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# Oral administration of submerged cultivated *Grifola frondosa* enhances phagocytic activity in normal mice

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## Abstract

*Grifola frondosa* fruiting body (Maitake) has been used as a dietary supplement due to its anti-tumour and immunomodulatory properties. The aim of this study was to evaluate the immunomodulatory effects of orally administered submerged cultivated *G. frondosa* mixture, including both mycelium and culture broth, in a healthy murine model. Composition analyses showed that submerged cultivated *G. frondosa* mixture contained only 32.48% carbohydrate, which was less than half of fruiting bodies. The content of adenosine, a potential immunomodulatory agent in medicinal mushrooms, was 2.8 mg g$^{-1}$. After feeding 8-week-old female BALB/cByJ mice with AIN-93G diet containing 0% (C), 1% (G1), 3% (G3) or 5% (G5) (wt/wt) *G. frondosa* mixture for 31 days, neither body weight nor the outward appearance of organs showed any significant difference among different diet groups. Splenocyte subpopulation, mitogen-activated cytokine release and splenic NK activity were not affected by *G. frondosa* administration, either. On the other hand, the phagocytic activity was enhanced in leucocytes of groups G3 and G5, without exerting detectable levels of serum pro-inflammatory cytokines. These results suggested that oral administration of submerged cultivated *G. frondosa* mixture may enhance host innate immunity against foreign pathogens without eliciting adverse inflammatory response.

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## Introduction

*Grifola frondosa* is a Basidiomycete fungus belonging to the order Aphyllopherales, and family Polyporaceae. Numerous bioactive polysaccharides from fruiting body and submerged cultivated mycelium of *G. frondosa* have been reported to enhance innate and cell-mediated immune responses in animals and man (Mizuno et al 1986; Ohno et al 1986; Shigesue et al 2000; Kodama et al 2003a).

The cultivation of *G. frondosa* to produce fruiting body is a long-term process requiring one to several months for the first fruiting body to appear. Nowadays many attempts are being made through submerged culture to accelerate the process and to result in biomass and exopolysaccharide yields within a few days (Zhang et al 2002; Lee et al 2004; Bae et al 2005). Recently, our laboratory also demonstrated the process for submerged culture of *G. frondosa* mycelium. It was found that the hot-water-soluble polysaccharides of mycelium enhanced the release of TNF-$\alpha$, IL-6 and IFN-$\gamma$, as well as phagocytic activity and NK cytotoxicity in human blood in-vitro (Wu et al 2006).

Most of the current commercial medicinal mushroom products, such as *Ganoderma lucidum* and *Cordyceps sinensis*, are made of submerged cultivated mycelium and culture broth, which is rich in protein, nucleosides and other nutrients. In addition, biological response modifiers (BRM), such as bestatin (Ishizuka et al 1992), lactic-acid-producing bacteria (Lee & Lee 2005) and soluble glucan from the lichenized fungus (Stuelp-Campelo et al 2002), have been reported to enhance phagocytic activity in animal models. Therefore, it is worth studying whether submerged cultivated *G. frondosa* mixture, including both mycelium and culture broth, has similar biological effect to other known BRM in-vivo.

Adenosine is a bioactive ingredient in medicinal mushrooms, such as *Ganoderma* spp. (Gao et al 2007), *Cordycepes sinensis* (Leung et al 2006) and *Antrodia cinnamomea* (Lu et al 2006). Adenosine is also an endogenous modulator of innate immunity (Hasko & Cronstein 2004). It regulates neutrophil function in opposing fashion through the ligation of A1 (immunostimulatory) and A2A (immunosuppressive) receptors. This counter-regulatory effect suggests that adenosine enhances the inflammatory response at sites where adenosine is present in low concentrations, while it has anti-inflammatory effect at sites where bacteria infect (Hasko & Cronstein 2004). Although adenosine widely exists in medicinal mushrooms, there is no information regarding its content in fruiting bodies or submerged cultivated mycelium or culture broth of *G. frondosa*.

It is important to point out that although *G. frondosa* is considered as a dietary supplement and taken orally by man, information regarding its immunological effects has seldom been obtained in healthy animal models involving oral administration. Most reports have either been conducted in-vitro or involve administration by intraperitoneal injection into tumour-bearing mice. In addition, most in-vivo studies have focused on the immunomodulatory function of fruiting body rather than submerged cultivated *G. frondosa* mycelium or culture broth. In this study, we investigated the effect of oral ingestion of submerged cultivated *G. frondosa* mixture, including both mycelium and culture broth, on the innate immunity in BALB/c mice through several immune functional assays. This investigation provided direct evidence that oral administration of submerged cultivated *G. frondosa* mixture enhanced phagocytic activity of blood leucocytes.

## Materials and Methods

### Microorganisms and media

*Grifola frondosa* TSRI01 was isolated from the mountainous district in Taiwan. The stock medium was made up of potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) and 0.5% yeast extract (Difco Laboratory, Detroit, MI). The stock slants were incubated at 28°C for 7 days and then stored at 4°C. The seed culture medium was composed of 2% glucose, 0.15% $KH_2PO_4$ and 0.02% $MgSO_4 \cdot 7H_2O$ (all from Merck), 0.4% yeast extract, 0.2% malt extract and 0.2% peptone (all from Difco). The fermentation medium was made up of 1% glucose, 0.8% yeast extract, 0.15% $KH_2PO_4$, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.2% $(NH_4)_2SO_4$ (Wu et al 2006).

### Preparation of submerged cultivated *G. frondosa* mixture

*G. frondosa* TSRI01 was initially grown on stock medium in a Petri dish at 28°C for 7 days and then transferred to the seed culture medium by punching out a 10-mm diameter of mycelia from the agar plate culture with a sterilized cutter. The primary seed culture experiments were performed in 500-mL Hinton flasks containing 100 mL of the seed culture medium and incubated at 28°C on a rotary shaker at 110 rev $min^{-1}$ for 6 days. The secondary seed culture experiments were performed in 1-L Hinton flasks containing 300 mL of fermentation medium after inoculating with 10% (v/v) of the primary seed culture under the conditions described above. Fed-batch fermentation was then performed in a 100-L fermentor (Bioengineering AG, Wald, Switzerland) containing 50 L of fermentation medium after inoculating with 5% (v/v) of the secondary seed culture. Fermentation was performed at 28°C, aeration rate 1.0 vvm, agitation speed 150 rev $min^{-1}$ and initial pH 5.4. When glucose concentration was less than 0.3%, glucose solution (35%, w/v) was then fed into the fermentor until glucose content was back to about 1%. The biomass reached 12 g $L^{-1}$ after a 10-day culture (Wu et al 2006). The obtained culture mixture, including mycelium and culture broth, was sprayed to dry and ground to powder.

### Proximate composition of *G. frondosa* mixture

The moisture, carbohydrate, protein, fat, fibre, reducing sugar and ash of dried culture mixture were determined according to the CNS (Chinese National Standard) method stipulated by the Bureau of Standards, Metrology and Inspection, Taiwan, ROC.

### Molecular mass determination of polysaccharides

Dried culture mixture was extracted with 10-fold (v/w) 0.1 M $Na_2CO_3$ at 90°C for 1 h followed by centrifugation at 8000 *g* for 20 min. Ethanol 80% (final concentration, v/v) was then added to the supernatant and kept at −20°C for 2 h. Polysaccharides precipitate was recovered by centrifugation of the ethanol mixture at 8000 *g* for 20 min. The molecular mass of polysaccharides was estimated by Waters 2690 GPC (Gel Permeation Chromatography) system (Waters Co., Milford, MA) with Ultrahydrogel Linear column (7.8 × 300 mm). The mobile phase was 0.1 M $NaNO_3$ (containing 0.02% $NaN_3$), column temperature was 48°C, flow rate was 0.1 mL $min^{-1}$ and injection volume was 10 $\mu$L. The elute was monitored by a refractive index detector (Waters 2410 refractometer). A set of pullulan standards P-400, P-200, P-100, P-50, P-20, P-10 and P-5 (Shodex Standard P-82; Macherey–Nagel, Germany) was used for calibration of the columns. Therefore, the molecular mass parameters shown are relative to the pullulan reference.

### Analysis of adenosine

Dried culture mixture was first extracted with 10-fold (v/w) water at 90°C for 1 h followed by centrifugation at 8000 *g* for 20 min. The content of adenosine in the obtained supernatant was analysed using HPLC. The HPLC system consisted of Waters Model Alliance 2690 and 996 photodiode array detector set at 254 nm wavelength. The separations were achieved with a 15-$\mu$L loop, and a reversed-phase column (Merck LiChrospher 100 RP-18, 5 $\mu$m, 4.0 × 250 mm i.d.; Darmstadt, Germany) followed by linear gradient elution using eluents A and B (A: 100% MeOH; B: 0.1 N $NH_4H_2PO_4$ (pH 3.0)) according to the following profile: 0 ∼ 20 min, 5% A, 95% B; 20 ∼ 30 min, 15% A, 85% B. The flow rate was kept at 0.8 mL $min^{-1}$.

### Animals feeding and grouping

Female BALB/cByJ mice (8 weeks) were purchased from National Laboratory Animal Center (Taipei, Taiwan) and were housed individually in a room maintained at 25 ± 2°C with a 12-h light–dark cycle. Mice were acclimatized for 1 day to American Institute of Nutrition 93G (AIN-93G) diet. Following the acclimatization period, mice were allocated randomly to the AIN-93G diet only (C) or to the AIN-93G diet containing 1% (G1), 3% (G3) or 5 % (G5) (wt/wt) *G. frondosa* for 31 days. The formulations of diets are shown in Table 1. Animals had free access to diet and distilled water throughout the acclimatization and experimental periods. All procedures were approved by Animal Care and Use Committee, Chia-Nan University, Tainan, Taiwan.

### Body weight and outward appearance of lymphoid organs

At the end of the experimental period, body weight and food intake were determined for each mouse. The outward appearance of spleen, liver and thymus were examined, after sacrifice by cervical dislocation.

### Sampling

At the end of the experimental period, mice were anaesthetized with $CO_2$ to permit sampling of blood from the orbital plexus for phagocytosis analysis, after which they were killed by cervical dislocation. Spleens were removed aseptically, and single splenocyte suspension in RPMI 1640, which contained 10% fetal bovine serum, 1 M HEPES, 2 mM glutamine, 100 U mL$^{-1}$ penicillin and 100 mg mL$^{-1}$ streptomycin (Invitrogen Life Technologies), was obtained by passage through a stainless mesh (200 mesh). Red blood cells (RBC) were lysed with lysis buffer (0.15 M $NH_4Cl$, 0.1 mM $Na_2EDTA$, 10 mM $KHCO_3$) on ice for 5 min. After washing with PBS, splenocytes were employed for three analyses: mitogen-stimulated cytokine production, NK cytotoxicity and lymphocyte subpopulation analysis.

**Table 1** The nutrient percentage composition of diets used in the study

| Ingredient | Content (%) | | | |
|---|---|---|---|---|
| | C | G1 | G3 | G5 |
| *G. frondosa* mixture | 0 | 1 | 3 | 5 |
| Corn starch | 52.95 | 51.95 | 49.95 | 47.95 |
| Casein | 20.0 | 20.0 | 20.0 | 20.0 |
| Sucrose | 10.0 | 10.0 | 10.0 | 10.0 |
| Soy bean oil | 7.0 | 7.0 | 7.0 | 7.0 |
| Alphacel | 5.0 | 5.0 | 5.0 | 5.0 |
| Mineral[a] | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin[a] | 1.0 | 1.0 | 1.0 | 1.0 |
| L-Cystine | 0.3 | 0.3 | 0.3 | 0.3 |
| Choline bitartrate | 0.25 | 0.25 | 0.25 | 0.25 |
| *t*-Butylhydroquinone | 0.0014 | 0.0014 | 0.0014 | 0.0014 |

C, control; G1, diet containing 1% *G. frondosa* mixture; G3, diet containing 3% *G. frondosa* mixture; G5, diet containing 5% *G. frondosa* mixture.

[a]Based on AIN-93G formula (Reeves 1997).

### Splenic lymphocyte subpopulation analysis

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against murine B220, CD3 and CD4 as well as phycoerythrin (PE)-conjugated monoclonal antibody against murine CD8 or isotype-matched, non-reactive negative control mAb (Beckman Coulter, Fullerton, CA) were used to stain isolated splenocytes according to the manufacturer's instruction. Stained cells were analysed in duplicate by flow cytometry (Coulter EPICS XL, Beckman Coulter).

### Cytokine secretion from stimulated splenocytes

Splenocytes were cultivated in 24-well plates at $4 \times 10^6$ cells/mL in a 37°C 5% $CO_2$ incubator. LPS (1 $\mu$g mL$^{-1}$, *E. coli* O55:B5; Fluka Chemie AG, Buchs, Switzerland) was used as a mitogen to stimulate B cells and macrophages. After 24 h, TNF-$\alpha$ and IL-6 were measured in the supernatant using ELISA tests (BD, Franklin Lakes, NJ). PHA (Sigma Chemical, St Louis, MO), which is a T cell mitogen, was added at a concentration of 25 $\mu$g mL$^{-1}$ in separate wells. After 48 and 72 h, IL-6 and IFN-$\gamma$ were measured from supernatant by ELISA tests, respectively.

### Cytotoxicity against NK-sensitive YAC-1 cells

Splenic NK cytotoxicity against YAC-1 target cells was assessed by a flow cytometry assay (Chang et al 1993) using the DIO membrane dye (Molecular Probes, Eugene, OR) to stain live YAC-1 cells and propidium iodide nuclear dye to stain dead cells. Briefly, mouse splenic effector cells ($5 \times 10^5$ per well) were mixed with DIO-labelled YAC-1 cells (effector cells:target cells = 50:1, to achieve the optimal effect) and then counter staining solution (propidium iodide) was added and incubated at 37°C for 4 h. Each sample was investigated in duplicate by flow cytometry, a standard technique for the examination of NK activity. During data acquisition, a live gate was set in the FL1 histogram on the green fluorescent target cells to discriminate effector and target cells.

### Phagocytic activity of leucocytes

The phagocytosis of FITC-labelled *E. coli* (FITC-*E. coli*) by peripheral blood leucocytes was determined by flow cytometry using PHAGOTEST (Orpegen Pharma, Heidelberg, Germany). Briefly, heparinized whole blood (100 $\mu$L) was cooled on ice for 10 min, 20 $\mu$L of ice-cold FITC-*E. coli* was then added and mixed. The tested tubes were then incubated at 37°C, while the corresponding negative controls were maintained on ice. After 10 min, all tubes were transferred back on ice and 100 $\mu$L of ice-cold quenching solution was added for suppression of signals from adherent, non-phagocytized bacteria. After several washing steps, red blood cells were lysed, followed by incubation with DNA staining solution to exclude aggregation artifacts from bacteria or cells. Each sample was investigated in duplicate by flow cytometry, a standard technique for the examination of phagocytosis (Lehmann et al 2000). The live gate was set in the red

fluorescence histogram on those events that had at least the same DNA content as a human diploid cell and the analysis was performed on 15 000 leucocytes. WinMDI 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA) was used for determination of phagocytic activity.

### Statistical analysis

All statistical analyses were performed using SAS system Version 8.0 under Microsoft Windows (SAS Institute Corp., Cary, NC). Group means were subjected to analysis of variance followed by Tukey's Studentized Range test, if justified by the statistical probability ($P < 0.05$).

## Results

### Composition analysis of submerged cultivated *G. frondosa* mixture

The proximate composition of the submerged cultivated *G. frondosa* mixture, including both mycelium and culture broth, is shown in Table 2. It has been reported that the fruiting bodies of *G. frondosa* contain 4.4–6.5% ash, 1.5–4.5% fat, 13.1–18.4% protein and 70.6–80.8% carbohydrate based on dry weight (Tabata et al 2004). The protein content in the current study (45%) was about 3-fold of that in fruiting bodies. This may be attributed to the residual yeast extract remaining in culture medium. Consequently, the carbohydrate content of submerged cultivated *G. frondosa* mixture was less than half of those in fruiting bodies.

**Table 2** Proximate composition of submerged cultivated *G. frondosa* mixture

| | Content (%) |
|---|---|
| Moisture | 7.97 ± 0.08 |
| Ash | 6.62 ± 0.06 |
| Protein | 45.07 ± 0.97 |
| Fat | 7.73 ± 0.05 |
| Carbohydrate | 32.48 ± 0.54 |
| Fibre | 0.13 ± 0.01 |
| Reducing sugar | 3.04 ± 0.05 |

The molecular mass distribution of polysaccharides of *G. frondosa* mixture was further determined by GPC as described in Materials and Methods. There was broad molecular mass distribution with two major peaks at ~ 240 kDa and ~ 30 kDa using pullulan as reference (data not shown).

Adenosine, another immunomodulator in medicinal mushrooms, was further analysed using HPLC as described in Materials and Methods. Figure 1 shows the HPLC profile of the dried submerged cultivated *G. frondosa* mixture used in this study. The adenosine peak was detected at retention time 9.642 min, and its content was calculated as 2.8 ± 0.3 mg (g dry weight)$^{-1}$ as compared with standard curve. The other peaks may represent bases, or other nucleosides, such as uracil, cytidine or inosine, commonly found in medicinal mushrooms (Gao et al 2007).

### Growth indices and organ appearance

No statistically significant difference in body weight gain or food intake was observed among different diet groups. Furthermore, no abnormal clinical signs, behaviour or organ appearance were detected in any of the tested groups (data not shown). Considered together, *G. frondosa* feeding did not induce any apparent toxicity in mice.

### Splenic lymphocyte subpopulations and mitogen-stimulated cytokine release

Given no significant effect of dietary *G. frondosa* on mice body or organ appearance, it would be intriguing to further investigate its effect on splenocyte subtypes or immunoactivity using flow cytometry. Splenic lymphocyte subpopulation is shown in Table 3. $CD3^+$ T cells and $B220^+$ B cells were not significantly influenced by *G. frondosa* mixture feeding. Similarly, the ratio of $CD4^+$ T cells (helper T cells) to $CD8^+$ T cells (cytotoxic T cells) did not significantly differ among diet groups.

The immunoactivity of T cells or macrophages was measured as cytokine release in PHA- and LPS-stimulated

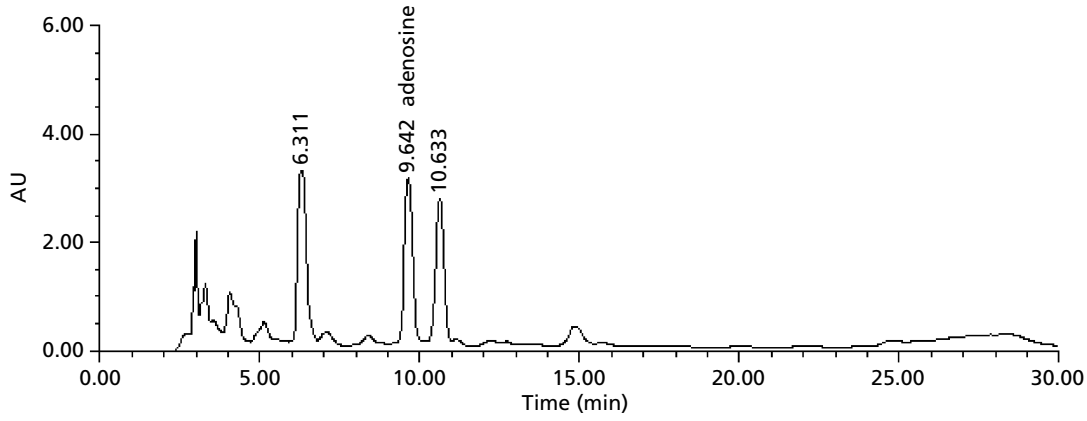


**Figure 1** HPLC analysis of adenosine in submerged cultivated *G. frondosa* mixture.

**Table 3** Effect of intake of submerged cultivated *G. frondosa* mixture on lymphocyte subpopulations of splenocytes

| | C | G1 | G3 | G5 |
|---|---|---|---|---|
| $B220^+$ (%) | 44.4 ± 4.7 | 46.1 ± 4.0 | 45.6 ± 3.5 | 45.4 ± 4.5 |
| $CD3^+$ (%) | 41.9 ± 6.2 | 44.8 ± 3.0 | 42.4 ± 2.6 | 41.3 ± 1.5 |
| $CD4^+/CD8^+$ | 2.3 ± 0.6 | 2.5 ± 0.7 | 2.3 ± 0.5 | 2.2 ± 0.4 |

Values are mean ± s.d., n = 12. Within each row, values are not different ($P < 0.05$) according to Tukey's Studentized Range test.
C, control; G1, fed diet containing 1% *G. frondosa* mixture; G3, fed diet containing 3% *G. frondosa* mixture; G5, fed diet containing 5% *G. frondosa* mixture for 31 days.

splenocytes, respectively. Without mitogen stimulation, there was no detectable cytokine in the cultured splenocyte supernatant regardless of diet groups (data not shown). Table 4 shows that with LPS ($1\,\mu g\,mL^{-1}$) stimulation for 24 h, IL-6 production from macrophages was elevated in all groups of mice without significant difference among diet groups. Similar results were obtained for TNF-$\alpha$ production in LPS-activated splenocytes (data not shown).

Furthermore, Th-2 specific IL-6 and Th-1 specific IFN-$\gamma$ production were analysed in PHA-treated splenocytes. When splenocytes were cultured in the presence of PHA ($25\,\mu g\,mL^{-1}$) for 48 h, IL-6 production ranged from $266 \pm 258\,pg\,mL^{-1}$(group C) to $404 \pm 321\,pg\,mL^{-1}$(group G5), with no significant difference among diet groups. Similarly, IFN-$\gamma$ production after stimulation ranged from $6990 \pm 1634\,pg\,mL^{-1}$(group C) to $10713 \pm 1894\,pg\,mL^{-1}$ (group G5), without significant difference among diet groups.

### Cytotoxicity against NK-sensitive YAC-1 cells

It has been reported that intraperitoneal injection of the extract of *G. frondosa* fruiting body to tumour-bearing and normal mice could enhance NK cytotoxicity (Kodama et al 2003a, b, 2004, 2005). To test whether oral administration of submerged cultivated *G. frondosa* also exerted a similar effect, splenic NK cytotoxicity was measured using an optimal ratio of E:T (50:1). Table 5 shows that splenic NK-mediated YAC-1 lysis ranged from 26.1 ± 6.8% (Group C) to 30.3 ± 6.4% (Group G5) without pronounced difference among diet groups.

### Phagocytosis of murine leucocytes

Based on the observation that dietary *G. frondosa* mixture did not exhibit any detectable activity or subtype change for splenocytes, we turned to analyse the immunoactivity in the blood of *G. frondosa*-fed mice. Table 5 demonstrates the phagocytic activity of leucocytes from mice blood. The uptake of *E. coli* by leucocytes increased from 9.1 ± 2.9% (group C) to 14.1 ± 3.8% and 13.3 ± 2.9% in groups G3 and G5, respectively ($P < 0.05$). However, the leucocyte phagocytic activity in group G1 mice did not differ from that of group C. To test whether the increased phagocytic activity correlated with elevated pro-inflammatory cytokine production, TNF-$\alpha$, IL-1, IL-6 and IFN-$\gamma$ levels were determined in sera. None of the tested mice produced detectable levels of cytokines in serum (data not shown).

## Discussion

Numerous studies have confirmed that the polysaccharides isolated from *G. frondosa* fruiting bodies have prominent beneficial effects on immune function (Suzuki et al 1985; Ohno et al 1995, 1996; Adachi et al 1998; Kubo & Nanba 1998). In this research, the submerged cultivated *G. frondosa* mixture, including mycelium and culture broth, was spray dried and used as a dietary supplement to feed mice to mimic the natural route for human usage. The approximate composition analysis showed significant differences in the levels of fat, protein and carbohydrate between submerged cultivated *G. frondosa* mixture and fruiting bodies. This indicates that the immunomodulatory effects of submerged cultivated *G. frondosa* mixture used in this study may not result from polysaccharides only.

Adenosine has been considered as a potential immunomodulatory and neuroprotective agent for 30 years (Hasko et al 2004). It also serves as an endogenous regulator of innate immunity (Hasko & Cronstein 2004). Adenosine exerts anti-inflammatory effects on a variety of cells, including mast cells, macrophages and neutrophils (Cronstein 1994; McCallion et al 2004). It also works as an immunostimulator in mast cells and neutrophils (Hasko & Cronstein 2004). Adenosine is an active component in medicinal mushrooms, such as *Ganoderma* spp. (Gao et al 2007), *Cordycepes sinensis* (Leung et al 2006) and *Antrodia cinnamomea* (Lu et al 2006). It has been reported that the fruiting bodies

**Table 4** Effect of intake of submerged cultivated *G. frondosa* mixture on cytokine production in mitogen-activated splenocytes of BALB/cByJ mice

| | C | G1 | G3 | G5 |
|---|---|---|---|---|
| LPS-stimulated IL-6 ($pg\,mL^{-1}$) | 10568 ± 5213 | 8246 ± 4781 | 10471 ± 5057 | 9771 ± 4676 |
| PHA-stimulated IL-6 ($pg\,mL^{-1}$) | 266 ± 258 | 275 ± 237 | 352 ± 282 | 404 ± 321 |
| PHA-stimulated IFN-$\gamma$ ($pg\,mL^{-1}$) | 6990 ± 1634 | 8477 ± 1420 | 11279 ± 2560 | 10713 ± 1894 |

Values are mean ± s.d., n = 12. Within each row, values are not different ($P < 0.05$) according to Tukey's Studentized Range test.
C, control; G1, fed diet containing 1% *G. frondosa* mixture; G3, fed diet containing 3% *G. frondosa* mixture; G5, fed diet containing 5% *G. frondosa* mixture for 31 days.

**Table 5** Effect of submerged cultivated *G. frondosa* mixture on splenic NK cell cytotoxicity and phagocytic activity in BALB/cByJ mice

| | C | G1 | G3 | G5 |
|---|---|---|---|---|
| YAC-1 cell lysis (%)[a] | 26.1 ± 6.8 | 28.4 ± 6.6 | 28.0 ± 6.9 | 30.3 ± 6.4 |
| Phagocytic activity (%)[b] | 9.1 ± 2.9 | 10.6 ± 3.7 | 14.1 ± 3.8* | 13.3 ± 2.9* |

C, control; G1, fed diet containing 1% *G. frondosa* mixture; G3, fed diet containing 3% *G. frondosa* mixture; G5, fed diet containing 5% *G. frondosa* mixture for 31 days.

[a]Splenocytes were cultured with DIO-labelled YAC-1 tumour cells at an E:T ratio of 50:1 for 4 h, and NK cell cytotoxicity was then determined by flow cytometry as described in Materials and Methods. Data represent mean ± s.e.m., n = 12. Values are not different ($P < 0.05$) according to Tukey's test.

[b]The percentages of phagocytosing leucocytes in various groups were determined by PHAGOTEST kit and flow cytometry as described in the Materials and Methods. Data represent mean ± s.d., n = 12.

*$P < 0.05$ represents significant differences compared to the control (C) according to Tukey's Studentized Range test.

and submerged cultivated mycelium of *C. sinensis* contains 264.6 and 1116.8 $\mu$g adenosine/g of dry weight, respectively (Leung et al 2006). Our study reported that submerged cultivated *G. frondosa* mixture was composed of 2.8 mg adenosine (g dry weight)$^{-1}$, which was more than double that in *C. sinensis*. This suggested that, in addition to polysaccharides, adenosine may also serve as an important immunomodulatory component in submerged cultivated *G. frondosa* mixture.

This study demonstrated that mice fed 3% (G3) and 5% (G5) *G. frondosa* mixture for 31 days exerted significantly higher phagocytic activity in blood leucocytes. It has been demonstrated that orally delivered, fungus-derived soluble glucans can be absorbed by intestinal epithelial cells and gut-associated lymphoid tissue (GALT) cells. The glucans are then translocated into systematic circulation and protect animals from subsequent exposure to fungal and bacterial pathogens (Rice et al 2005). It is therefore possible that the plateau effect on groups G3 and G5 may be due to the intestine absorption limit of *G. frondosa* mixture.

It is well-known that IFN-$\gamma$ can work as an immunomodulator to enhance phagocytic activity. However, no detectable pro-inflammatory cytokine release was found in sera taken from tested mice. This result indicates that oral ingestion of *G. frondosa* mixture did not cause systematic inflammatory response in-vivo, while augmenting microbicidal effects. This result also supports the previous notion that the elevated phagocytic activity in leucocytes is not contributed to by indirect IFN-$\gamma$ activation (Wu et al 2006).

The separation of T helper cells into Th1 and Th2 according to their cytokine profiles and differential effects in cell-mediated (Th1) and humoral immunity (Th2) is very useful in the understanding of the immune mechanism. IL-2 and IFN-$\gamma$ are considered to be the products of Th1 cells, whereas IL-4, IL-6, and IL-10 are products of Th2 cells (Mosmann & Coffman 1989). In this study, it was found that oral administration of submerged cultivated *G. frondosa* mixture did not affect the level of cytokine release from LPS- or PHA-stimulated splenocyte culture (Table 4). This result indicated that neither cell-mediated immune response (Th1) nor humoral immunity (Th2) was enhanced by oral administration of *G. frondosa* mixture. It is noteworthy that these effects varied considerably among the mice in each treatment group. Orally administered *Ganoderma lucidum* has also been found to exert variable effects on mitogen-activated splenocytic cytokine release in each treatment group, but significantly elevated cytokine release was noted at high dose (500 mg kg$^{-1}$) (Kohguchi et al 2004). In contrast, intraperitoneal administration of D-Fraction of *G. frondosa* fruiting bodies has been demonstrated to evoke Th-1-dominant immune reactions in tumour-bearing mice (Inoue et al 2002) but to result in the enhancement of humoral immunity in normal mice (Kodama et al 2004).

It is well-known that Th1 cytokine production is correlated with T lymphocyte proliferation in adaptive immunity and NK cytotoxicity in innate immunity. Since neither elevated Th1 cytokine nor enhanced NK cytotoxicity was observed, these two results agree with each other. Furthermore, splenic lymphocyte cell surface antigen detection revealed no difference in the ratio of T cells to B cells or Th cells to Tc cells among different diet groups. Therefore, the immunomodulatory effect of submerged cultivated *G. frondosa* mixture is presumably on non-specific innate immune reactions rather than on lymphocytes that introduce specific acquired immunity.

The effect of oral administration of submerged cultivated *G. frondosa* mixture on splenocytes was neither pronounced nor consistent between mice, even in the same diet group, rendering its mechanism in immunomodulation less clear than those found in the literature. The discrepancies could be explained by using different experimental design, oral feeding versus intraperitoneal injection, crude culture mixture versus polysaccharide extract and different analytic parameters. The importance of using an experimental model that does not bypass the gut barrier is emphasized in this study, which mimics the human situation of consumption of *G. frondosa* as a dietary supplement. In conclusion, our studies showed evidence of enhanced phagocytic activity by oral administration of submerged cultivated *G. frondosa* mycelium and culture broth.

## Conclusion

This report demonstrates the effect of oral administration of submerged cultivated *G. frondosa* mixture, including both

mycelium and culture broth, on innate immunity in healthy mice. The phagocytic activity was enhanced in leucocytes of BALB/cByJ mice fed 3% and 5% *G. frondosa*, without exerting detectable level of sera pro-inflammatory cytokines. These results suggest that submerged cultivated *G. frondosa* mixture may enhance host innate immunity against foreign pathogens without eliciting an adverse inflammatory response.

## References


Adachi, Y., Ohno, N., Yadomae, T. (1998) Activation of murine kupffer cells by administration with gel-forming (1 → 3)-beta-D-glucan from Grifola frondosa. *Biol. Pharm. Bull*. **21**: 278–283

Bae, J. T., Sim, G. S., Lee, D. H., Lee, B. C., Pyo, H. B., Choe, T. B., Yun, J. W. (2005) Production of exopolysaccharide from mycelial culture of Grifola frondosa and its inhibitory effect on matrix metalloproteinase-1 expression in UV-irradiated human dermal fibroblasts. *FEMS Microbiol. Lett*. **251**: 347–354

Chang, L., Gusewitch, G. A., Chritton, D. B., Folz, J. C., Lebeck, L. K., Nehlsen-Cannarella, S. L. (1993) Rapid flow cytometric assay for the assessment of natural killer cell activity. *J. Immunol. Methods* **166**: 45–52

Cronstein, B. N. (1994) Adenosine, an endogenous anti-inflammatory agent. *J. Appl. Physiol*. **76**: 5–13

Gao, J. L., Leung, K. S., Wang, Y. T., Lai, C. M., Li, S. P., Hu, L. F., Lu, G. H., Jiang, Z. H., Yu, Z. L. (2007) Qualitative and quantitative analyses of nucleosides and nucleobases in Ganoderma spp. by HPLC-DAD-MS. *J. Pharm. Biomed. Anal*. **44**: 807–811

Hasko, G., Cronstein, B. N. (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol*. **25**: 33–39

Hasko, G., Sitkovsky, M. V., Szabo, C. (2004) Immunomodulatory and neuroprotective effects of inosine. *Trends Pharmacol. Sci*. **25**: 152–157

Inoue, A., Kodama, N., Nanba, H. (2002) Effect of maitake (Grifola frondosa) D-fraction on the control of the T lymph node Th-1/Th-2 proportion. *Biol. Pharm. Bull*. **25**: 536–540

Ishizuka, M., Abe, F., Abe, S., Uchida, K., Ikeda, T., Ito, N., Aoyagi, K., Yamaguchi, H., Takeuchi, T. (1992) Enhancement of host resistance to opportunistic infections by ubenimex (bestatin). *Adv. Exp. Med. Biol*. **319**: 193–200

Kodama, N., Komuta, K., Nanba, H. (2003a) Effect of maitake (Grifola frondosa) D-Fraction on the activation of NK cells in cancer patients. *J. Med. Food* **6**: 371–377

Kodama, N., Kakuno, T., Nanba, H. (2003b) Stimulation of the natural immune system in normal mice by polysaccharide from maitake mushroom. *Mycoscience* **44**: 257–261

Kodama, N., Murata, Y., Nanba, H. (2004) Administration of a polysaccharide from Grifola frondosa stimulates immune function of normal mice. *J. Med. Food* **7**: 141–145

Kodama, N., Asakawa, A., Inui, A., Masuda, Y., Nanba, H. (2005) Enhancement of cytotoxicity of NK cells by D-Fraction, a polysaccharide from Grifola frondosa. *Oncol. Rep*. **13**: 497–502

Kohguchi, M., Kunikata, T., Watanabe, H., Kudo, N., Shibuya, T., Ishihara, T., Iwaki, K., Ikeda, M., Fukuda, S., Kurimoto, M. (2004) Immuno-potentiating effects of the antler-shaped fruiting body of Ganoderma lucidum (Rokkaku-Reishi). *Biosci. Biotechnol. Biochem*. **68**: 881–887

Kubo, K., Nanba, H. (1998) Modification of cellular immune responses in experimental autoimmune hepatitis in mice by maitake (Grifola frondosa). *Mycoscience* **39**: 351–360

Lee, Y., Lee, T. S. (2005) Enhancement in ex vivo phagocytic capacity of peritoneal leukocytes in mice by oral delivery of various lactic-acid-producing bacteria. *Curr. Microbiol*. **50**: 24–27

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J., Yun, J. W. (2004) Submerged culture conditions for the production of mycelial biomass and exopolysaccharides by the edible Basidiomycete Grifola frondosa. *Enzyme Microb. Technol*. **35**: 369–376

Lehmann, A. K., Sornes, S., Hastensen, A. (2000) Phagocytosis: measurement by flow cytometry. *J. Immunol. Methods* **243**: 229–242

Leung, P. H., Zhang, Q. X., Wu, J. Y. (2006) Mycelium cultivation, chemical composition and antitumour activity of a Tolypocladium sp. fungus isolated from wild Cordyceps sinensis. *J. Appl. Microbiol*. **101**: 275–283

Lu, M. K., Cheng, J. J., Lai, W. L., Lin, Y. R., Huang, N. K. (2006) Adenosine as an active component of Antrodia cinnamomea that prevents rat PC12 cells from serum deprivation-induced apoptosis through the activation of adenosine A(2A) receptors. *Life Sci*. **79**: 252–258

McCallion, K., Harkin, D. W., Gardiner, K. R. (2004) Role of adenosine in immunomodulation: review of the literature. *Crit. Care Med*. **32**: 273–277

Mizuno, T., Ohsawa, K., Hagiwara, N., Kuboyama, R. (1986) Fractionation and characterization of antitumor polysaccharides from Maitake, Grifola fondosa. *Agric. Biol. Chem*. **50**: 1679–1688

Mosmann, T. R., Coffman, R. L. (1989) Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol*. **7**: 145–173

Ohno, N., Adachi, Y., Suzuki, I., Sato, K., Oikawa, S., Yadomae, T. (1986) Characterization of the antitumor glucan obtained from liquid cultured Grifola frondosa. *Chem. Pharm. Bull*. **34**: 1709–1715

Ohno, N., Asada, N., Adachi, Y., Yadomae, T. (1995) Enhancement of LPS triggered TNF-alpha (tumor necrosis factor-alpha) production by (1- > 3)-beta-D-glucans in mice. *Biol. Pharm. Bull*. **18**: 126–133

Ohno, N., Egawa, Y., Hashimoto, T., Adachi, Y., Yadomae, T. (1996) Effect of beta-glucans on the nitric oxide synthesis by peritoneal macrophage in mice. *Biol. Pharm. Bull*. **19**: 608–612

Reeves, P. G. (1997) Components of the AIN-93 diets as improvements in the AIN-76A diet. *J. Nutr*. **127**: 838S–841S

Rice, P. J., Adams, E. L., Ozment-Skelton, T., Gonzalez, A. J., Goldman, M. P., Lockhart, B. E., Barker, L. A., Breuel, K. F., DePonti, W. K., Kalbfleisch, J. H., Ensley, H. E., Brown, G. D., Gordon, S., Williams, D. L. (2005) Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge. *J. Pharmacol. Exp. Ther*. **314**: 1079–1086

Shigesue, K., Kodama, N., Nanba, H. (2000) Effects of maitake (Grifola frondosa) polysaccharide on collagen-induced arthritis in mice. *Jpn. J. Pharmacol*. **84**: 293–300

Stuelp-Campelo, P. M., de Oliveira, M. B. M., Leao, A. M. A. C., Carbonero, E. R., Gorin, P. A. J., Iacomini, M. (2002) Effect of a soluble $\beta$-D-glucan from the lichenized fungus Ramalina celastri on macrophage activity. *Int. Immunopharmacol*. **2**: 691–698

Suzuki, I., Itani, T., Ohno, N., Oikawa, S., Sato, K., Miyazaki, T., Yadomae, T. (1985) Effect of a polysaccharide fraction from Grifola frondosa on immune response in mice. *J. Pharmacobiodyn*. **8**: 217–226

Tabata, T., Yamasaki, Y., Ogura, T. (2004) Comparison of chemical compositions of Maitake (Grifola frondosa (Fr.) S. F. Gray) cultivated on logs and sawdust substrate. *FSTR* **10**: 21–24

Wu, M.-J., Cheng, T.-L., Cheng, S.-Y., Lian, T.-W., Wang, L., Chiou, S.-Y. (2006) Immunomodulatory properties of Grifola frondosa in submerged culture. *J. Agric. Food Chem*. **54**: 2906–2914

Zhang, Y., Mills, G. L., Nair, M. G. (2002) Cyclooxygenase inhibitory and antioxidant compounds from the mycelia of the edible mushroom Grifola frondosa. *J. Agric. Food Chem*. **50**: 7581–7585