

Immunomodulatory effects of *Agaricus blazei* Murill in Balb/cByJ mice

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Background and Purpose: *Agaricus blazei* Murill has been reported to possess biological effects that include immunomodulatory and tumoricidal activities, but its potential effects have not been systematically analyzed in vivo. We evaluated the immunomodulatory effects of *A. blazei* in Balb/cByJ mice.

Methods: 160 male Balb/cByJ mice were divided into four groups and treated with various quantities of intragastric *A. blazei* extract or distilled water for 8 to 10 weeks. Nine parameters, relating to general immune function or adaptive immunity against immunogen chicken oval albumin, were determined.

Results: The results revealed that mice receiving *A. blazei* extract exhibited significantly greater serum immunoglobulin G levels, increased T-cell numbers in spleen, and elevated phagocytic capability compared with controls. Consumption of *A. blazei* was also associated with significant increases in oval albumin-specific serum immunoglobulin G level, delayed-type hypersensitivity, splenocyte proliferation rate, and tumor necrosis factor- α secretion by splenocytes.

Conclusions: Consumption of *A. blazei* extract was associated with significant enhancement of seven out of nine immune functions tested. We conclude that *A. blazei* Murill possesses a wide range of immunomodulatory effects in vivo.

Key words: Agaricus; Inbred BALB C mice; Plant extracts; Spleen; T-lymphocytes

Introduction

The edible mushroom *Agaricus blazei* Murill is a native fungus originating from Sao Paulo in southeastern Brazil, and is now widely used as a food and medicinal tea worldwide. The reason for the popularity of this basidiomycete fungus is its claimed preventive and therapeutic effects on many diseases and illnesses, including cancer, diabetes, physical and emotional stress, hyperlipidemia, high cholesterol levels, arteriosclerosis and chronic hepatitis [1,2]. Indeed, both organic and aqueous extracts of *A. blazei* were demonstrated to contain antimutagenic protective activities [3-7], whereas the mycelial fractions of the mushroom inhibited the

cytopathic effect exerted by western equine encephalitis virus in Vero cells [8]. Many polysaccharides and proteoglycans isolated from *A. blazei* were demonstrated to possess antitumor activities [2,9-13].

To protect against infections, the human body uses both innate immunity, including physical barriers, phagocytes and soluble mediators (e.g., complements), and adaptive immunity, including antibodies and cytotoxic T cells, to destroy pathogenic invaders and also prevent reinfections. In immunologic studies, *A. blazei* has been shown to potentiate a wide range of immune activities, including the activation of the complement system through the alternative pathway in human serum [14]; the induction of macrophages to secrete tumor necrosis factor- α (TNF- α), interleukin (IL)-8, and nitric oxide [15]; the stimulation of mouse Thy1.2-, L3T4-, and Lyt2-T lymphocyte subsets to proliferate [16]; the promotion of antibody-producing

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cell production in mice [17]; and the suppression of peripheral blood mononuclear cell proliferation and IL-2, IL-4, and interferon-gamma mRNA syntheses activated by phytohemagglutinin (PHA) [18]. The former two effects are associated with innate immune function, whereas the latter three events are involved in adaptive immunity. Therefore, *A. blazei* possesses many bioactivities that may confer potential as a preventive and therapeutic agent.

Although *A. blazei* has been demonstrated to regulate certain immune activities, its immunomodulatory effects have never been systematically analyzed *in vivo*. In the present study, we evaluated the influence of *A. blazei* on the immunity of Balb/cByJ mice.

Methods

Materials and mice

The standard chemicals and reagents were obtained from Sigma (St. Louis, MO, USA), unless otherwise specified. All reagents were used according to the recommendations of the manufacturers. *Agaricus blazei* Murill was the product of Toyo Hakko Co. Ltd. (Obu, Aichi, Japan).

The procedure of preparing *A. blazei* extract began with grinding the appropriate amount of dried mushrooms, followed by hot water (80–85°C) extraction for 30 min and filtration twice to remove debris. The mushroom extract solution was then concentrated by a rotary vacuum evaporator into an extract paste (2 kg of dried mushrooms made 1 kg of paste with moisture content below 30%). Bacterial count and heavy metal analysis were routinely performed and yielded a negative detection of any microbe including *Escherichia coli* in 1 g of extract paste. The same amount of paste contained less than 20 ppm of heavy metal, which excluded lead and arsenate (data not shown).

Amino acid composition analysis revealed that 1 g of paste contained the following major amino acids (in mg): alanine, 16.0; arginine, 9.6; aspartic acid, 10.8; glycine, 8.8; histidine, 2.8; lysine, 7.6; phenylalanine, 4.4; praline, 9.2; and serine, 6.0. According to the dry weight of soluble material (Brix), the paste was diluted with double distilled water (endotoxin-free ddH₂O; Sigma) to the concentration of Brix equal to 12 (designed as the 3N concentration). At this concentration, one mL of *A. blazei* extract had the energy of 0.3 kcal and contained 47 mg protein, 12 mg fat, 2 mg carbohydrate, 0.1 mg sodium, and 15 mg ash. The prepared 3N extract was then sealed in glass bottles, sterilized at 105°C for

10 min, and stored or diluted further to the 0.45N and 0.6N solutions for feeding the mice.

A total of 160 male Balb/cByJ mice aged 3–4 weeks were purchased from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and transported to the animal facility of Chung Shan Medical University (CSMU) in Taichung, Taiwan, where the animals were further quarantined and maintained until use. The experimental procedure, including the number of animals, protocols, and schedules, was approved by the Committee of Experimental Animals of CSMU prior to acquisition of the mice.

All mice were weighed weekly as a check on health status. At the age of 7–8 weeks, the mice were randomly divided into four groups with 40 animals each (Groups A to D), and fed respectively with 1 mL of ddH₂O, 0.45N, 0.6N, or 3N extract once per day until to the end of the experiment. The feeding of *A. blazei* solutions was achieved by intragastric administration with a blunt-ended, ball tip, and curved needle connected to a 1-mL syringe. All experiments used 20 mice from each of Groups A to D at the same time, but data were collected from only 10 mice and then subjected to comparisons among different groups by *t* test. In all cases, differences with a value of $p < 0.05$ were considered significant.

General immune function testing

Determination of serum immunoglobulin G levels

To reveal the influence of *A. blazei* on serum immunoglobulin G (IgG) levels, 20 mice from each group were fed with ddH₂O or different concentrations of *A. blazei* extract for 8 weeks before blood collection (0.1–0.2 mL) by retro-orbital puncture with heparinized microhematocrit glass capillaries (Brand, Wertheim, Germany). The serum samples were then isolated and diluted 10⁴- to 10⁵-fold with phosphate-buffered saline (PBS), followed by enzyme-linked immunosorbent assay (ELISA) determination of IgG concentrations with a commercial kit (Bethyl Lab, Montgomery, TX, USA). The mean with its standard error derived from the IgG concentrations of 10 mice in each group was obtained for statistical analysis and comparisons with data from other groups.

Phagocytosis assay

Eighty mice were divided into 4 groups for feeding with ddH₂O or different concentrations of the extract. However, only 10 mice from each group were subjected to blood drawing (0.1 mL, mixed with an equal amount

of anticoagulant) before and after 8-week feeding with the mushroom extract. The whole blood sample in the test tube was mixed with 25 mL of *Staphylococcus aureus* suspension (10^8 colonies/mL), incubated at room temperature for 30 min, and blood smears were made on glass slides and stained with Liu's staining solution. For each group of mice, a total of 250 polymorphonuclear lymphocytes (PMNL) [5 PMNL per field of 5 randomly selected fields for every 10 mice] were examined under microscope ($\times 1000$, oil-immersion) and the average bacterial number phagocytosed by a PMNL was determined.

Determination of TNF- α secreted by splenocytes

TNF- α secreted from mouse splenocytes were determined. As in the IgG experiment, the same number of mice from the four groups were given water or *A. blazei* extract for 9 weeks. Their spleens were then surgically removed and washed in 1 mL of RPMI-1640 medium supplemented with 100 U/mL of penicillin and 100 μ g/mL of streptomycin (tissue culture medium and reagents were from Gibco, Grand Island, NY, USA). Splenocytes were subsequently isolated from the spleens and subjected to centrifugation on 2 mL of Ficoll-Paque Plus reagent (Amersham Biosciences, Uppsala, Sweden) at 1500 rpm for 20 min, followed by washing with 40 mL of RPMI-1640 medium, and re-suspended in 2 mL of the same medium for viable cell counting. Approximately 6×10^5 live splenocytes were stimulated with 10 μ g/mL of PHA for 72 h before ELISA determination of the TNF- α concentration (Endogen, Woburn, MA, USA). The concentration determination employed splenocyte culture supernatants that were diluted 2-fold with the medium prior to the assay.

Flow cytometric determination of various cell types in spleen

The purified splenocytes were also subjected to flow cytometric determination (FACSCalibur equipped with an argon laser and the Cell Quest software; Becton Dickinson, San Jose, CA, USA) of various cell populations. For this, Cy-Chrome-conjugated CD3, fluorescein isothiocyanate-conjugated CD19, and phycoerythrin-conjugated major histocompatibility complex (MHC) class II molecule antibodies were used (all from PharMingen Co., San Diego, CA, USA). The percentages of T-cell-, B-cell-, and MHC class II-positive antigen-presenting cells in 10^4 splenocytes were thus determined.

Adaptive immune function testing

To test whether *A. blazei* can affect adaptive immunity in Balb/cByJ mice, we injected 0.2 mL of oval albumin (OVA; Sigma-Aldrich, St Louis, MO, USA) solution (1 g in 10 mL of PBS) emulsified 1:1 (v/v) in complete Freund's adjuvant (Sigma-Aldrich) into the mouse peritoneal space after 4-week feeding with ddH₂O or various concentrations of the extract solutions. Twenty mice from each group were used. Fed continually with *A. blazei* extract, every mouse was immunized again with 0.2 mL of OVA solution (0.3 mg/mL, emulsified 1:1 in incomplete Freund's adjuvant; Sigma-Aldrich) two weeks later to boost its adaptive immunity against OVA.

Determination of OVA-specific immunoglobulin M and IgG levels in blood

Whole blood samples (0.1 to 0.2 mL) were collected by retro-orbital puncture of *A. blazei* extract-administered, OVA-immunized mice at four time points; namely, one-day before injection of OVA and 3 days, 2 weeks, and 4 weeks after booster injection of OVA. The blood samples collected from 20 mice from each group at these time points underwent serum isolation and were saved at -80°C until the immunoglobulin M (IgM)/IgG concentrations were determined. Since the purified OVA-specific IgG and IgM were not available for standard curve plotting, the titer of every serum sample was determined with ELISA assay. The titering of IgG or IgM was achieved by diluting the serum samples to the dilutions that exhibited similar ELISA readings. ELISA determination used the following protocol. Briefly, 100 μ g/mL of OVA solution was loaded in each well of the 96-well flat-bottom plate, incubated overnight at 4°C , the OVA solution washed off with PBS and loaded again with 200 μ L of BSA solution (1% in PBS), and then further incubation done for 2 h at room temperature. After washing off the BSA solution, mouse serum samples, which had been appropriately diluted (IgM, diluted 10- and 20-fold; IgG, diluted 10- to one-million fold), were added and incubated for 2.5 h, washed with PBS, and reacted with horseradish peroxidase-conjugated rabbit anti-mouse IgM or IgG antibody (both from Bethyl Lab) for 1 h at room temperature. Colorimetric quantitation was accomplished by reacting with the peroxidase substrate, followed by measurement using an ELISA reader.

Evaluation of delayed-type hypersensitivity of OVA-sensitized mice

One week after the booster injection of OVA, delayed-type hypersensitivity against the immunogen in the same

groups of mice was tested. For this, a volume of 50 μL OVA solution (10 μg in PBS) or the same volume of PBS was injected epidermally into every left or right, respectively, hind footpad of 10 animals from each group. The thickness (in mm) of the injected footpad was measured by a dial thickness gauge before and 72 h after the OVA challenge to determine the degree of swelling, as a measure of the immunogen-elicited delayed-type hypersensitivity [19].

Assay of splenocyte proliferation and TNF- α secretion induced by OVA

The same set of animals used in the previous two tests was sacrificed for spleen isolation after 10-week feeding with *A. blazei* extract. The splenocytes were purified as previously described and subsequently subjected to OVA-stimulated proliferation assay with XTT reagent (Cell Proliferation Kit, Biological Industries Co., Kibbutz Beit Haemek, Israel). Briefly, 1×10^5 splenocytes (in 100 μL) were seeded in the wells of a 96-well plate, OVA was then added (final concentration, 500 $\mu\text{g}/\text{mL}$) or mock-treated, and the mixture incubated at 37°C under 5% carbon dioxide for 3 days and then reacted with 50 μL of XTT solution. The supernatants of the fast-proliferating cells, presumably OVA-specific spleen T cells, showed darker blue color which could be quantified by an ELISA reader (optical density absorption at 450 nm). The isolated splenocytes were also tested for the influence of *A. blazei* on TNF- α secretion induced by OVA. In a well of the 24-well culture plate, 6×10^5 splenocytes were maintained in 600 μL of RPMI-1640 medium with or without 500 $\mu\text{g}/\text{mL}$ of OVA added, and the supernatant was removed for TNF- α concentration determination by ELISA procedure as in the previous protocol) after 72 h incubation at 37°C and under 5% carbon dioxide.

Results

In the present study, several experiments were designed to investigate the influence of *A. blazei* Murill on the immunity of the Balb/cByJ mouse. We sought to determine whether certain fundamental nonspecific immune reactions and also (OVA-induced) adaptive immune functions in mice ingesting the mushroom extract were different from those in mice fed only with ddH₂O.

General immune function testing

Feeding mice with *A. blazei* extract for 8 weeks (Groups B to D) increased the levels of serum IgG significantly

compared with mice fed only with ddH₂O (Group A), with Group C mice showing the highest average IgG level (Fig. 1). The same groups of animals were continuously given ddH₂O or various concentration of the extract for an additional week before the phagocytic assay was performed. In control mice, on average 5.7 *S. aureus* cells were engulfed by a PMNL (data not shown). After 8 weeks, in samples from 10 ddH₂O- or *A. blazei* extract-fed animals the mean number of bacterial cells phagocytosed by a PMNL was 7.1, 10.3, 14.2, and 18.1 in Groups of A to D, respectively (data not shown). Fig. 2 shows the increases calculated from separately dividing the average bacterial cell numbers engulfed by a PMNL of the 10 animals in Groups A to D by the average cell numbers from the 10 mice in the Control Group. There was a significant, dose-dependent increase in phagocytosis with increasing doses of *A. blazei* extract (Groups B to D).

Levels of TNF- α released from splenocytes on PHA stimulation in mice fed with *A. blazei* extract for 9 weeks were slightly elevated (Fig. 3), but not significantly so compared with controls. FACSscan results (Fig. 4) showed that the CD3⁺ T-cell populations, but not the CD19⁺ B-cells or MHC class II-positive antigen-presenting cells, were significantly increased in Groups B to D compared with controls.

In summary, we detected that consuming *A. blazei* for 8 to 9 weeks improved general immune function in mice as evidenced by increases in serum IgG levels, improved PMNL phagocytic capability, and greater splenic T-cell production.

Adaptive immune function testing

In order to investigate the influence of *A. blazei* on adaptive immunity, we immunized Balb/cByJ mice with

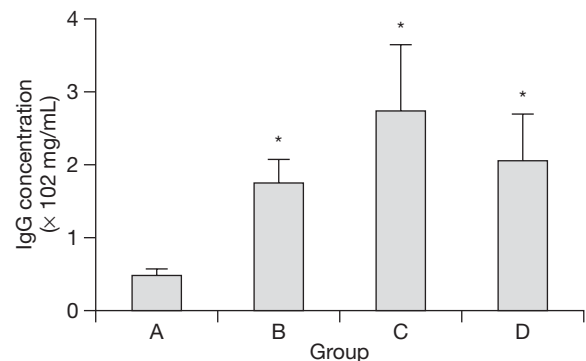


Fig. 1. Effect of treatment with *Agaricus blazei* extract on mean serum immunoglobulin G (IgG) levels in mice (n = 10 per group). * $p < 0.05$ vs control (Group A) [t test].

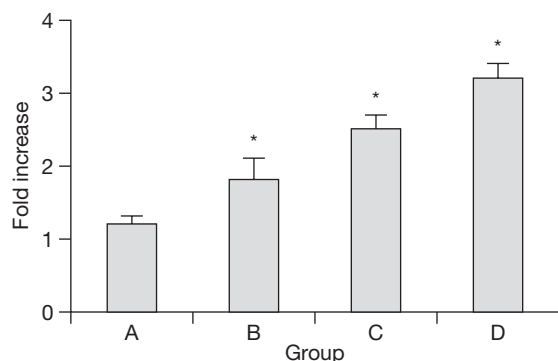


Fig. 2. Effect of treatment with *Agaricus blazei* extract on phagocytotic activity in mice. * $p < 0.01$ vs control (Group A) [t test].

a common chicken protein OVA. The results showed no difference in levels of serum IgM against OVA at 17, 28, or 42 days after sensitization with the immunogen among different groups of mice fed with ddH₂O or various concentrations of *A. blazei* extract (data not shown). Unlike IgM, the average OVA-specific IgG titer in mice fed with 0.6N (Group C, the highest) or 3N extract (Group D, the second highest) was significantly higher than that of the control group (Group A) at 28 days post-immunization of mice with the protein (Fig. 5). OVA-elicited delayed-type hypersensitivity also revealed significant differences in mice fed with *A. blazei* extract vs controls in the mean thickness of mouse footpad (Fig. 6). Epidermal injection of control solution (PBS) into the right hind footpads of mice did not cause any swelling at the site (data not shown), suggesting that mouse delayed-type hypersensitivity elicited by OVA could be modulated by *A. blazei*.

In OVA-immunized mice fed *A. blazei* extract for 10 weeks, OVA-specific splenocytes, presumably T-cell

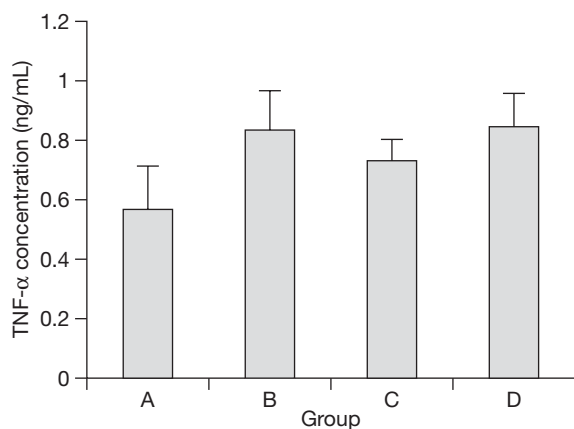


Fig. 3. Effect of treatment with *Agaricus blazei* extract on tumor necrosis factor- α (TNF- α) secretion by stimulated murine splenocytes.

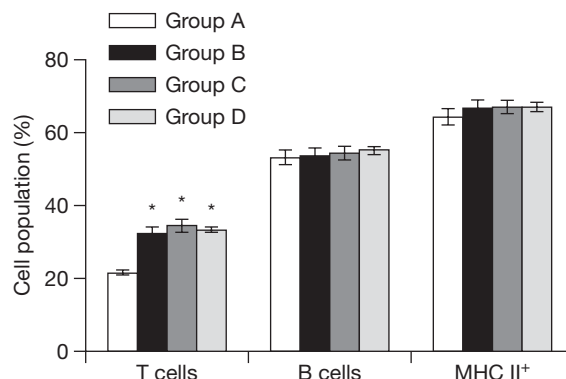


Fig. 4. Populations of T cells (CD3⁺ cells), B cells (CD19⁺ cells), and antigen-presenting cells (MHC class II-positive cells) in spleens of mice treated with *Agaricus blazei*. * $p < 0.01$ vs control (Group A) [t test].

populations, were stimulated, with those animals (Group C) fed with 0.6N extract reaching a significantly higher level compared with controls (Fig. 7). Moreover, all splenocyte samples isolated from mice in Groups B, C, and D had significant increases in TNF- α secretion upon stimulation with OVA, with Group C mice having the highest levels (Fig. 8).

Discussion

A. blazei is an edible mushroom that is believed to possess immune-enhancing properties, although no relevant experimental evidence was previously available. Since it has been widely used in foods and medicinal teas worldwide as well as in Taiwan, we tested its immunomodulatory capacity according to the guideline and protocol published in the Health Food Control Act of the Taiwanese Department of Health, ROC (available

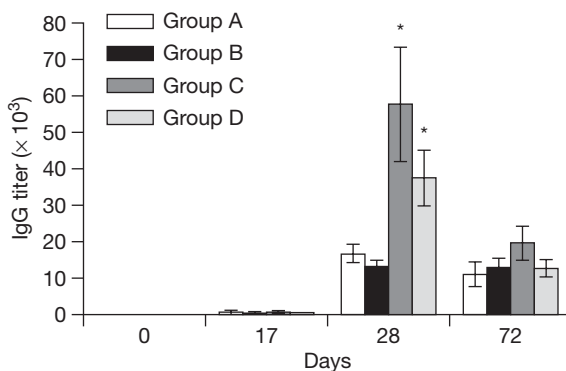


Fig. 5. Effect of treatment with *Agaricus blazei* extract on oval albumin (OVA)-specific immunoglobulin G (IgG) levels in mice (mean and standard error of 3 determinations). * $p < 0.05$ vs control (Group A) [t test].

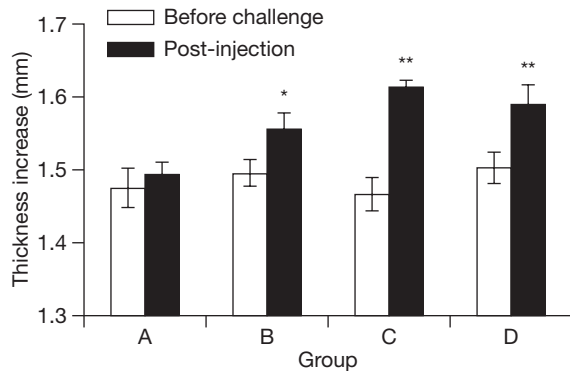


Fig. 6. Effect of treatment with *Agaricus blazei* extract on delayed-type hypersensitivity in oval albumin-immunized mice (mean and standard deviation of 10 measurements). * $p < 0.05$, ** $p < 0.01$ vs control (Group A) [t test].

from: www.doh.gov.tw/cht/List.aspx?show=show&goon=1&dept=L). In the previous pilot study, testing of the safety of the 3N *A. blazei* extract in 105 animals found no evidence of harm after intragastric administration daily for a period of 8 weeks. Similarly, the same concentration of *A. blazei* extract has been used to feed or inject mice without causing toxicity to the animals (Yoshikazu Inoue, personal communication).

The present study demonstrates that innate immunity is greatly improved in mice administered *A. blazei* extract, as evidenced by significant increases in serum IgG levels, PMNL phagocytic capability, and spleen T-cell populations compared with control animals. *A. blazei* also modulated adaptive immune functions in mice, as shown by significant elevation of immunogen (OVA)-specific IgG titers (humoral immunity) and effects on cellular immunity, which included stronger delayed-type hypersensitivity, higher splenocyte (presumably T-cell) proliferation rates, and increased OVA-stimulated TNF- α secretion by

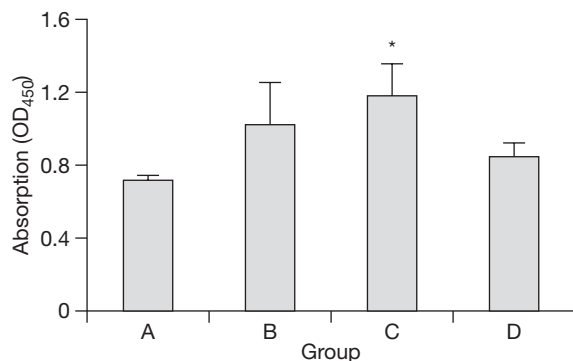


Fig. 7. Splenocyte proliferation assay in oval albumin-immunized mice (mean and standard deviation of 3 experiments). * $p < 0.05$ vs control (Group A) [t test].

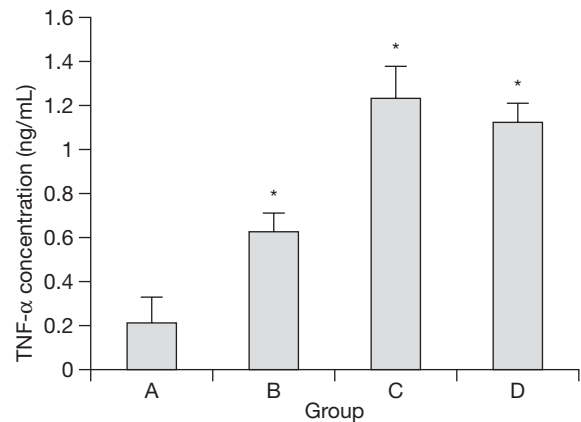


Fig. 8. Effect of treatment with *Agaricus blazei* extract on tumor necrosis factor-alpha (TNF- α) secreted by oval albumin-stimulated murine splenocytes (mean and standard deviation of 3 assays). * $p < 0.01$ vs control (Group A) [t test].

splenocytes. It is known that delayed-type hypersensitivity is the result of macrophage-mediated tissue inflammatory response driven by T-helper Type 1 (Th1) cells [20]. Therefore, it is noteworthy that the elevated levels of OVA-induced TNF- α (Fig. 8), a pro-inflammatory cytokine produced mainly by Th1 cells, correlated with the degrees of mouse footpad swelling (caused by OVA-elicited delayed-type hypersensitivity) in mice fed with *A. blazei* extract (Fig. 6). Since pro-inflammatory cytokines have been shown to enhance phagocytosis of *S. aureus* [21] and *Mycobacterium tuberculosis* [22] by PMNL, it is also possible that the greater phagocytic capability in *A. blazei* extract-ingested mice (Fig. 2) is due to the fact that the mushroom stimulates TNF- α secretion in vivo (Fig. 3 and Fig. 8).

Results for 5 of the increased innate and adaptive immune parameters (Fig. 1, Fig. 5, Fig. 6, Fig. 7, and Fig. 8) indicated that the greatest effect was achieved by feeding mice with 1 mL of 0.6N *A. blazei* extract daily (Group C), with no further increase at the higher dose. However, it is inconsistent that the increased serum IgG levels (Fig. 1) were not associated with elevated B-cell proliferation rates (Fig. 4) in mice fed with *A. blazei*. This may be explained by an increased efficiency of antibody secretion by B cells in the *A. blazei*-fed mice. This hypothesis seems to be consistent with the previous study [17], in which *A. blazei* extract promoted antibody production but failed to increase CD19⁺ cells in mice. Furthermore, the populations of B cells, T cells, and MHC class II-positive antigen-presenting cells were determined only in the spleen, and not in other lymphoid organs, and the cell number changes in this organ might

not correlate with the changes in the systemic concentrations of soluble factors such as antibodies. This might be the reason for detecting significant increases in total IgG (Fig. 1 and Fig. 5) and spleen T-cell proliferation rate (Fig. 7), without increases in MHC class II-positive splenocytes to present antigens to B and T cells, respectively, in mice treated with *A. blazei* extract (Fig. 4).

We therefore conclude that *A. blazei* significantly increased: 1) serum IgG levels; 2) T-cell populations; and 3) PMNL phagocytic activity in mice. *A. blazei* was also associated with significantly increased levels of OVA-specific serum IgG, delayed-type hypersensitivity, splenocyte proliferation, and TNF- α secretion by splenocytes. Of the nine specific items relating to general and adaptive immunities we tested, seven parameters were significantly enhanced in mice fed with *A. blazei* extract compared with control animals, suggesting that the mushroom does possess the overall capability of modulating immune function in the animals tested. These benefits might be replicated in humans taking sufficient *A. blazei* for an appropriate period of time. However, the clinical immunomodulatory potential of *A. blazei* requires further study.

Acknowledgments

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