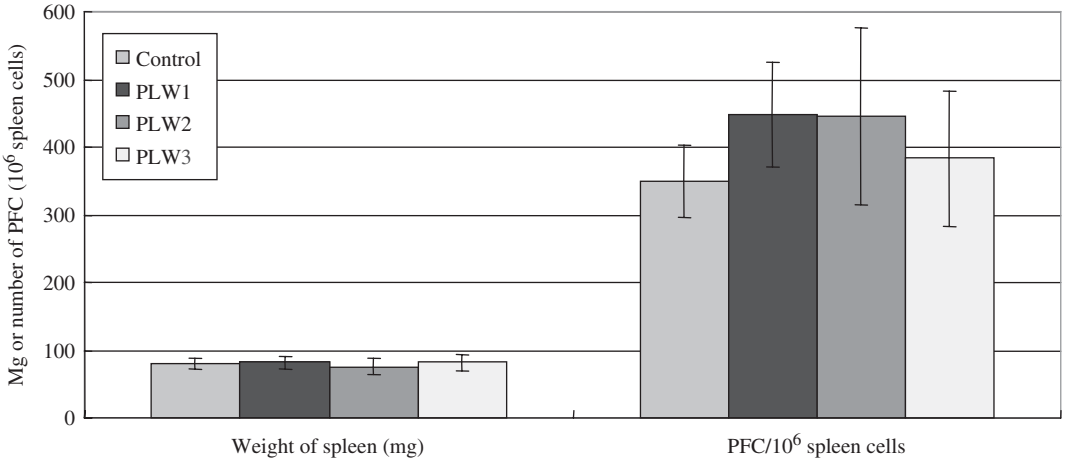

Figure 4. Effect of PLW oral administarion for 19 days on the weight of spleen and the number of PFC in normal mice. Each value represents the mean ± SD ($n = 8$).

To analyze how PLW administration altered immune responses, we decided to assay 2 weeks after the last MMC treatment. At this time, PFC production is beginning to recover and the condition of the mouse is closest to that of the immunodeficient patient. The number of PFC per 10^6 spleen cells against SRBC antigen was lower in the MMC-treated groups than in the controls. However, the numbers almost doubled in PLW-treated groups when compared with MMC-controls, while there were no differences in spleen weights of the two groups. The increase was not dose-dependent with the most effective dosage being PLW 2 g/kg. The production of the 4 g/kg group was lower than that of the 2 g/kg group.

Since PLW administration enhanced antibody production (mainly IgM), it was possible that IgG would be elevated if the isotype switched from IgM to IgG in the immunoglobulin. In spleens harvested from mice 4 days after immunization, the population of IFNγ or IL-4 antigen positive cells was lower in the MMC-control than the control. However, these populations tended to recover with PLW administration. IFNγ antigens are present on IFNγ- producing T lymphocytes and IL-4 antigens are present on IL-4-producing T lymphocytes, while CD3 antigens only exist on the cell surface membrane of T lymphocytes. The production of IL-4 plays a role in B cell differentiation and it has been suggested that the augmentation of antibody response that occurs with PLW results from expansion of IL-4-producing cells. Since IFNγ- augments tumoricidal activity (35) and induces functional maturation of dendritic cells (10,11), the augmenting effect of PLW may result from expansion of IFNγ-producing T lymphocytes, as has been reported in normal mice (18,19).

The destruction of balance in the body leads to immune disease (20–22) and PLW has the ability to change Th1/Th2 balance and thus augment antibody production in immunodeficient mice. Since PLW augments the immune system, it may be used to prevent various diseases. In correlation, it may be viable for strengthening the immunodeficent condition of cancer patients. However, further investigation of the immunopotentiating activity of PLW compounds is necessary.

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Received December 6, 2005; accepted January 11, 2007