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Extract of Medicinal Mushroom *Agaricus blazei* Murill Enhances the Non-specific and Adaptive Immune Activities in BALB/c Mice

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Abstract. *Agaricus blazei Murill (AbM) is traditionally used against a wide range of conditions such as ulcerative colitis, Crohn's disease, foot-and-mouth disease and chronic hepatitis C infection. In this study, we evaluated the immunomodulatory effects of AbM. For the non-specific immune response experiments, a total of 40 female BALB/c mice were divided into control (group 1) and experimental (groups 2-4) groups of 10 animals each. Groups 2, 3 and 4 were orally-administered high (819 mg/kg), medium (273 mg/kg) and low (136.5 mg/kg) doses of AbM daily for six weeks and then six parameters related to non-specific immune response were detected. For the adaptive immune response experiments, 40 female mice were similarly divided into four groups. After six weeks of treatment, animals were immunized with the OVA immunogen. Two weeks later, splenocytes and sera were collected. Four parameters related to adaptive immune response were evaluated. We found that feeding mice with AbM extract increased the IgG level in serum, promoted phagocytosis of peritoneal macrophages and elevated the activity of Natural killer cells. We also found that the highest dose of AbM increased interleukin-2 (IL-2) levels in splenocytes and that a medium dose increased interferon-γ. The levels of interleukin-4 (IL-4) were reduced or unchanged. T-helper type 1 cytokine levels were increased. AbM increased the humoral immune response and also affected the cellular immune response. These results provide evidence that AbM can modulate innate and adaptive immunity.*

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The practice of complementary and alternative medicine (CAM) is increasing in industrialized nations (1-9). Studies on cost effectiveness indicate that CAM therapy can potentially contribute to a reduction in medical care costs (10, 11). An increasing number of hospitals in industrialized countries have begun to integrate CAM practice into patient care. For example, in the USA, more than 37% of hospitals introduced one or more CAM therapies in 2008 (12). There is an intense effort to identify natural compounds which may have therapeutic potential. Due to their rich composition, including minerals, essential amino acids, vitamins and fibers, mushrooms have been used as an important nutritional food and form of therapy worldwide, especially in Asian countries, since ancient times (13, 14). Their use in the Western hemisphere has been slightly increasing over the past few

decades (15, 16). One type of mushroom that has received attention is *Agaricus blazei* Murrill (AbM) known in Brazil as Cogumelo do sol or medicinel, in Japan as Himematsutake, Agarikusutake or Kawarihiratake, and in China as Ji Song Rong (11, 17). It is widely used today in Asian countries both as an edible mushroom, considered a functional food, and as natural therapy in the form of a medicinal extract, mostly for prevention and treatment of atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis and cancer (18).

Mushroom constituents such as ergosterol, derived from an acid-treated fraction, may inhibit promotion or progression of tumors by exerting direct cytotoxicity against tumor cells, interfering with tumor angiogenesis, or up-regulating other non-immune tumor-supressive mechanisms (19, 20). Whole-mushroom extracts contain compounds that may modulate tumorigenesis at different stages and/or may act at the same stage but through different mechanisms. Thus, such compounds could potentially provide additive, or even synergistic, effects in the prevention and treatment of cancer (21).

A few researchers revealed that AbM has anti-mutagenic properties but the biological pathways and chemical substances involved in its pharmacological activities are still unknown. The purpose of this study was to take a closer look at and provide further insight into specific and non-specific immune response activated by *Agaricus blazei* Murrill.

Materials and Methods

Reagents and chemicals. Reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Enzyme-linked immunosorbent assay (ELISA) kits for cytokine measurements were purchased from BD Biosciences (San Diego, CA, USA). Fetal bovine serum (FBS) and cell culture supplies were purchased from Hyclone (Logan, UT, USA).

Preparation of AbM. AbM powder obtained from Chang Gung Biotechnology Corporation, Ltd. (Taipei, Taiwan, R.O.C.) was thoroughly mixed with distilled water at 60˚C for 10 min, then cooled to room temperature and left for 5 h with stirring at 200×*g* to form solutions of low (136.5 mg/kg), medium (273 mg/kg) and high (819 mg/kg) concentrations which were 1-, 2- and 6-fold that of human daily intake, respectively. The AbM supernatant solution was filtered and freeze-dried. This AbM was stored at –50˚C until use (18).

Animals and treatment doses. A total of 40 female BALB/c mice of six weeks of age were used. Animals were housed singly in cages. Mice were maintained under standard pathogen-free conditions with a 12-hour light/dark cycle at a temperature and relative humidity range of 20±2˚C and 75±15%, respectively. They were fed orally with Laboratory Rodent Diet 5001 manufactured by PMI Nutrition International (St. Louis, MO, USA) throughout the study. Animals used in the present study were maintained in accordance with the guidelines approved by the National Science Council of the Republic of China and the Committee for the Purpose of Control and Supervision of Experiments on Animals. Experiments were performed according to law, regulations and guidelines for animal experiments in Taiwan, which are in agreement with the declaration of Helsinki. For the non-specific immune response experiments, female mice were randomized and divided into control (group 1) and experimental (groups 2-4) of 10 animals per group. Groups 2, 3 and 4 were orally-administered high, medium and low doses of AbM daily by oral gavage for six weeks. Control mice received distilled water (vehicle) by oral gavage. All mice were examined weekly as a check on health status. For the adaptive immune response experiments, 40 female mice of six weeks of age were similarly divided into four groups, each consisting of ten mice. Groups 2, 3 and 4 were administered by oral gavage high, medium and low doses of AbM, daily for six weeks as described above. After six weeks of treatment, animals were immunized by intraperitoneally injections with 2 μg of OVA w/w Complete Freunds' Adjuvant (Sigma-Aldrich, St. Louis, MO, USA). The boosting injection (6 μg of OVA w/w incomplete Freunds' Adjuvant) was given two weeks later. Splenocytes and sera were collected two weeks after the secondary immunization for measurement of OVA-specific antibody.

Determination of serum immunoglobulin G and M levels for non-specific immune response. Effects of AbM on serum IgG and IgM levels were determined in 10 mice from each group receiving distilled water or different dose treatments of AbM orally for six weeks, as described above. Whole blood was collected *via* orbital puncture with heparinized microhematocrit glass capillaries at the beginning, the end of the third week and the end of the experiments (the sixth week). The samples were diluted with PBS and IgG and IgM levels were quantified by an ELISA kit (Bethyl Lab, Montgomery, TX, USA).

Preparation of splenocytes. Murine splenocytes were prepared as previously described (22) with minor modifications. Mice were sacrificed and spleens were surgically removed and minced at room temperature in RPMI-1640 medium. The suspension was filtered through a nylon mesh and centrifuged at 1500 ×*g* for 5 min. Erythrocytes were then lysed by the addition of ACK buffer (150 mM NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2). The splenocytes were grown in RPMI-1640 medium supplemented with 10 mM HEPES (pH 7.0), non-essential amino acids, 50 μM β-mercaptoethanol, 0.03% L-glutamine, 50 μg/ml gentamycin and 10% heat-inactivated (56˚C, 30 min) fetal bovine serum. A splenocyte suspension at 5×10^6 cells/ml was then prepared.

ELISA assays for quantitative determination of cytokines for non-specific immune response. Approximately 2×10^6 cells/ml splenocytes isolated from the spleen of control and AbB treated mice were incubated with Concanavalin A (Con A) (final concentration 5 μg/ml) in 24-well culture plates at 37˚C in 5% CO_2. After 48 h, plates were centrifuged at 1400×*g* for 5 min and supernatants were collected for the determination of INF-γ, IL-2, IL-4 levels using an ELISA kit (Rapidbio Lab., West Hills, CA, USA) according to the manufacturer's instructions. The optical density was measured using an ELISA reader with a wavelength of 450 nm.

Splenocyte proliferation assay for non-specific immune response. Following six weeks of treatments, spleens were collected from mice under aseptic conditions in Hank's balanced salt solution (HBSS; Sigma), minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension, and the erythrocytes were then lysed with ammonium chloride (0.8%, w/v).

After centrifugation (380×*g* at 4˚C for 10 min), the pelleted cells were washed three times in PBS, and resuspended in complete medium. Cell numbers were counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as previously described (23). Briefly, splenocytes from each mouse were seeded into four wells of a 96-well flat-bottom microtiter plate at 5×10^6 cell/ml in 100 µl of complete medium. Con A (final concentration 5 µg/ml) or lipopolysaccharide (LPS; final concentration 10 µg/ml) were added, and then medium was added, to a final volume of 200 µl. The plates were incubated at 37˚C in a humid atmosphere with 5% CO_2. After 44 h, 50 µl of 3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (2 mg/ml), Promega's CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit, was added to each well and incubated for 4 h. The plates were centrifuged (1400×*g*, 5 min) and the untransformed MTT was removed carefully by pipetting. Subsequently, 150 µl of a DMSO working solution (192 µl DMSO with 8 µl 1 N HCl) was added to each well, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min. The stimulation index (SI) was calculated based on the following formula: SI=the absorbance value for mitogen-stimulated-cultures divided by the absorbance value for non-stimulated cultures.

Natural killer (NK) cell cytotoxicity assay. Approximately 1×10^7 splenocytes/ml were isolated from the spleen of control and BALB/c mice administered AbM. YAC-1 cells (1×10^5 cells/ml) in 15-ml tubes were washed twice with serum-free RPMI-1640 medium then PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) was added to the cells, which were mixed thoroughly for 2 min at 25˚C before adding phosphate-buffered saline (PBS). After 1 min, 4 ml RPMI-1640 medium were added and the cells were centrifuged for 10 min at 1200 ×*g* and 25˚C. Approximately 1×10^5 YAC-1 cells were placed onto 96-well plates in 100 µl medium then 1×10^7 cells/ml (100:1), 5×10^6 (50:1) and 2.5×10^6 (25:1) splenocytes were added to each well and mixed with 3,3'-Dioctadecyloxacarbocyanine perchlorate (DiOC18)-labeled target cells at effector-to-target (E:T) ratios of 100:1, 50:1, and 25:1. Propidium iodide (PI) was added to the culture medium to allow for the determination of YAC-1 cell death after 3 h incubation at 37˚C in 5% CO_2. Determination of the NK cell cytotoxicity after 12 h was measured using the LIVE/DEAD® cell-mediated cytotoxicity kit for animal cells (Molecular Probes, Carlsbad, CA, USA) (24). The percent age cytotoxicity was assessed by flow cytometric analysis. Lysed (PI^+, $DiOC18^+$) and viable ($DiOC18^+$ and PI^-) YAC-1 cells were identified by their dual- or single-positive staining. Total cytotoxicity was determined as: [%experimental cytotoxicity] – [%background cytotoxicity in control YAC-1 cultures].

Phagocytosis assay of peritoneal macrophages for non-specific immune response. Peritoneal macrophages were isolated from mice at the end of six weeks of AbM treatment. Briefly, the mouse peritoneal cavity was carefully exposed without disrupting blood vessels and 2 to 3 ml of RPMI was slowly injected. The lavage was sucked back and cultured in tissue culture flasks for 2 h at 37˚C under 5% CO_2 to allow adherence of macrophages. The non-adherent cells were removed and flasks were washed three times with HBSS. The adherent cells were harvested from the flask using a rubber policeman and were resuspended in 10% fetal calf serum (FCS)-RPMI-1640 medium. Approximately 1×10^5 cells/well were added to 50 µl of RPMI-1640 medium supplemented with 50 µg gentamicin sulfate, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum, then mixed with 50 µl of *Escherichia coli*-Fluorescein isothiocyanate (FITC), according to the manufacturer's instructions (PHAGOTEST kit; ORPEGEN Pharma Gesellschaft fur biotechnologische, Heidelberg, Germany) and shaken in a shaker bath for 30 min at 37˚C. Cells then were centrifuged at 1500 ×*g* for 5 min. Flow cytometric analysis was conducted for each sample (FACS Calibur; Becton Dickinson, USA). Fluorescence data were collected on 10^5 cells and analyzed as described previously (25, 26).

Flow cytometric determination of various cell types in spleen for non-specific immune response. Leukocytes from spleen of AbM-treated mice and normal control groups were isolated and processed for immunofluorescence (IF) staining of cell surface antigens for flow cytometric analysis. Briefly, single-cell suspensions of harvested leukocytes were prepared at 2×10^7 cells/ml in staining buffer (10% FCS in PBS) and pre-incubated with 1 µg of the 2.4 G2 antibody for 5 to 10 min on ice prior to staining. Fifty microliters of cell suspension (equal to 10^6 cells) were dispensed into each tube along with a previously determined optimal concentration of cell surface-specific antibody in 50 µl of staining buffer. Each test tube was mixed gently and incubated for 30 min in the dark in an ice bath. Cells were stained with fluorochrome-labeled antibodies of the appropriate isotype control for non-specific binding. Cells stained in the absence of primary antibodies served as negative controls. After the incubation period, cells were washed by adding 2 ml of staining buffer, followed by centrifugation for 7 min (at 300 to 400×*g*) at 4˚C. The washing was repeated twice. Cell pellets were suspended in 500 µl of staining buffer and analyzed on a BD LSR II flow cytometer (BD Biosciences).

Measurement of OVA-specific IgG, IgM and IgE. Whole-blood samples (0.1 to 0.2 ml) were collected by retro-orbital puncture of immunized mice at four weeks after booster injection of OVA. OVA-specific IgG, IgM and IgE antibodies in sera were detected by an indirect ELISA. Microtiter plate wells (Nunc, USA) were coated with 100 µl OVA solution (25 µg/ml in 50 mM carbonate–bicarbonate buffer, pH 9.6) for 24 h at 4˚C. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween) and then blocked with 5% FCS/PBS at 37˚C for 2 h. After three washings, 100 µl of a series of diluted sera samples (initial dilution 1:50) or 0.5% FCS/PBS as control were added to wells. The plates were then incubated for 2 h at 37˚C, and then washed three times. Aliquots of 100 µl of rabbit horseradish peroxidase conjugated anti-mouse IgG, IgM or IgE diluted 1:10000, were added to each plate. The plates were further incubated for 2 h at 37˚C. After washing, the peroxidase activity was assayed as follows: 100 µl of substrate solution (10 mg of *O*-phenylenediamine and 37.5 µl of 30% H_2O_2 in 25 ml of 0.1 M citrate–phosphate buffer, pH 5.0) was added to each well. The plate was incubated for 10 min at 37˚C, and the enzyme reaction was terminated by adding 50 µl/well of 2 N H_2SO_4. The optical density was measured in an ELISA reader at 490 nm, and where sets of sera samples were subjected to within and between group comparisons, ELISA assays were performed on the same day for all of the samples.

Splenocyte proliferation assay for adaptive immune response. Spleens were collected from the OVA-immunized mice. All the protocols were the same for proliferation as the non-specific response procedures described above.

Cytokine analysis of cultured supernatants of splenocytes for determination of the adaptive immune response. Splenocytes (2×10^6

Table I. *Effect of treatment with Agaricus blazei Murill (AbM) extract on mean serum immunoglobulin G and M levels in mice.*

Groups (n=10)	IgG (g/ml)		IgM (g/ml)	
	Before experiment	After treatment	Before experiment	After treatment
Normal control	303.31±44.62	1083.66±62.95	156.73±46.29	171.55±74.71
Low dose	310.84±76.48	1160.21±34.13[a]	161.02±26.18	152.33±69.59
Medium dose	351.70±62.85	1251.89±30.59[ab]	177.17±97.30	128.03±36.84
High dose	304.81±74.09	1295.88±74.70[ab]	133.92±32.84	152.79±47.86

[a]$p<0.05$ *vs.* normal control group; [b]$p<0.05$ *vs.* low-dose treatment group.

cells/well) from immunized mice prepared as described above were incubated with Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml) or OVA (final concentration 30 μg/ml), in 24-well culture plates at 37˚C in 5% CO_2. After 48 h, the plates were centrifuged at 1400 ×*g* for 5 min and culture supernatants were collected for the determination of INF-γ, IL-2, IL-4 levels. The presence of INF-γ, IL-2, IL-4 in the cultured supernatants of splenocytes were determined using a mouse ELISA kit (Rapidbio Lab.) according to the manufacturer's instructions. The optical density was measured using an ELISA reader with wavelength of 450 nm.

Flow cytometric analysis of various cell types in spleen for determination of adaptive immune response. Leukocytes from spleens of AbM-treated mice and normal control groups were isolated and processed for IF staining of cell surface antigens for flow cytometric analysis. The methods were the same for flow cytometric analysis of various cell types in spleen tissue for determination of non-specific immune response as discussed above.

Statistical analysis. Statistical analysis for comparison between two groups was performed using the Student's *t*-test. Data are expressed as the mean±standard deviations. Values of $p<0.05$ were considered as being significant.

Results

In this study, we designed several experiments to investigate the influence of AbM on the immunity of the mice. We tried to determine whether certain fundamental non-specific immune reactions and also OVA-induced adaptive immune functions in mice ingesting the mushroom extract were different from those of mice fed only with ddH_2O.

Serum immunoglobulin G and M levels of indicators of the non-specific immune response. Feeding mice with AbM extract for six weeks increased serum IgG levels significantly compared with those fed only ddH_2O. The IgG levels were dependent on treatment doses (Table I). After treatment, the IgG levels of the high-dose group reached 4.26-fold that before the experiment. Before the experiment, the IgM levels were no different among all the treatment groups (Table I). Feeding mice with AbM extract for six weeks did not increase serum IgM levels significantly compared with those fed only ddH_2O.

Table II. *The level of serum cytokine in mice stimulated by Concanavalin A (ConA) and fed with three different doses of Agaricus blazei Murill (AbM).*

Group (n=10)	IL-2 (pg/ml)	IL-4 (pg/ml)	IFN-γ (pg/ml)
Normal control	2685.27±827.13	223.45±47.97	3025.09±1196.97
Low dose	3139.67±765.21	195.89±38.19	4045.56±1371.61
Medium dose	3570.35±1344.51	171.05±37.96[a]	4856.47±1723.45[a]
High dose	3961.91±954.13[a]	173.89±27.99[a]	4140.46±1299.44

[a]$p<0.05$ *vs.* normal control group.

Effects of AbM on cytokine levels in the non-specific immune response. IL-2 levels did not differ among all treatment groups and the control group except IL-2 was elevated at the high AbM dose when stimulated by ConA ($p<0.05$) (Table II). IL-4 levels on the other hand were significantly reduced by AbM at medium and high doses as compared with control mice (Table II). The amount of IFN-γ was significantly elevated in splenocytes at the medium AbM dose when compared with the control group (Table II).

Effects of splenocyte proliferation in the non-specific immune response. The three different AbM doses significantly increased splenocyte proliferation is response to ConA stimulation (Table III). There were no differences in proliferation among the three different dose treatments of AbM. With the exception of the medium dose, treatment with AbM significantly increased in response to splenocyte proliferation LPS stimulation (Table III).

Effects of AbM on NK cell cytotoxicity. To investigate whether AbM acts on NK cell activity, splenocytes from AbM-treated and untreated (control) groups were isolated and NK cell activity was determined. Data in Table IV indicate that at a target cell ratio of 25:1, YAC-1 cells were targeted by NK cells only after intragastric treatment with medium AbM dose. The three different treatment doses were all effective at a target ratio of 50:1 and 100:1, but these effects were not dose-dependent (Table IV).

Table III. *Splenocyte proliferation of mice fed with different doses of Agaricus blazei Murill (AbM).*

Group (n=10)	Con A (SI)	LPS (SI)
Normal control	2.03±0.26	2.51±0.24
Low dose	2.27±0.21[a]	2.83±0.32[a]
Medium dose	2.35±0.26[a]	2.78±0.36
High dose	2.40±0.37[a]	2.92±0.44[a]

SI: Stimulation index. [a]$p<0.05$ *vs*. normal control group.

Table IV. *Effect of Agaricus blazei Murill (AbM) therapy on splenic Natural killer (NK) cytotoxic activity (%) in BALB/c mice.*

Group (n=10)	E/T ratio		
	25.0	50.0	100.0
Normal control	7.46±3.11	21.15±3.71	40.20±3.67
Low dose	8.36±3.82	25.87±3.55[a]	55.70±14.03[a]
Medium dose	11.08±2.99[a]	25.60±2.89[a]	49.60±5.26a
High dose	9.24±3.63	25.52±2.45[a]	49.52±5.31[a]

E/T ratio: Effector-to-target ratio; [a]$p<0.05$ *vs*. normal control group.

Table V. *Phagocytosis of Fluorescein isothiocyanate (FITC)-labeled Escherichia coli with increasing doses of Agaricus blazei Murill (AbM) at Multiplicity of infection (MOI)=1, 5 or 25. AbM did not show a concentration-dependent phagocytosis.*

Group (n=10)	MOI=1	MOI=5	MOI=25
Normal control	1.18±0.93	7.30±2.65	21.25±6.21
Low dose	1.18±0.64	14.14±3.88[a]	31.10±9.81[a]
Medium dose	2.78±1.34[a]	15.84±5.75[a]	35.93±15.14[a]
High dose	1.48±0.53	16.27±7.91[a]	33.63±9.32[a]

[a]$p<0.05$ *vs*. normal control group.

Table VI. *Effect of Agaricus blazei Murill (AbM) treatments on T- and B-lymphocytes (T-helper and T-cytotoxic) subpopulation in BALB/c mice.*

Group (n=10)	CD4/CD3	CD8/CD3	CD3/CD45	CD19/CD45
Normal control	31.13±1.72	16.40±2.06	51.54±3.83	43.29±3.69
Low dose	31.98±1.94	16.99±1.30	53.77±4.45	41.85±4.41
Medium dose	31.22±2.66	16.28±2.39	53.63±5.40	41.97±5.44
High dose	31.31±1.36	16.38±1.73	52.12±4.79	43.64±4.41

[a]$p<0.05$ *vs*. normal control group.

Effects of AbM on phagocytosis of peritoneal macrophages. After six weeks, in samples from control, low, medium or high dose treatment groups, the mean number of phagocytosed bacterial cells with detection through flow cytometry was on average 5.3, 7.8, 9.0 and 8.4 *Escherichia coli* cells individually at multiplicity of infection (MOI) of 25. AbM induced phagocytosis activation. There was no significant dose-dependent increase in phagocytosis with increasing dose of AbM. At an MOI of 5 or more, all doses of AbM induced increased phagocytosis (Table V).

Effects of AbM on cell type distribution in spleen. BALB/c mice were administered AbM intragastrically (136.5 mg/kg, 273 mg/kg or 819 mg/kg) or physiological saline for six weeks. FACSccan results (Table VI) showed that T- and B-cell percentages were not significantly affected by AbM nor was the CD4/CD8 ratio.

Effects of AbM on OVA-specific IgG, IgM and IgE. The OVA-specific IgG, IgM and IgE antibody levels in the serum were measured by indirect ELISA method two weeks after the last immunization. The serum IgG levels in mice immunized with OVA were significantly enhanced by low, medium and high doses of AbM when compared with the unimmunized control or immunized control groups (Table VII) ($p<0.05$), without significant difference between doses. OVA-specific IgM levels were significantly elevated only in mice treated by low doses of AbM compared with the immunized control group ($p<0.05$). There were no significant differences between the effects of high or medium AbM doses (Table VII) ($p>0.05$). Data in Table VII indicate that AbM did not alter IgE levels.

Effects of AbM on splenocyte proliferation and adaptive immune response. The effects of different doses of AbM on splenocyte proliferative response to ConA, LPS and OVA stimulation are shown in Table VIII. ConA stimulation, and three different doses of AbM treatment induced significant splenocyte proliferation as compared with control groups. Effects of AbM were not dose-dependent. AbM was only effective at the low and medium doses at increasing splenocyte proliferation when incubated with LPS. AbM did not alter the effects of OVA on splenocyte proliferation.

Effects of AbM on cytokine levels of the cultured supernatant of splenocytes and the adaptive immune response. Splenocytes from immunized mice after exposure to different doses of AbM extract were stimulated with Con A and levels of IL-2, IL-4 and IFN-γ in supernatant from splenocytes incubated with Con A, LPS or OVA from AbM-treated mice were evaluated. Low and medium doses of AbM increased IL-2 levels in the presence of Con A as compared with the control groups. Effects were not dose-dependent (Table IX). AbM did not alter effects of LPS on IL-2 (Table IX). Effects of AbM

Table VII. *Effect of treatment with Agaricus blazei Murill (AbM) extract on mean serum immunoglobulin G, M and E levels in immunized mice.*

Group (n=10)	IgG	IgM	IgE
Normal control, unimmunized	0.034±0.004	0.032±0.004	0.013±0.003
Normal control, immunized	0.468±0.056[a]	0.309±0.110[a]	0.071±0.042[a]
Low dose	0.630±0.150[ab]	0.491±0.181[ab]	0.051±0.034[a]
Medium dose	0.661±0.126[ab]	0.406±0.139[a]	0.056±0.023[a]
High dose	0.627±0.123[ab]	0.417±0.146[a]	0.051±0.028[a]

[a]$p<0.05$ *vs.* normal control, unimmunized; [b]$p<0.05$ *vs.* normal control, immunized.

Table VIII. *Splenocyte proliferation of immunized mice fed with different doses of Agaricus blazei Murill (AbM).*

	SI		
Group (n=10)	ConA 5.0 μg/ml	LPS 10.0 μg/ml	OVA 25.0 μg/ml
Normal control w/o immunized	1.53±0.15	2.06±0.33	0.81±0.06
Normal control w/w immunized	1.50±0.25	1.76±0.26[a]	1.04±0.12[a]
Low dose	2.09±0.27[ab]	2.40±0.58[b]	1.13±0.23[a]
Medium dose	1.95±0.29[ab]	2.06±0.32[b]	1.03±0.12[a]
High dose	1.86±0.28[ab]	2.02±0.33	1.12±0.21[a]

[a]$p<0.05$ *vs.* normal control unimmunized; [b]$p<0.05$ *vs.* normal control immunized; ConA: concanavalin A; LPS: lipopolysaccharide; OVA: ovalbumin; SI: stimulation index.

Table IX. *The expression levels of interleukin-2 (IL-2) (pg/ml) in splenocytes from Agaricus blazei Murill (AbM)-treated mice incubated with ConA, LPS or OVA.*

Group (n=10)	Con A	LPS	OVA
Normal control, unimmunized	1082.9±313.7	11.9±7.6	7.9±6.6
Normal control, immunized	1289.2±416.8	19.8±13.3	10.9±7.8
Low dose	1869.9±480.6[ab]	15.8±7.1	27.2±14.5[ab]
Medium dose	1877.4±439.0[ab]	24.4±9.4[ac]	26.5±21.7[a]
High dose	1581.5±373.5[a]	22.2±19.5	14.5±5.2[acd]

[a]$p<0.05$ *vs.* normal control unimmunized; [b]$p<0.05$ *vs.* normal control immunized; [c]$p<0.05$ *vs.* low-dose treatment group; [d]$p<0.05$ *vs.* medium-dose treatment group.

on OVA stimulation increased IL-2 levels only at the low AbM dose (Table IX). AbM (low and medium doses) increased levels of IFN-γ in splenocytes treated with ConA when compared with control groups. The effects of AbM were not dose-dependent. The highest AbM dose did not elevate the IFN-γ level (Table X). AbM did not affect IFN-γ levels in splenocytes incubated with LPS or OVA (Table X). AbM had no effects on IL-4 levels under any of the treatment conditions (Table XI).

Effects of AbM on different spleen cell types and the adaptive immune response. AbM did not significantly alter the percentages of T-helper cell (CD4/CD3), T-cytotoxic cells (CD8/CD3), T-cells (CD3/CD45) or B-cells (CD19/CD45) as compared with the two control groups (Table XII).

Discussion

Mushroom extract may inhibit tumor promotion or progression by having direct or indirect cytotoxic effects by up-regulating specific and non-specific immune responses. In the current study, we found that feeding mice with an AbM extract increased serum IgG levels but not IgM levels for the non-specific and specific immune responses.

Table X. *The expression levels of interferon-γ (IFN-γ) (pg/ml) in splenocytes from Agaricus blazei Murill (AbM)-treated mice incubated with ConA, LPS or OVA.*

Group (n=10)	ConA	LPS	OVA
Normal control, unimmunized	1877.4±747.6	19.3±24.0	5.8±8.4
Normal control, immunized	2176.0±898.3	4.8±5.5	2.5±5.3
Low dose	3436.8±1012.6[ab]	14.7±15.1	5.8±11.1
Medium dose	3166.5±936.6[ab]	13.7±17.6	8.3±13.0
High dose	2874.6±997.0[a]	17.7±28.3	9.5±10.5

[a]$p<0.05$ *vs*. normal control unimmunized; [b]$p<0.05$ *vs*. normal control immunized; [c]$p<0.05$ *vs*. low-dose treatment group; [d]$p<0.05$ *vs*. medium-dose treatment group.

Table XI. *Effect of treatment with Agaricus blazei Murill (AbM) on mean Interleukin 2 (IL-4) (pg/ml) levels in splenocytes stimulated with Con A, LPS or OVA.*

Group (n=10)	ConA	LPS	OVA
Normal control, unimmunized	102.0±26.6	5.7±9.3	18.8±28.4
Normal control, immunized	134.7±46.5	16.5±22.5	16.1±21.9
Low dose	104.9±50.2	10.9±18.8	14.2±17.8
Medium dose	132.9±40.0	11.2±22.2	14.9±27.7
High dose	119.6±55.7	7.7±15.4	11.7±19.9

Under stimulation by ConA for the non-specific immune response, the level of IL-2 in splenocytes was increased only on high dose AbM treatment but IFN-γ was increased only at a medium AbM dose. The synthesis of IFN-γ could lead to an attenuation of tissue inflammation. Splenocyte proliferation in mice fed with different doses of AbM showed that responses to ConA or LPS stimulation had significant effects as compared with the control groups.

Results from the flow cytometric assays indicated that AbM promoted phagocytosis by peritoneal macrophages and elevated the activity of NK cells.

CD19 is an activated B-cell marker and B-cell differentiation requires the interaction of various cytokines, which come from macrophages or T-cell secretions. In the present study, AbM did not affect B-cells. FACScan results also showed that AbM treatment did not increase $CD3^+$ T-cell populations. These results differ from those reported by Chan and colleagues (27). After OVA immunization, T-cell and B-cells were still not significantly increased in treatment groups compared with the controls. Our results show that AbM triggers innate immunity as evidenced by significant increases in serum IgG, IL-2 and IFN-γ levels, and of phagocytosis and NK activity.

AbM would appear to exert its immunostimulant actions primarily by induction of innate immunity mediated by phagocytes. The activation appears to be mediated by cytokines that stimulate the Th1 immune response and NK cell activity. In addition to the influence of AbM on promoting the induction of Th1 immune response, this mushroom extract can also induce production of antibodies. Based on these observations, we conclude that AbM increases the humoral immune response and affects the cellular immune response.

Table XII. *The proportion of splenocyte subsets of immunized BALB/c mice fed with different doses of Agaricus blazei Murill (AbM).*

Group (n=10)	CD4/CD3	CD8/CD3	CD3/CD45	CD19/CD45
Normal control, unimmunized	28.9±4.1	15.0±4.0	45.3±6.4	48.5±5.9
Normal control, immunized	25.4±3.7	14.4±2.7	42.1±4.4	51.8±4.5
Low dose	25.8±4.8	13.6±2.8	43.0±6.4	50.8±5.8
Medium dose	25.7±3.7	13.6±3.0	40.6±5.3	53.7±5.0
High dose	28.5±4.9	14.9±3.0	44.8±8.0	50.3±6.2

The reports on the effects of AbM on the inflammatory processes have not been consistent with respect to the direction of effects. These differences may be due to the type of extract used, mushroom cultivation and natural process of genetic recombination and mutation. Such differences could change mushroom chemical composition and impact on the effects of AbM on the immune system. Clinical trials are needed in order to compare the activities of isolated compounds with the whole mushroom extracts and to determine whether AbM has any therapeutic efficacy.

Conflicts of Interests

The Authors have no conflicts of interests.

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