

## Immunomodulatory Activity of the Water Extract from Medicinal Mushroom *Inonotus obliquus*

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The immunomodulatory effect of aqueous extract of *Inonotus obliquus*, called as Chaga, was tested on bone marrow cells from chemically immunosuppressed mice. The Chaga water extract was daily administered for 24 days to mice that had been treated with cyclophosphamide (400 mg/kg body weight), immunosuppressive alkylating agent. The number of colony-forming unit (CFU)-granulocytes/macrophages (GM) and erythroid burst-forming unit (BFU-E), increased almost to the levels seen in non-treated control as early as 8 days after treatment. Oral administration of the extract highly increased serum levels of IL-6. Also, the level of TNF- $\alpha$  was elevated by the chemical treatment in control mice, whereas was maintained at the background level in the extract-treated mice, indicating that the extract might effectively suppress TNF- $\alpha$  related pathologic conditions. These results strongly suggest the great potential of the aqueous extract from *Inonotus obliquus* as immune enhancer during chemotherapy.

**KEYWORDS:** Bone marrow, Cytokine, Hematopoiesis, Immune modulator, *Inonotus obliquus*

*Inonotus obliquus*, a white rot fungus in the family *Hymenochaetaceae* has only generative hyphae with no clamp connections and pore instead of gills. It has been known as the clinker polypore because the fruitless form of a fruiting body resembles clinkers, the irregular lumps of black material that remains after coal has been burned, and also known as Chaga throughout the Russia (Wasser, 2002). It is a parasitic fungus growing on birch, alder, beech and other hardwood trees throughout Russia, North America, Eastern Europe, and Japan. Since ancient times, Chaga has been a medicinal fungus in Siberian folk medicine to treat a variety of pharmacological activities such as stomach disease, liver-heart problems, blood purification, and pain relief. It has been also used as Russian and Siberia folk remedy for cancers, including inoperable breast cancer, lip cancer, gastric, parotid gland, pulmonary, stomach, skin, and rectal cancers, and Hodgkin's disease. Today, Chaga is well known for its antimicrobial, antiviral, antitumor activity (Borchers *et al.*, 1999). Therefore, scientific research regarding the effects of Chaga has been centered around its common folk uses.

Many bioactive compounds have been reported from the Chaga mushroom. The active substances among these are thought to be oxygenated triterpenes (Park *et al.*, 2004). The mushroom contains large amounts of betulin or betulinic acid, a chemical that is being studied for use as a chemotherapeutic agent because of its anti-cancer properties and also the full spectrum of immune-stimulating phytochemicals found in other medicinal mushrooms

such as maitake and shitake mushrooms. Although Chaga mushroom may turn out to be one of the most useful medicinal mushrooms, its biological action as immune-modulator has not yet been validated (Kim *et al.*, 2005).

Chemotherapy and/or radiotherapy often results in hematopoietic and immune dysplasia as hematopoietic stem cells are damaged during the procedure, and subsequently, committed hematopoietic and immune cells are depleted. Consequently, patients often experience anemia, lymphocytopenia, thrombocytopenia, and/or granulocytopenia, leading to serious and lethal infections and increasing the mortality and morbidity of these patients. Chemotherapeutic agent affects almost all subpopulations undergoing cell division, including early blasts, present in the bone marrow. Therefore, how rapidly patients recover from chemotherapy and/or radiotherapy greatly depends on the percentage of resting stem cells remaining after such treatment. As a means to protect stem cells or help damaged stem cells to recover, the use of biological response modifiers (BRMs) has received attention. Various compounds, especially carbohydrates isolated from mushrooms, yeasts, and plants were reported to affect bone marrow and peripheral blood cells, and to induce hematopoiesis (Hofer *et al.*, 1993). For example, a single peritoneal injection of Scleroglucan, derived from *Sclerotium glaucum*, enhanced the bone marrow cellularity (Pretus *et al.*, 1991), and OL-2 from *Omphalia lapidescens* increased the number of lymphocytes and various immune cells in both the peritoneum and the spleen (Ohno *et al.*, 1993). Intravenous injections of glucan-F, a soluble glucan, increased the overall number of granulocyte/macrophage (GM)-col-

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ony forming units (CFU) as well as the erythroid CFU in irradiated mice (Patchen and MacVitte, 1986). The underlying mechanism of these observations is not yet clear.

In this study, we tested whether this mushroom extract could be used to overcome the immune-suppressive situation caused by chemical-induced bone marrow damage. It was found that, in chemical-treated mice, the oral administration of the Chaga mushroom extract increased the number of CFU-GM and erythroid burst-forming units (BFU-E) almost to the normal levels. It also appeared to induce the proliferation of the cells committed to splenocyte because the number of cells was highly increased. Increased levels of chemically protective cytokines including IL-6 and TNF- $\alpha$ , which are well known to stimulate stem cell recovery and hematopoietic regeneration after bone marrow damage (Antman *et al.*, 1988), were also observed in the serum of chemically immunosuppressed mice treated with the Chaga mushroom water extract. Our data suggests the potential of the Chaga mushroom extract as an effective BRM and its possible use as an immune enhancer in immunocompromised and immunosuppressed individuals.

## Materials and Methods

### Preparation of the water extract from *Inonotus obliquus*.

The Chaga mushroom was obtained from Korean Ginseng Corp. (Seoul, Korea). The mushrooms were cut into pieces, dried and stored at room temperature in air-tight containers. Because of hard and woody nature of the Chaga mushroom, it was cut into pieces as small as possible after weighing, soaked in distilled water, boiled and cooled down to 60°C. Dried mushrooms (20 g) were extracted with 100 ml of water twice for 5 hr. The two water-soluble fractions were combined, centrifuged, and filtered through ADVANTEC filter paper, No. 2 (Toyo, Tokyo, Japan). The water-soluble fraction was obtained by centrifugation after filtration. The supernatant was concentrated into dryness using a rotary evaporator. The brown powder was obtained by freeze-drying the concentrates. The yield of dried water extract was approximately 12%. The resulting brown powder was weighed and dissolved in phosphate-buffered saline (PBS) for oral administration for mice.

**Limulus test.** Endotoxin in the Chaga mushroom water extract (CW) was assayed under endotoxin-free experimental conditions using a Limulus Amebocytes Lysate (LAL) Pyrogen kit (BioWhittaker, Walkersville, MD). Experiments were performed according to the manufacturer's protocol. Briefly, 100  $\mu$ l of standard reagent, CW or phosphate-buffered saline (PBS) was mixed with 100  $\mu$ l of LAL reagent and was incubated for 1 hr at 37°C. Each tube was then examined for gelation. The quantity of endot-

oxin in the Chaga water extract was less than 0.015 EU/mg

**Animals experiment.** Female BALB/c mice, 8 to 10 weeks of age and weighing 18 to 20 g, were purchased from the Seoul National University Laboratory Animal Center. Mice were housed either in conventional cages or, after cyclophosphamide treatment, in positive pressure lamina air flow microisolators (Lab Products, Maywood, NJ) on cob chip contact bedding. The animals were housed under specific pathogen-free conditions according to the institutional guidelines. Research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council. Mice were divided into 3 groups and injected subcutaneously with 400 mg of cyclophosphamide (Cy, Endoxan-Asta<sup>TM</sup>, Laboratoires Asta-Medica, M $\acute{e}$ rignac, France) per kg body weight. In the first group, mice were treated with Cy, and then 10 mg of the Chaga extract per animal was orally administered every day for 24 days. In the case of the second group, Cy-treated mice were the control administered with PBS. The third group was non-treated and administered with PBS. Each group consisted of nine mice. Three mice from each group were sacrificed by cervical dislocation on days 8, 16, and 24 after irradiation for hematological and bone marrow evaluation. The parameters analyzed included the number of the CFU in the bone marrow cells and serum levels of cytokines.

**Colony-forming assay.** Bone marrow cells from intact mice were drawn by flushing femoral bones with an IMDM medium and the femoral bone marrow cells were placed in triplicate at a concentration of  $1 \times 10^5$  cells/ml in semisolid methylcellulose supplemented with 1% methylcellulose in IMDM, 15% fetal bovine serum (FBS),  $10^{-6}$  M  $\beta$ -mercaptoethanol, 10 ng/ml IL-3, 50 ng/ml SCF, and 3 units/ml erythropoietin (Stem Cell Technologies, Vancouver, British Columbia, Canada) as a source of colony-stimulating activity. Plates were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Colonies were counted under the microscope 10 days after cultivation. The number of colonies consisting of more than 50 cells was counted, and the average number of colonies and standard errors were calculated from triplicate. The different colonies were identified and counted based on their morphological characteristics as referenced by Murine Hematopoietic Cell Colony Photographs from Stem Cell Technologies. BFU-E is light or pinkish brown color, small in size, dense, and irregular in shape. Colorless colonies with a dark, dense core or those consisting of granular or foamy cells were counted as CFU-GM.

**Splenocytes proliferation and TNF- $\alpha$  production.** On day 24, mice administered CW and PBS after cyclophos-

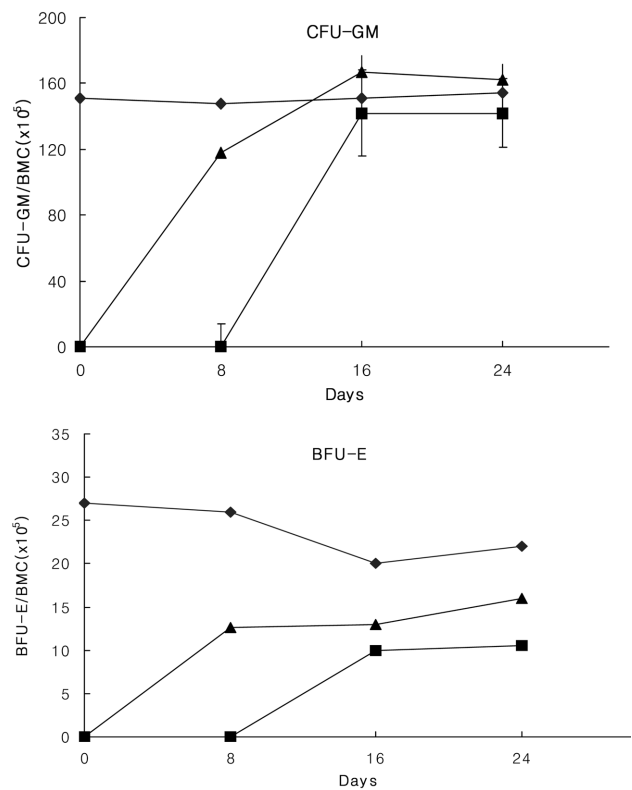
phamide treatment were sacrificed. Spleens were removed in sterile conditions and splenocytes were collected. Then the cells were suspended in culture medium (RPMI-1640 medium contained with 10% FBS) at a concentration of  $1 \times 10^7$  cells/l. One hundred microliter of the cell suspension and 100  $\mu$ l of concanavalin A (ConA, Pharmacia/LKB Biotech, Piscataway, NJ) with final concentration of 3 mg/l were seeded to a 96-well culture plate simultaneously. Triplicates were designed. The cultures were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 3 days. Two hours before completion, 10  $\mu$ l of MTT (5 g/l) was added to each well. The absorbance was measured on an EJ301 ELISA Microwell Reader (Wuxi Keda, China) at 570 nm. The results were described as the average of triplicate absorbances. Splenocytes isolated from ConA-sensitized mice were cultured in the presence of different concentration of the Chaga mushroom water extract for the assessment of TNF- $\alpha$ .

**Cytokine assay.** ELISA kits for TNF- $\alpha$  and IL-6 in serum and splenocyte culture were purchased from Endogen (Woburn, MA), and levels of the cytokines were measured according to the manufacturer's instruction.

**Statistical analysis.** Data are expressed as means  $\pm$  SEM. Statistical significance was determined using a Student's *t* test.

## Results and Discussion

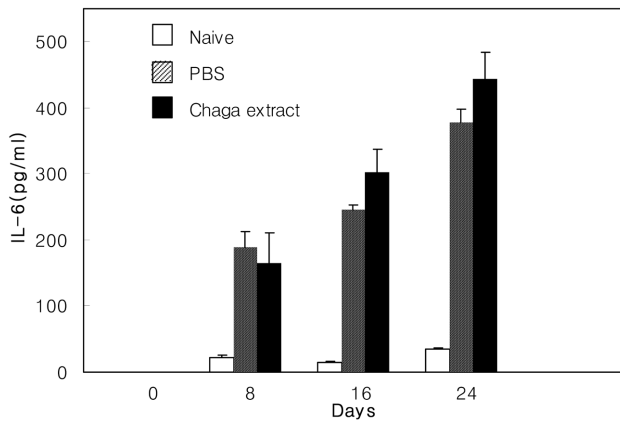
**Effect of the Chaga mushroom water extract (CW) on hematopoietic stem cells in chemically immunosuppressed mice.** Because rapidly-dividing hematopoietic stem cells in the bone marrow are the major damaged cells by chemical treatment, the effects of CW were first tested on the number of the CFU in the bone marrow cell population. Bone marrow cells were isolated on days 8, 16, and 24 after treatment, and were seeded at an average of  $10^5$  cells per plate in the methylcellulose assay system. To determine which CFU was affected by CW, BFU-E and CFU-GM were determined by morphological characteristics. In PBS-treated control mice, neither CFU-GM nor BFU-E were formed properly and their numbers remained significantly lower than non-treated mice. In contrast, the number of CFU-GM in CW-treated mice was comparable with that in non-treated normal mice at all time points (Fig. 1). The number of BFU-E in CW-treated mice at day 8 was approximately one-half of that in non-treated mice, but it reached almost the normal level at day 24. Our results suggested that the water extract from *I. obliquus* is a very effective BRM that has significant biological effects on the bone marrow. The Chaga extract helped chemical treated mice to rapidly recover the cells that are capable of forming a colony. The daily adminis-



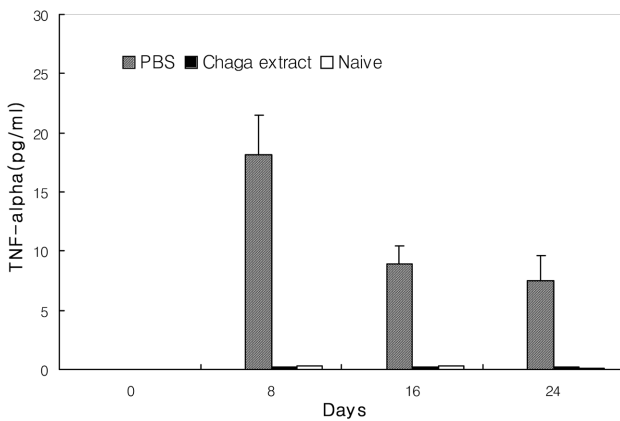
**Fig. 1.** The effects of Chaga extract on colony-forming units in cyclophosphamide-treated mice. Bone marrow cells ( $1 \times 10^5$ /well) from PBS- or Chaga extract-administrated mice were plated on methylcellulose containing growth factors on days 8, 16, and 24 after chemical treatment. After 10 days, the total colony number was counted: (◆), naive; (▲), PBS; (■), Chaga extract. Colony type was classified by morphological observation. Data are expressed as means  $\pm$  SEM from three independent experiments.

tration of Chaga extract for 24 days increased the number of CFU to an almost normal level.

**Cytokine levels in serum of the mice treated with the CW.** It was previously reported that IL-6 might be involved in the recovery of blood cells from the immunosuppressive condition (Zeidler *et al.*, 1992). The effects of the CW on IL-6 was tested in cyclophosphamide-treated mice. Level of the cytokine in the serum was measured using the commercially available kits at days 8, 16, and 24. As clearly shown in Fig. 2, level of IL-6 was significantly increased in CW-treated mice over time. In contrast, the level of TNF- $\alpha$ , whose level had been increased after cyclophosphamide-treatment, was decreased over time (Fig. 2). These results suggested that Chaga mushroom extract could increase serum levels of chemoprotective cytokines, while decreasing the level of chemical-induced TNF- $\alpha$  (Fig. 3). Chemotherapy is known to result in serious dysregulation of cytokine expression (Neta and



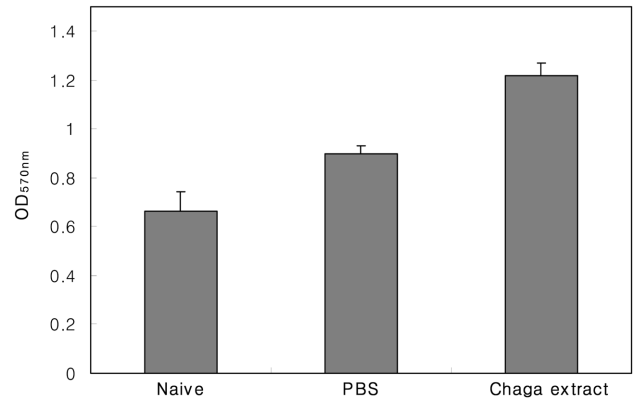
**Fig. 2.** IL-6 production in the serum of cyclophosphamide-treated mice after administration of the Chaga mushroom extract. Serum levels of IL-6 on days 8, 16, and 24 were analyzed using ELISAs for naive, PBS-, Chaga extract-treated mice. Data are expressed as means  $\pm$  SEM of three mice.



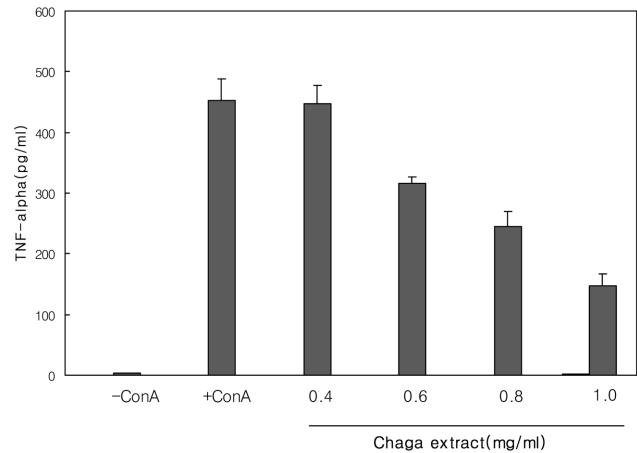
**Fig. 3.** TNF- $\alpha$  production in the serum of cyclophosphamide-treated mice after administration of the Chaga mushroom extract. Serum levels of IL-6 on days 8, 16, and 24 were analyzed using ELISAs for naive, PBS-, Chaga extract-treated mice. Data are expressed as means  $\pm$  SEM of three mice.

Oppenheim, 1988). The Chaga extract increased the levels of IL-6 over the 24-day period. This protein is well known to play an important role in the proliferation and differentiation of various immune cells. TNF- $\alpha$ , which is increased as a consequence of tissue injury and anemia due to chemical treatment (Neta *et al.*, 1992), is thought to be a key mediator for the pathogenesis of chemotherapeutic damage (Neta *et al.*, 1986). It is interesting to find that CW significantly reduced the level of TNF- $\alpha$ . All these data suggested that the CW is able to modulate the dysregulation of cytokine production in chemotherapeutic damage.

**Effects of the CW on splenocyte proliferation.** T lymphocyte function was evaluated based on Concanavalin-A



**Fig. 4.** Effects of the Chaga mushroom water extract on splenocyte proliferation induced by ConA. Data are presented as mean OD values from triplicate wells  $\pm$  standard error.



**Fig. 5.** TNF- $\alpha$  secretion in cyclophosphamide-treated mice. Splenic levels of TNF- $\alpha$  in accordance with the concentrations were analyzed using ELISAs for naive, PBS-, Chaga mushroom water extract-treated mice. Data are expressed as means  $\pm$  SEM of three mice.

induced splenocyte proliferation. The CW resulted in a significant increase in cell proliferation in treated mice when compared with control and naive animals (Fig. 4). This result clearly shows that stimulation of splenic lymphocyte proliferation is induced by Chaga extracts, thereby affecting immune function.

**Effects on the splenic TNF- $\alpha$  level by the CW.** The amount of TNF- $\alpha$  of splenocyte induced by ConA remarkably decreased by the treatment of the CW in a dose dependent manner as shown in Fig. 5. The ability of CW to modulate mitogen-driven splenocyte cell proliferation and ConA-induced TNF- $\alpha$  secretion indicates the suitability of Chaga mushroom for further investigation in relation to their molecular mechanisms of action in TNF- $\alpha$  driven immunological diseases, particularly autoimmune

diseases such as psoriasis, rheumatoid arthritis, and type 1 (autoimmune) diabetes.

In conclusion, all these results suggest that the water extract of Chaga mushroom is a very potent immune modulator that recovers the bone marrow system damaged by chemotherapy. It also suggests that the immunomodulatory activity of the water extract may be due to the potentiation of the host immune system through the regulation of cytokines in the cytokine network. Therefore, the Chaga mushroom water extract shows a great potential as a supplement or a major therapeutics in immunocompromised or immunosuppressed individuals whose bone marrow system is damaged.

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