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INVITED REVIEW

Antiinflammatory and Immunomodulating Properties of Fungal Metabolites

Cristina Lull,[1] Harry J. Wichers,[1] and Huub F. J. Savelkoul[2]

[1]*Agrotechnology and Food Innovations, Wageningen University and Research Center, Bornsesteeg 59, 6708 PD Wageningen, The Netherlands*
[2]*Cell Biology and Immunology Group, Wageningen University and Research Center, Marijkeweg 40, 6709 PG Wageningen, The Netherlands*

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We discuss current information on the ability of extracts and isolated metabolites from mushrooms to modulate immune responses. This can result in a more enhanced innate and acquired disease resistance. The major immunomodulating effects of these active substances derived from mushrooms include mitogenicity and activation of immune effector cells, such as lymphocytes, macrophages, and natural killer cells, resulting in the production of cytokines, including interleukins (ILs), tumor necrosis factor alpha (TNF)-α, and interferon gamma (INF)-γ. In particular, the ability of selective mushroom extracts to modulate the differentiation capacity of $CD4^+$ T cells to mature into T_H1 and/or T_H2 subsets will be discussed. As a consequence these extracts will have profound effects in particular diseases, like chronic autoimmune T_H1-mediated or allergic T_H2-mediated diseases. Immunosuppressive effects by mushroom components have also been observed. The therapeutic effects of mushrooms, such as anticancer activity, suppression of autoimmune diseases, and allergy have been associated with their immunomodulating effects. However, further studies are needed to determine the molecular mechanisms of the immunomodulating effects of mushrooms metabolites both individually and in complex mixtures, for example, extracts.

INTRODUCTION

The number of different mushroom species on earth is estimated at 140 000, of which may be only 10% are known. Meanwhile, of those approximately 14 000 species that we know today, about 50% are considered to possess varying degrees of edibility, more than 2000 are safe, and about 700 species are known to possess significant pharmacological properties [1, 2, 3, 4]. Mushrooms have long been attracting a great deal of interest in many areas of foods and biopharmaceuticals. They are well known for their nutritional and medicinal values [1, 4, 5, 6, 7, 8, 9]. In accordance to Breene [10] the gross composition of mushrooms is water (90%), and from the dry matter: protein (10%–40%), fat (2%–8%), carbohydrates (3%–28%), fiber (3%–32%), and ash (8%–10%) (the ash percentage is the fraction of dry matter that remains after incineration of the organic material in a sample, and is mainly composed of salts, metals, and so forth). Many species of mushrooms are cultivated worldwide. Global production increased to about 6.2 million tons in 1997, with a more than 12% increase annually from 1981 to 1997 [11]. Mushroom extracts have been increasingly sold as dietary supplements. The market value of mushroom dietary supplement products worldwide is about US$5–6 billion per year [12].

Medicinal mushrooms have an established history of use in traditional oriental therapies. Historically, hot-water-soluble fractions (decoctions and essences) from medicinal mushrooms were used as medicine in the Far East, where knowledge and practice of mushroom use primarily originated [4, 13, 14]. Mushrooms such as *Ganoderma lucidum* (Reishi), *Lentinus edodes* (Shiitake), *Inonotus obliquus* (Chaga), and many others have been collected and used for hundreds of years in Korea, China, Japan, and eastern Russia [4].

Mushroom metabolites are increasingly being utilized to treat a wide variety of diseases, particularly as they can be added to the diet and used orally, without the need to go through phase-I/II/III trials as an ordinary medicine, and they are considered as a safe and useful approach for disease treatment. A lot of scientific

Correspondence and reprint requests to Huub F. J. Savelkoul, Cell Biology and Immunology Group, Wageningen University and Research Center, Marijkeweg 40, 6709 PG Wageningen, The Netherlands; huub.savelkoul@wur.nl

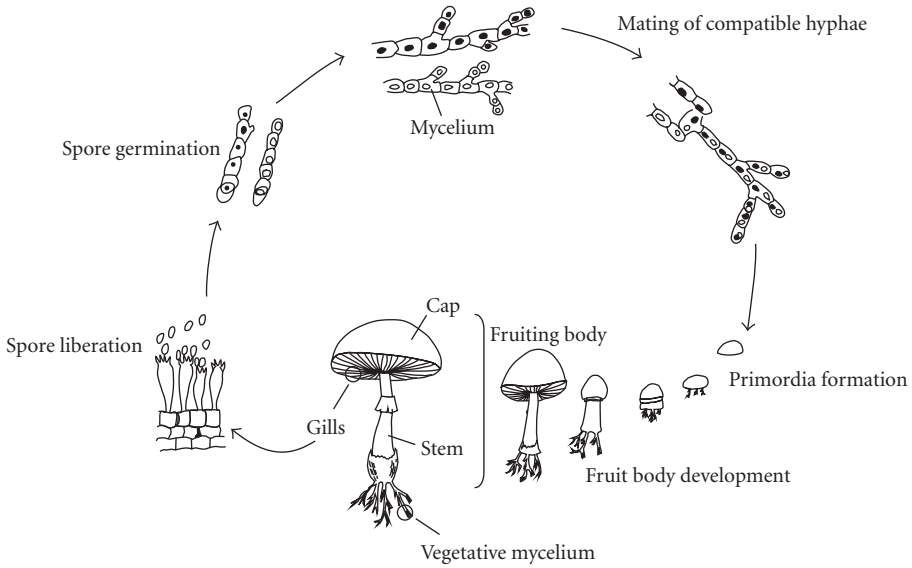


Figure 1. Diagrammatic representation of mushroom life cycle.

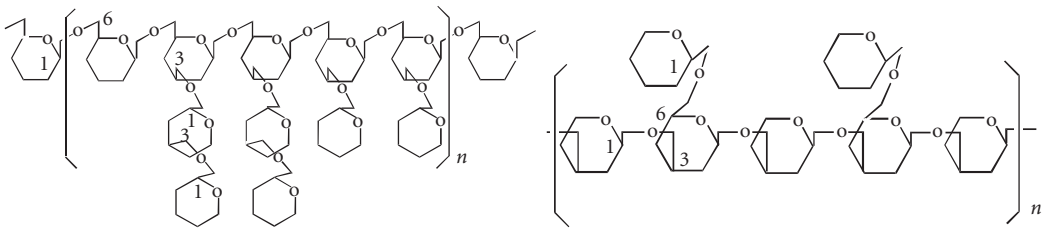
investigations have been performed to discover possible functional properties, which could be efficient in possible treatments of diseases like allergic asthma [15, 16, 17], food allergy [18, 19], atopic dermatitis [20], inflammation [21, 22], autoimmune joint inflammation such as rheumatoid arthritis [23], atherosclerosis [24, 25], hyperglycemia [26], thrombosis [27], human immunodeficiency virus (HIV) infection [28, 29], listeriosis [30], tuberculosis [31], septic shock [32], and cancer [33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55].

In the last years many researchers have studied the possibility that extracts and isolated metabolites from mushrooms stimulate or suppress specific components of the immune system. Immunomodulators can be effective agents for treating and preventing diseases and illnesses that stem from certain immunodeficiencies and other depressed states of immunity [56]. Synonymous terms for immunomodulators include biological response modifiers, immunoaugmentors, or immunorestoratives [57]. Those metabolites which appear to stimulate the human immune response are being sought for the treatment of cancer, immunodeficiency diseases, or for generalized immunosuppression following drug treatment, for combination therapy with antibiotics, and as adjuvants for vaccines [58]. Those metabolites that suppress immune reactions are potentially useful to mitigate autoimmune or certain gastrointestinal tract diseases (eg, Crohn's) [59].

At least 651 species and 7 infraspecific taxa representing 182 genera of hetero- and homobasidiomycetes mushrooms contain antitumor or immunostimulating metabolites [4]. Bioactive metabolites can be isolated from fruiting bodies (Figure 1), pure culture mycelia, and culture filtrate (culture broth). Nowadays many attempts are being made to obtain bioactive metabolites from mycelia through submerged fermentation culture. The cultivation of mushrooms to produce fruiting bodies is a long-term process requiring from one to several months for the first fruiting bodies to appear. The growth of mushroom cell cultures in submerged conditions in a liquid culture medium accelerates the process, resulting in biomass yield within a few days and allows to obtain standardized nutriceutical substances.

Several major substances with immunomodulatory and/or antitumor activity have been isolated from mushrooms. These include mainly polysaccharides (in particular β-D-glucans (Figure 2)), polysaccharopeptides (PSP), polysaccharide proteins, and proteins. Furthermore, other bioactive substances, including triterpenes, lipids, and phenols, have been identified and characterized in mushrooms with proven medicinal properties. The major immunomodulating effects of these active substances derived from mushrooms include mitogenicity and activation of immune cells, such as hematopoietic stem cells, lymphocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells, resulting in the production of cytokines. The therapeutic effects of mushrooms, such as anticancer activity, suppression of autoimmune diseases, and allergy have been associated in many cases with their immunomodulating effects.

Whilst it is known that mushroom extracts have immunomodulatory and/or antitumor activity, the standard approach has been to isolate, characterize, and administer the pure active constituents. However, different components in a mushroom extract may have synergistic activities [49, 60]. There are several reports of mushrooms containing more than one polysaccharide with antitumor activity. The responses to different polysaccharides are likely to be mediated by different cell surface receptors, which may be present only on specific subsets of cells and may



(a) (b)

Figure 2. Repeating unit of immunomodulatory β-glucans (a) from *Grifola frondosa* (D-fraction, MW: 1000 kD) and (b) from *L edodes* (lentinan, MW: 500 kD).

Table 1. Immunomodulatory activities of mushroom compounds on hematopoietic stem cells.

Species	Compound	Immune effects	Reference
Grifola frondosa	MD-fraction	↑ BMCs growth and differentiation into CFU-GM ↑ recovery of CFU-GM response after DOX induced hematopoietic suppression	[61]
Lentinus lepideus	PG101	↑ CFU-GM, BFU-E, IL-1β, IL-6, GM-CSF ↓ TNF-α in irradiated mice	[63]
Sparassis crispa	SCG	↑ granulocytes, monocytes, $\gamma\delta$T cells and NK1.1 cells in the peripheral cells in CY-induced leukopenia	[64]

trigger distinct downstream responses. A combination of such responses involving different cell subsets could conceivably provide greater tumor inhibition than could be induced by a single polysaccharide [49].

EFFECTS OF MUSHROOM METABOLITES ON HEMATOPOIETIC STEM CELLS

Various metabolites, especially carbohydrates isolated from mushrooms, were reported to affect bone marrow cells (BMCs), and to induce hematopoiesis (Table 1). Recently, Lin et al [61] reported that Maitake MD-fraction (obtained by further purification of D-fraction), an extract isolated from the fruit body of *Grifola frondosa* whose active component is an isolated β-glucan, a protein-bound polysaccharide compound, caused direct enhancement of the colony-forming units-granulocytes/macrophages (CFU-GM) response of BMCs progenitors and enhanced recovery of the CFU-GM response after doxorubicin (DOX) induced hematopoietic suppression. These studies suggest that MD-fraction has the potential to reduce hematopoietic suppression induced by chemotherapy.

PG101, a water-soluble extract that consists of protein-bound polysaccharides, isolated from cultured mycelia of *Lentinus lepideus* [62], is a potent immune modulator that recovers the radiation-damaged bone marrow system very efficiently. In PG101-treated mice, the number of CFU-GM and erythroid burst-forming units (BFU-E) were increased to almost the levels seen in nonirradiated control as early as 8 days after irradiation. Radiation is known to result in serious dysregulation of cytokine expression. PG101 increased the levels of IL-1β, IL-6, and granulocyte macrophage-colony-stimulating factor (GM-CSF) over the 24-day period. PG101 significantly reduced the level of TNF-α. TNF-α, which is increased as a consequence of tissue injury and anemia due to radiation, is thought to be a key mediator for the pathogenesis of radiation damage. Thus, PG101 showed great potential as a supplement or a major therapeutics in immunocompromised or immunosuppressed individuals whose bone marrow system is damaged [63].

SCG, a β-(1→3)-D-glucan with β-(1→6) branches isolated from fruit bodies of *Sparassis crispa*, enhanced the hematopoietic response in cyclophosphamide- (CY-) induced leukopenic mice by intraperitoneal routes over a wide range of concentrations. Monocytes and granulocytes in the peritoneal cavity, liver, spleen, and bone marrow recovered faster than in the control group. The ratio of NK cells and $\gamma\delta$T cells in the liver, spleen, and peritoneal cavity was also increased. These results suggest the usefulness of *S crispa* in cancer immunotherapy [64].

EFFECTS OF MUSHROOM METABOLITES ON THE INNATE IMMUNE SYSTEM

Macrophages

The recognition of microbes by macrophages and neurophilic granulocytes leads to phagocytosis of the microbes and activation of the phagocytes to kill the ingested microbes. Recognition is mediated by toll-like receptors (TLR) that are specific for different components of microbes. TLR-2 binds lipogycans, TLR-4 binds bacterial

lipopolysaccharide (LPS), TLR-5 binds flagellin, and TLR-9 binds unmethylated CpG nucleotides present in bacteria. As a consequence of recognition and phagocytosis several enzymes are activated, including oxidases and inducible nitric oxide synthase (iNOS), resulting in the production of bacteriocidal reactive oxygen intermediates (ROI) and nitric oxide (NO).

The effects of mushroom extracts and metabolites on macrophages have been extensively studied in vitro and in vivo. Some mushroom metabolites activate macrophages to produce various mediators, even in normal mice. Activities are summarized in Table 2.

Water extracts of the mycelial culture and fruiting bodies of *Agaricus blazei* Murill induced TNF-α secretion by macrophages derived from rat bone marrow. Fractions B-4 and B-5 obtained from ethanol precipitation of fruiting bodies markedly induced TNF-α secretion. Similar effects were observed in IL-8 secretion by macrophages. Regarding NO, fraction B-5 induced a significant increase in NO secretion and fractions B-4 and B-6 slightly induced NO secretion. Northern blot analysis showed that the increases in cytokine and NO secretion were due to an increase in cytokine mRNAs or NO synthase mRNA [65]. Thus *A blazei* Murill contains certain components which activate macrophages contributing to the immune response in vitro.

Wang et al [66] reported that after treatment of macrophage cultures with a polysaccharide from fresh fruiting bodies of *G lucidum*, the levels of IL-1β, TNF-α, and IL-6 were 5.1-, 9.8-, and 29-fold higher than in cultures of untreated cells. In addition, the release of INF-γ from T lymphocytes was also greatly enhanced in the presence of this polysaccharide. This proinflammatory cytokine response is suggested to facilitate the antitumor activity of this extract.

Grifolan (GRN), an antitumor β-glucan isolated from *G frondosa* induced the release of IL-1, IL-6 and TNF-α from macrophages [67, 68]. Ishibashi et al [69] reported that an insoluble as well as a high-molecular-mass soluble form of GRN are required for TNF-α production by macrophages.

The effect of Maitake D-fraction was studied by Sanzen et al [70] on the iNOS-mediated NO production in RAW264.7 macrophages with special reference to antitumor activity of MD-fraction against human hepatoma-derived huH-1 cells and the data suggested that MD-fraction is a novel inducer for iNOS which contributes at least in part to antitumor activity of MD-fraction.

Kodama et al [30] examined the effects of Maitake D-fraction on the treatment of *Listeria*-infected mice in combination with vancomycine (VCM). In mice administered with both D-fraction and VCM, macrophages produced 2.7 times as much IL-1β as that of nontreated control mice. The bactericidal activity of splenic T cells was also enhanced by 2.6 times of that of nontreated control mice. These results suggest a clinical benefit of D-fraction in the case of antibacterial treatment for patients with high risks.

Monocytes/macrophages seem to be the major target cell type responsive to PG101. Jin et al [62] proposed that PG101 interacts with macrophages or related cells and results in the activation of the transcription factor nuclear factor kappa B (NF-κB), which sets off a series of reactions producing a variety of proinflammatory and antiinflammatory cytokines (TNF-α, IL-1β, IL-10, IL-12, GM-CSF, IL-18) in a sequential manner. Inflammatory-cytokine-induced phosphorylation of a degradative motif in IκB triggers IκB proteolysis, liberating NF-κB from the inactive heterodimer and NF-κB transcription which in turn prevents cytokine-induced death of inflammatory cells. Despite its significant biological effect on various cytokines, PG101 remained nontoxic in both rats and human peripheral blood mononuclear cells (hPBMCs) even at a biological concentration approximately 20 times greater. PG101 demonstrates great potential as a therapeutic immune modulator.

A galactomannan isolated from a polar extract of *Morchella esculenta* carpophores enhanced macrophage activation. At 3.0 μg/mL the galactomannan polysaccharide (about 2.4% protein) increased NF-κB-directed luciferase expression in THP-1 human monocytic cells to levels of 50% of those achieved by maximal activating concentration (10.0 μg/mL) of LPS [71].

By administering PL, an acidic polysaccharide isolated from *Phellinus linteus*, the production of NO and tumoricidal activity were increased in murine peritoneal macrophages in vivo and in vitro. PL has been claimed to cause the inhibition of tumor growth and metastasis of murine B16F10 melanoma cells [72]. Such properties of PL may be related to its ability to induce the production of the tumoricidal effector molecule NO through protein tyrosine kinase (PTK) and protein kinase C (PKC) [73]. Considering the main role that proinflammatory cytokine production plays in the pathogenesis of septic shock, Kim et al [32] examined how the in vivo administration of PL can modulate circulating cytokine responses in LPS-treated mice. Administration of PL in vivo decreased IL-2, IFN-γ, and TNF-α production in splenocytes and enhanced spontaneous cell apoptosis in macrophages and lymphocytes stimulated with LPS in vitro. Thus, part of the antiinflammatory effects of PL treatment in vivo may result from the enhanced apoptosis of a portion of the activated macrophages and lymphocytes. The ability of PL to significantly reduce TNF-α production indicates the potential of the polysaccharides in possible therapeutic strategies that are based on down regulation of TNF-α [32].

The methanol extract of fruit bodies of *Cordyceps pruinosa* inhibited IL-1β, TNF-α, NO, and prostaglandin E_2 (PGE_2) in vitro and in vivo. The extract inhibited these inflammatory mediators in LPS-stimulated murine macrophage cell line RAW264.7 and primary macrophages, by suppressing gene expression of IL-1β, TNF-α, iNOS, and cyclooxygenase-2 (COX-2) through the inhibition of NF-κB activation. Administration of the extract significantly decreased the plasma level of these

Table 2. Immunomodulatory activities of mushroom products on macrophages.

Species	Product	Immune effects	Reference
G frondosa	D-fraction	↑ IL-1β	[30]
L lepideus	PG101	↑ TNF-α , IL-1β, IL-10, IL-12, GM-CSF, IL-18	[62]
A blazei	Water extracts mycelia and fruit bodies	↑ TNF-α	[65]
	Fractions B-4 and B-5	↑ TNF-α , IL-8, NO	
G lucidum	Polysaccharide	↑ IL-1β , TNF-α, IL-6	[66]
G frondosa	GRN	↑ IL-1, IL-6, TNF-α	[67, 68, 69]
G frondosa	MD-fraction	↑ iNOS	[70]
M esculenta	Galactomannan	↑ macrophage activity	[71]
P linteus	PL	↑ NO ↓ IL-2, IFN-γ, and TNF-α production in splenocytes ↓ apoptosis of a portion of the activated macrophages and lymphocytes in LPS-treated mice	[72, 73]
C pruinosa	Methanol extract	Inhibit IL-1β, TNF-α , NO, PGE_2	[74]
S aspratus	Fucogalactan	↑ TNF-α, NO	[75]
A cylindracea	Ubiquitin-like peptide	↑ NO	[77]
T mongolicum	Lectins (TML-1, TML-2)	↑ TNF-α, Nitrite ions	[78]

inflammatory mediators in LPS-injected mice. These results suggest that the *C pruinosa* methanol extract suppresses inflammation through suppression of NF-κB-dependent inflammatory gene expression, suggesting that the *C pruinosa* extract may be beneficial for treatment of endotoxin shock or sepsis [74]. Also, the methanol extract of fruit bodies of *Pleurotus florida* showed anti-inflammatory and antiplatelet-aggregating activities but the exact mechanism for these activities is unknown [21].

A fucogalactan, isolated from *Sarcodon aspratus*, elicited the release of TNF-α and NO in macrophages of mice in vitro. TNF-α production induced with 50 μg/mL of fucogalactan was significantly higher than that induced by lentinan (500 μg/mL) by approximately 4.3-fold. Mizuno et al [75] suggested that the immunomodulating activity of this fucogalactan on TNF-α and NO productions might contribute to antituor activity in tumor-bearing hosts as well as various immunomodulating effects.

In mice treated with an immunosuppressive carcinogen, administration of a mushroom-enriched diet containing *L edodes*, *G frondosa*, and *Pleurotus ostreatus* restored the normal level of the chemotactic activity of macrophages and the capability of lymphocytes to proliferate in response to mitogen [76].

Proteins and peptides from mushrooms are also known to activate macrophages. A ubiquitin-like peptide isolated from fruiting bodies of the mushroom *Agrocybe cylindracea* enhanced NO production in murine peritoneal macrophages with a potency comparable to that of LPS [77]. Two lectins isolated from the mushroom *Tricholoma mongolicum* (TML-1 and TML-2) stimulated the production of nitrite ions and TNF-α by macrophages in normal and tumor-bearing mice [78].

Natural killer cells

Natural killer cells are a class of lymphocytes that rapidly respond to intracellular infections with viruses or bacteria, by killling the infected cells and by producing the macrophage-activating cytokine, IFN-γ.

Some mushroom metabolites exhibit stimulating effects on NK cells (Table 3). Innate immunity is in the critical arms of immune surveillance against tumor development. Moreover, in the innate immune system, NK cells, which do not express T-cell receptors that recognize specific peptides presented on the major histocompatibility complex (MHC), rather than T cells, seem well suited for this role. NK cells can recognize the surface changes that occur on a variety of tumor cells and virally infected cells [79]. NK cells have two relevant functions, related to the natural immune response against pathogens [80]. One is cytotoxicity, mediated by the recognition and lysis of target cells such as virus- and bacteria-infected cells. The second NK cells function is to produce cytokines such as IFN-γ, TNF-α, and GM-CSF, that can modulate natural and specific immune responses. Additionally, infected or activated DCs and macrophages produce cytokines and chemokines such as IFN-α/β, IL-12, IL-15, and IL-18 that stimulate NK cells to rapidly produce other cytokines (including IFN-γ, TNF-α, and GM-CSF) and chemokines (such as ATAC/lymphotactin, mig, and MIP-1α) [81].

Kodama et al [41, 82] monitored levels of NK cell cytotoxic activity in cancer patients receiving D-fraction. Elevated levels of cytotoxic activity were maintained for one year. To elucidate the mechanisms underlying long-term activation of NK cells during treatment with D-fraction, the authors examined tumor volume and levels of IFN-γ and TNF-α in MM46-bearing C3H/HeN mice to which D-fraction was administered for 19 days. D-fraction

Table 3. Immunomodulatory activities of mushroom products on NK cells.

Species	Compound	Immune effects	Reference
G frondosa	D-fraction	↑ TNF-α, IFN-γ released from spleen cells; TNF-α expressed in NK cells in tumor-bearing mice ↑ NK cells activity (INF-γ) indirectly through IL-12 produced by macrophages and DCs in normal mice	[41, 82, 83, 84]
A blazei	Hot-water extract	↑ NK activity of spleen cells in naïve BALB/c mice	[85]
A blazei	*n*-hexane Dichloromethane Methanol	Maintain NK activity of spleen cells in tumor-bearing mice	[87]
A blazei	ABMK	↑ NK activity on cancer patients	[88]

markedly suppressed tumor growth, corresponding with increases in TNF-α and IFN-γ released from spleen cells and a significant increase in TNF-α expressed in NK cells. Furthermore, D-fraction increased macrophage-derived IL-12, which serves to activate NK cells. Thus, NK cells are not only responsible for the early effects of D-fraction on tumor growth, but also for the long-term tumor-suppressive effects of D-fraction through increased IL-12 released from macrophages. D-fraction was capable of enhancing and maintaining peripheral blood NK cell activity in patients with lung and breast cancer [41]. In addition, Maitake D-fraction, stimulated the natural immunity related to the activation of NK cells indirectly through IL-12 produced by macrophages and DCs in normal mice [83]. IFN-γ production by splenic NK cells increased significantly 3 days after D-fraction administration. In a recent study, Kodama et al [84] reported the activation of macrophages and DCs in normal mice as well. Therefore, administration of D-fraction to healthy individuals may serve to prevent infection by microorganisms.

Treatment with hot-water extracts of *A blazei* fruiting bodies increased NK activity of spleen cells in naïve BALB/c mice [85]. In meth A-bearing BALB/c mice, the same extracts enhanced the induction of antigen-specific cytotoxic T lymphocytes (T_C) and IFN-γ production. Up regulation of NK and T_C activity is triggered by IL-12-dependent activation [86]. It is not yet clear whether oral administration of *Agaricus* extracts enhances IL-12 production in vivo [85].

Ehrlich-carcinoma-bearing mice treated with the *n*-hexane, dichloromethane, or methanol extracts from *A blazei* fruiting bodies were able to maintain the NK activity of spleen cells during the first 10 days after tumor implantation. The NK activity of these groups was similar to that of normal controls and higher than that of tumor-bearing mice treated with water. The results of NK activity on the 30th day after the injection of tumor cells suggest that none of the three extracts was able to maintain the lytic activity against Yac-1 target cells. It is possible that after 30 days the production of soluble factors like prostaglandins, TGF-β, or IL-10 by Ehrlich carcinoma cells was enough to prevent the increase of NK activity by the *n*-hexane extract [87].

Ahn et al [88] investigated the beneficial effects of the consumption of an extract of *A blazei* Murill Kyowa (ABMK) on immunological status and qualities of life in cancer patients undergoing chemotherapy. They observed that NK cell activity was significantly higher in the ABMK-treated group and suggested that ABMK treatment might be beneficial for gyneacological cancer patients undergoing chemotherapy.

The medicinal fungus water extract (FWE) consists of equal amounts of *Coriolus versicolor*, *Cordyceps sinensis*, *L edodes*, *A blazei*, and *G lucidum*. Zhang et al [89] reported that FWE enhanced the phagocytosis of peritoneal macrophages, promoted NK activity in mice, and suppressed the growth of B-16 melanoma. FWE had significantly promoted mouse NK activity at the dose of 400 mg/kg, which suggests that FWE may possess the ability to activate NK to directly kill tumor cells, induce NK to secrete cytotoxic agents to elicit the apoptosis of tumor cells, or remove tumor cells by other pathways.

Dendritic cells

DCs are antigen-presenting cells (APC) with a unique ability to induce primary immune response of both helper (T_H) and T_C [90]. Beside activating naive T cells, DCs can directly activate naive and memory B cells. DCs at different stages of differentiation can regulate effectors of innate immunity such as NK cells and NK T cells. The induction of tumor immunity can be initiated by the effectors of innate immunity and further developed by cells of adaptive immunity, with DCs playing a central regulatory role.

Cao and Lin [91] studied the regulatory effects of Gl-PS, *G lucidum* polysaccharides (GLPS), on maturation and function of cultured murine bone-marrow-derived DCs in vitro. Gl-PS could promote not only the maturation of cultured murine bone-marrow-derived DCs, but also the immune response initiation induced by DCs.

PL induced maturation of bone-marrow-derived DCs and readies them for T-cell-mediated immune responses. PL significantly increased membrane molecules, including MHC class I, II, CD80, and CD86, and IL-12p70 in DCs. Also, PL markedly reduced the endocytic activity of DCs and augmented their capacity to promote the proliferation of naïve allogeneic T cells [92]. PL enhanced

the phenotypic and functional maturation of DCs *via* TLR-2- and/or TLR-4-mediated NF-κB, ERK, and p38 MAPK signal pathways. It is the first article reporting that a polysaccharide from mushrooms can activate a TLR signaling [93]. Kim et al [94] reported that the administration of PL induced antitumor and immunomodulating activities via maturation of $CD11c^+CD8^+$ DCs in tumor-bearing mice. The inhibitory effect of PL on the growth of MCA-102 tumor cells was associated with its immunoregulatory properties, including the induction of IL-12 and IFN-γ production leading to a T_H1 dominant state. Therefore, PL would be useful in preventing tumor growth, and it also has the advantage of having no side effects.

The existence of a strongly immunosuppressive state in cancer-bearing individuals inhibits DCs maturation. Kanazawa et al [95] reported that a protein-bound polysaccharide K (PSK) isolated from the cultured mycelium of *C versicolor* promoted both the phenotypic and functional maturation of DCs derived from human $CD14^+$ mononuclear cells. PSK has also been reported to resolve the immunosuppressive state of a cancer-bearing host and might be associated with DCs maturation directly [95]. Activities of mushroom metabolites on DCs are summarized in Table 4.

Complement

Activation of complement by either the classical or alternative pathway results in the generation of a wide spectrum of biological activities with the potential to modify immune responses [96, 97]. Particularly, the activation of complement via the alternative pathway is important in natural immunity to bacterial infections [98, 99].

Although there are a few reports concerning the relationship between complement-activating and tumor-regressing activity of glucan including lentinan, the positive correlation between the two activities was found by Okuda et al [100]. They observed a correlation between the ability to activate complement via the alternative pathway in vitro and inhibition of tumor growth in vivo. However, the opposite result, no correlation, was found by Hamuro et al [101]. Thus there is no consistent view on the correlation between the two antagonozing activities.

ABP-F and ABP-M, fine particles of *A blazei* Murill fruiting body and mycelium, respectively, prepared by mechanical disruption, activated the human complement system via the alternative pathway in human serum (Table 5). When particles from fruiting bodies of *A blazei* Murill (ABP-F) were reacted with human serum, the formation of complement-opsonized ABP, iC3b-ABP-F complexes, and binding of the complexes to human peripheral blood monocytes, were demonstrated in vitro by immunofluorescence. Further, the resident human peripheral nucleated cells incubated in the presence of iC3b-ABP-F complexes inhibited the proliferation of the human tumor cell line TPC-1 in vitro [102].

An alkali extract from cultured mycelium of *G lucidum* activated both classical and alternative pathways of complement [103]. Min et al [104] reported that triterpenoids such as ganoderiol F, ganodermanondiol, and ganodermanontriol from *G lucidum* had a potent anticomplement activity against the classical pathway with IC_{50} values of 4.8–4.17 μM. A clinical study in elderly patients with insomnia and palpitation has shown that taking *G lucidum* essence for 4–6 weeks increased their serum C3 levels [105].

Also, LELFD, a β-(1→3)-glucan, obtained from liquid-cultured mycelium of *G frondosa*, could activate the alternative complement pathway [106].

Anticomplementary activity of 61 strains of higher fungi from Korea was screened for immunostimulation [107]. Extracts from 11 of 61 strains, including 5 of *G lucidum*, 3 of *L edodes*, 2 of *Cordyceps sp*, and 1 of *Agaricus campestris*, showed higher anticomplementary activity than Krestin from *C versicolor*. The most potent anticomplementary activity was found with an extract from *L edodes* IY105, that reduced complement capacity by 31.7%.

EFFECTS OF MUSHROOM METABOLITES ON ADAPTIVE IMMUNE SYSTEM

T lymphocytes

T lymphocytes include T-helper (T_H) cells and cytotoxic T (T_C) cells. T_H cells interact with B cells and help them to divide, differentiate, and make antibody or interact with mononuclear phagocytes and help them destroy intracellular pathogens. T_H cells generate their effects by releasing soluble cytokines and/or by direct cell-cell interactions. The T_C cells destroy target host cells that have been infected by pathogens.

T_H cells

$CD4^+$ cells secrete a number of cytokines that are important in the activation of B and other T cells, as well as cells of the innate immune system. Based on the types of cytokines these $CD4^+$ cells produce, they are classified into a number of T_H types (0, 1, 2, or 3). T_H1 cells produce IL-2, IFN-γ, and TNF-β (LT), and introduce cellular immunity to mainly intracellular infections organisms. T_H2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, and activate humoral immunity, mainly directed against extracellular infections. Precursor or T_H0 cells produce IL-4 and IFN-γ concomitantly. Less is known about the physiological role of T_H0 type cells. Thymus-derived regulatory T-cell populations, including naturally occurring $CD4^+CD25^+$ T cells and inducible IL-10 or TGF-β-producing T_R/T_H3 cells, develop in the periphery from T_H cells depending on the tolerance-inducing microenvironment in which these T cells reside. By blocking activation of other lymphocytes and APC either directly (by CTLA4-CD28 interaction) or indirectly (by cytokines like IL-10 and TGF-β), these cells ensure self-tolerance mechanisms. In diseased states, however, the presence and/or

Table 4. Immunomodulatory activities of mushroom compounds on DCs.

Species	Compound	Immune effects	Reference
G lucidum	Gl-PS	↑ proliferation of one-way MLC induced by DC ↑ phenotypic and functional maturation of DC ↑ membrane molecules, including MHC I, II, CD80, and CD86, and IL-12p70 in DC ↓ endocytic activity of DC ↑ capacity of DC to promote the proliferation of naïve allogenic T cells and readies them for T-cell-mediated immune responses	[91]
P linteus	PL		[92]
C versicolor	PSK	Promoted both the phenotypic and functional maturation of DC derived from human $CD14^+$ mononuclear cells	[95]

Table 5. Immunomodulatory activities of mushroom compounds on complement.

Species	Compound	Immune effects	Reference
A blazei	Fine particles of fruiting body: ABP-F, and mycelium: ABP-M	Activation of the human complement system via the alternative pathway in human serum	[102]
G lucidum	Alkali extract	Activation of both classical and alternative pathways of complement	[103]
G lucidum	Triterpenoids	Anticomplement activity	[104]
G frondosa	LELFD	Activation of the alternative complement pathway	[106]

activity of these cells is often reduced leading to enhanced immunopathology, characteristic of chronic inflammatory diseases, like auto-immune and allergic diseases.

The downstream immune response is chosen depending on which subtype of T cell is activated, which means that the proportion of the activated sub-types influences phylaxis immunity and antitumor immunity. This control system is also affected by the production of IL-1β, IL-12, and IL-18 by APC [108, 109]. The development of T_H1 or T_H2 types from naïve cells to effector cells is regulated by the presence of specific cytokines in the microenvironment at the time of T cell priming. For the T_H1 type, IL-12 is a necessary cytokine of differentiation [110], whereas for the T_H2 type, IL-4 and IL-10 are critical [111]. Recent study shows that many immune disorders are attributable to the collapse of the system controlling the proportion of T_H1 to T_H2 cells [112]. Many diseases such as leprosy, allergy, multiple sclerosis, and responses to immunotoxic agents have pathology associated with aberrant T_H1 and T_H2 polarization. T_H1 cells may cause immunopathology and organ-specific autoimmune disease if dysregulated [113, 114, 115, 116]. Because cytokines produced by T_H2 cells, such as IL-4 and IL-5, can activate mast cells and eosinophils and in addition can result in elevated levels of IgE, they have been strongly implicated in atopy and allergic inflammation [117]. Restoration of the proper balance between T_H1 and T_H2 cells is generally considered essential in the treatment of tumors, which are generated when cellular immunity is affected by immunosuppressing factors.

Some mushroom polysaccharides might induce a type 1 immune response, whereas others favor a type 2 polarization [49, 118]. Borchers et al [49] reported that the limited data available to date do not allow one to determine whether mushroom polysaccharides do so independently of the animal strain or species and disease state investigated or whether the nature of their immunomodulatory effects depends on the model to a greater extent than has been appreciated to date.

Lentinan has been described as a T-cell-oriented adjuvant [119]. The skewing of T_H1/T_H1 balance to T_H1 by lentinan (Table 6) is directed through the distinctive production of IL-12 versus IL-6, IL-10, and PGE_2 by peritoneal macrophages, depending on intracellular glutathione redox status [120]. Based on the intracellular content of glutathione, two classes of macrophages have been proposed with diverse functional consequences: reductive macrophages with high, and oxidative macrophages with low glutathione levels.

Sclerotinia sclerotiorum glucan (SSG) from *Sclerotinia sclerotiorum* IFO 9395 induced the development of T_H1 cells via the IL-12 pathway [118].

Inoue et al [121] investigated the antitumor functions of D-fraction in relation to its control of the balance between T lymphocyte subsets T_H1 and T_H2. D-fraction decreased the activation of B cells and potentiated the activation of T_H cells, resulting in enhanced cellular immunity. It also induced the production of IFN-γ, IL-12p70, and IL-18 by whole spleen cells and lymph node cells, but suppressed that of IL-4. These results suggest that D-fraction establishes T_H1 dominance which induces cellular immunity in the population that was T_H2 dominated due to the presence of this particular carcinoma [121]. In a later study, Harada et al [122] reported that D-fraction induces the differentiation into T_H1 cells of $CD4^+$ T cells in tumor-bearing BALB/c mice

Table 6. Immunomodulatory activities of mushroom compounds on T cells.

Species	Compound	Immune effects	Reference
F velutipes	Fve	T_H1 response	[18]
S sclerotiorum IFO 9395	SSG	T_H1 response	[118]
L edodes	Lentinan	T_H1 response	[120]
G frondosa	D-fraction	Enhances T_H1 dominant response through enhancement of IL-12p70 and IFN-γ produced by activated DCs	[121, 122]
V volvacea	Vvo	↑ T_H1-specific cytokines (IL-2, IFN-γ , LT), T_H2-specific cytokine (IL-4), TNF-α, and IL-2R	[123, 124]

in which the T_H2 response was dominant through enhancement of IL-12p70 production by DCs, when the ratio of $CD8\alpha^+$ DCs to $CD8\alpha^-$ DCs increased. In addition, examination of the tumor rejection effect of D-fraction-stimulated DCs loaded with tumor antigen revealed that tumor growth is inhibited completely by activating $CD4^+$ T cells and $CD8^+$ T cells. Furthermore, the level of TNF-α, which is produced by activated macrophages and NK cells and is cytotoxic for tumor cells, increased by D-fraction-DCs injection, indicating that D-fraction enhanced the protective immunity by DCs loaded with tumor antigen through activating macrophages and NK cells. Although the action of D-fraction on DCs and its intracellular signal transduction pathway remain unclear, D-fraction may be a useful stimulator of DCs, which induce the differentiation of $CD4^+$ T cells to T_H1 cells [122].

Vvo, a fungal immunomodulatory protein (FIP) purified from the edible mushroom, *Volvariella volvacea*, induced most T_H1-specific cytokines (IL-2, IFN-γ, and LT) and one T_H2-specific cytokine (IL-4) within 4 hours in mouse spleen cells. This result indicates that Vvo principally acts on T_H1 cells and to a lesser extent on T_H2 cells in the early event of activation. It is known that IL-4 acts on B cells to induce activation and differentiation, leading in particular to the production of IgE. The lower effect of Vvo compared with other FIPs on the prevention of systemic anaphylaxis may be attributed to the elevated expression of IL-4 [123, 124].

Fve, a FIP isolated from the fruiting body of *Flammulina velutipes*, selectively stimulates a T_H1 response in hPBMCs [18]. Recently Hsieh et al [18] have characterized the immunomodulatory effects of Fve in more detail and investigated the prophylactic use of Fve via the oral route in a murine model of food allergy. They have demonstrated that oral administration of Fve during allergen sensitization could induce a T_H1-predominant allergen-specific immune response in mice and protect the mice from systemic anaphylaxis-like symptoms after subsequent oral challenge with the same allergen. It is worth noting that Fve could be administered orally and retain its activity, while most protein drugs cannot. This characteristic greatly promotes the potential of immunoprophylactic use of Fve [18]. Liu et al [16, 17] have demonstrated the efficacy of local nasal immunotherapy (LNIT) for group 2 allergen of house dust mite *Dermatophagoides-pteronyssinus-* (Dp2-) induced airway inflammation in mice, using Dp2 peptide and Fve or LZ-8, a FIP isolated from *G lucidum*.

B cells

Three polysaccharides isolated from *G lucidum*, two heteroglycans (PL-1 and PL-4) and one glucan (PL-3) enhanced the proliferation of T and B lymphocytes in vitro to varying contents and PL-1 exhibited an immune stimulating activity in mice [125].

PGL, a complex β-D-glucan, has a strong effect on suppressing the antibody production [126].

GLIS, a proteoglycan isolated from the fruiting body of *G lucidum*, is a B-cell stimulating factor. This compound stimulated B lymphocyte activation, proliferation, differentiation and production of immunoglobulins. The activation of B cells by GLIS may be associated with the expression of PKC α and PKC γ in B cells [127]. GLIS stimulated the proliferation of mouse spleen lymphocytes, resulting in a threefold to fourfold increase in the percentage of B cells. GLIS also activated mouse spleen lymphocytes, and most of the activated cells were B cells [127].

PL selectively activates murine B cells but not T cells [128]. Since PL cannot penetrate cells due to its large molecular mass (approximately 15 kD), this selectivity may be caused by the surface binding of this molecule to receptors specifically expressed on B-cells but not on T cells. The B-cell receptor, BCR, consists of surface immunoglobulin and CD79a-CD79b. Upon BCR ligation, the BCR-associated kinase Lyn phosphorylates CD79a-CD79b. In addition, coreceptors such as CD19 and CD38 positively regulate BCR signaling. Complement receptor CD11b-CR3, or Mac-1, is expressed on the surface of macrophages and NK cells and has been identified as the receptor of β-glucans [129]. Although PL and β-glucans show different specificities on B and T cells, they may use the same receptor on B cells. A further complete investigation of the membrane receptors of PL should shed light on its selectivity for B cells.

Table 7. Immunomodulatory activities of mushroom compounds on B cells.

Species	Compound	Immune effects	Reference
G lucidum	PL-1, PL-3, PL-4	↑ T and B lymphocytes proliferation ↑ antibodies	[125]
G lucidum	PGL	↓ antibody production	[126]
G lucidum	GLIS proteoglycan	↑ proliferation of mouse spleen lymphocytes ↑ B lymphocyte activation, proliferation, and differentiation and production of immunoglobulins	[127]
P linteus	PL	↑ murine splenic lymphocytes and activation of B cells	[128]
G lucidum	LZ-8	↓ antibody production	[130]
F velutipes	Fve	↓ antibody production	[131]

Evidence that FIPs suppress antibody production came from the result that the proportion of Arthus reaction-positive mice was reduced to 40% by LZ-8 [130]. Fve also suppressed antibody production as demonstrated by its effect in the hind paw edema test but the inhibition was not complete [131]. Activities are summarized in Table 7.

Figure 3 summarizes the targets for interaction between mushroom ingredients and various components of the adaptive immune system.

RECOGNITION AND RECEPTORS

Evidence for β-glucan receptor binding of immune cells

The innate immune system is the first line of defense against microbial invasion, and must immediately recognize and counter infections while the slower, more specific, adaptive response is mounted. The innate cellular response is comprised principally of phagocytic cells and is dependent on germline encoded receptors which recognize conserved microbial structures. The innate immune system identifies infectious agents or compounds by means of pattern-recognition receptors (PRR). These receptors recognize pathogen-specific macromolecules called pathogen-associated molecular patterns (PAMP).

Polysacharides cannot penetrate cells due to their large molecular mass, so the first step in the modulation of cellular activity is binding to immune cell receptors. Among all the immunomodulatory metabolites isolated from mushrooms, glucans and in particular β-glucans have been studied profoundly to identify its target receptor in immune cells. It has been postulated that glucans are fungal pattern-recognition molecules for the innate immune system [132, 133]. The mechanisms by which the innate immune system recognizes and responds to fungal cell wall carbohydrate is a very complex and multifactorial process [134]. The various activities of β-glucans may reflect the presence of multiple cellular targets or receptors [135]. To date several β-glucan receptors have been identified as candidates mediating these activities [136], namely, complement receptor 3 (CR3, $\alpha_M\beta_2$ integrin, or CD11b/CD18) [137], lactosylceramide [138], scavengers receptors [139], dectin-1 [140], and toll-like receptors TLR-2 and TLR-4 [141].

Dectin-1 is broadly expressed, with highest surface expression on populations of myeloid cells (monocyte/macrophage and neutrophil lineages) in the blood, bone marrow and spleen. DCs, and a sub-population of T cells , also expressed dectin-1 but at lower levels [142]. It is plausible that the expression of dectin-1, as a T-cell binding receptor, on a subset of T-cells may be part of a novel mechanism for the regulation of the T cell response by specific subsets of T cells as well as by APC [143].

Recently, Kim et al [93] have shown that PL, proteoglycan isolated from *P linteus*, could induce the phenotypic and functional maturation of DCs via TLR-2 and/or TLR-4. Shao et al [141] suggested that TLR-4 is also involved in GLPS-mediated macrophage activation. Rat antimouse TLR-4 monoclonal antibody (AB) inhibited the proliferation of BALB/c mouse B cells under GLPS stimulation. Combination of Abs against mouse TLR-4 and immunoglobulin achieved almost complete inhibition of GLPS-induced B-cell proliferation, implying that both membrane Ig abd TLR-4 are required for GLPS-mediated B cell activation.

Lowe et al [134] reported that a β-D-(1→3)- linked glucan polymer composed of seven glucose subunits is the minimum binding ligand for glucan PRR on a human monocyte cell line and indicated that all available monocyte glucan receptors will recognize the basic β-D-(1→3)-glucan structure with approximately the same affinity. However, as the glucan polymer becomes more complex it appears to be preferentially recognized by one glucan receptor versus another.

Additional studies are required to determine which receptor(s) are essential to the expression of the various immunobiological effects ascribed to β-glucans. The intracellular events that occur after glucan-receptor binding have not been fully determined. As long as it remains unclear what receptors are involved in and what downstream events are triggered by the binding of these glucans to their target cells, it will be difficult to make further progress in understanding their biological activities.

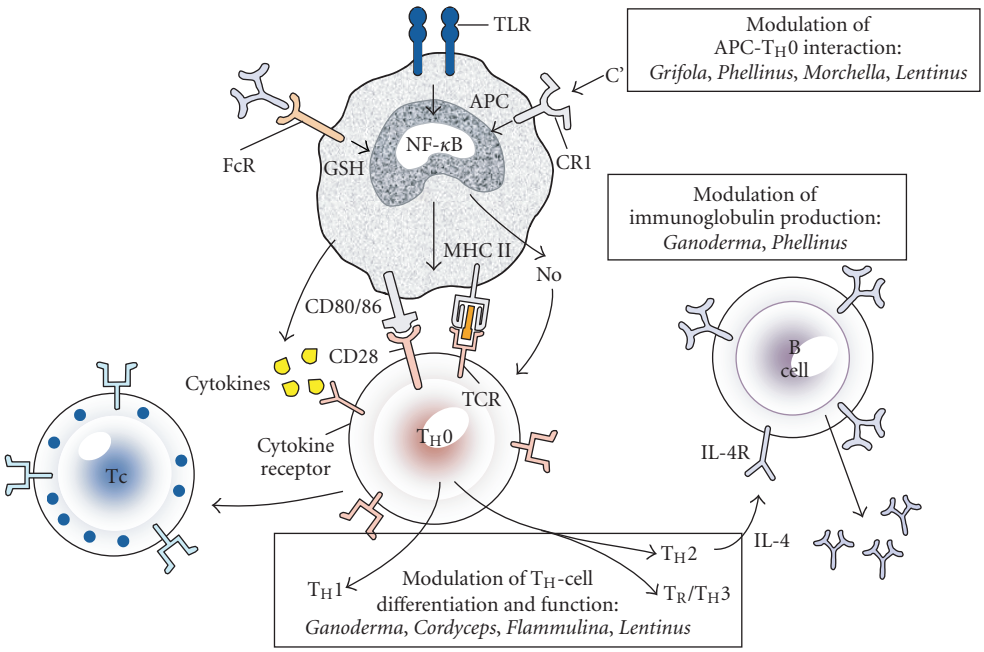


FIGURE 3. Schematic representation of the possible targets of the adaptive immune system for mushroom ingredients with immunomodulatory properties. APC: antigen-presenting cell; FcR: Fc receptor; TLR: Toll-like receptor; CR1: complement receptor type 1; C': activated complement; GSH: glutathione; MHC II: major histocompatibility complex class II; TCR: T-cell receptor; T_H: helper T cells; T_C: cytotoxic T lymphocytes; T_R: regulatory T cells; NO: nitric oxide; IL-4: interleukin-4; IL-4R: interleukin-4 receptor; CD: cluster designation.

CONCLUSIONS

The information presented here illustrates the distinct immunomodulatory properties associated with mushroom constituents. The discovery and identification of new safe drugs, without severe side effects, has become an important goal of research in the biomedical science. Medicinal effects have been demonstrated for many traditionally used mushrooms, with large differences in immunomodulatory properties. The species studied so far represent a vast source of immunomodulating and antitumor extracts and metabolites. Thus, the biochemical mechanisms that mediate the biological activity are still not clearly understood. Mushroom metabolites are known to stimulate different cells of the immune system. The major immunopotentiation effects of these active substances include mitogenicity, stimulation of hematopoietic stem cells, activation of alternative complement pathway, and activation of immune cells, such as T_H cells, Tc cells, B cells, macrophages, DCs, and NK cells.

Different profiles have been observed in relation to the activated immune cells, for example, GLPS activate mouse B cells and macrophages but not T cells [141], polysaccharides from *P linteus* can stimulate B cells, T cells, and macrophages [144], while lentinan is a stimulator of T cells and macrophages, but not B cells [145]. Some of them might promote a T_H1 response and others a T_H2 response [49]. In the particular case of glucans, despite the structural and functional similarities of some of them, they differ in their ability to elicit various cellular responses, particularly cytokine expression and production and in their effectiveness against specific tumors [5]. The relationship between polysaccharide origin, structure, and their immunomodulation activity remains to be further characterized [125, 146].

Mushroom products are obvious immunoenhancers that potentiate the immune system in multiple ways. Mushroom polysaccharides are among the emerging new agents that could directly support or enhance functional autologous hematopoietic stem cell recovery [61]. In preventive medicine, defense against invasion by foreign bodies is dependent on enhancing the natural immune system, including activation of macrophages and NK cells. Macrophages stimulated by mushroom products release several inflammatory cytokines, IL-1, IL-6, IL-8, TNF-α, and NO, all of which directly induce tumoricidal activity in macrophages. Macrophages produce also IL-1β, IL-10, IL-12, GM-CSF, and IL-18. In other cases mushroom extracts inhibit the production of NO, PGE_2, IL-1β, and TNF-α in LPS-stimulated macrophages and LPS-administer mice. This antiinflammatory effect occurs by down regulation of iNOS, COX-2, IL-1β, and TNF-α gene expression via the suppression of NF-κB activation. Thus, these mushroom extracts might be relevant for clinical use for inflammatory diseases, including endotoxemia or sepsis. Some mushroom metabolites like D-fraction

represent an important biological response modifier (BRM) due to the enhancement of NK cells activity in cancer patients. Mushroom polysaccharides induce regulatory effects on maturation and function of DCs and consequently enhance the capacity of DCs to promote the proliferation of naïve allogenic T cells and readies them for T-cell-mediated immune responses. Both classical and alternative pathways of complement have been activated by mushrooms and also anticomplementary activity has been detected in different mushrooms. T and B lymphocytes are also activated by mushrooms. Some mushroom polysaccharides stimulate the production of antibodies but others as PGL have a strong effect on suppressing the antibody production [126].

The immunomodulating action of mushroom metabolites is specially valuable as a means of prophylaxis, a mild and noninvasive form of treatment, prevention of metastatic tumors, and as a cotreatment with chemotherapy [4]. The enhancement or potentiation of host defense mechanisms has been recognized as a possible means of inhibiting tumor growth without harming the host, but other alternative mechanisms are possible, like targeting the *ras*-mediated signaling pathway [147]. Whether certain metabolites enhance or suppress immune responses can depend on a number of factors, including dose, route of administration, and timing of administrations of the compound in question. The type of activity these metabolites exhibit can also depend on their mechanism of action or the site of activity. Taken together, the present data suggest that mushroom extracts or metabolites should be selected and used properly for modulation of immune responses. Due to the differences in activities among various extracts and isolated metabolites, it is imperative to evaluate its biological properties before any suggestions for use of a particular product in clinical practice. For example, D-fraction enhanced rather than suppressed the development of collagen-induced arthritis (CIA) [148]. Administration of D-fraction stimulates immune function of normal and tumor-bearing mice [84]. GLIS from *G lucidum* has an effect on lymphocytes or purified B cells from tumor-bearing mice markedly stronger than on lymphocytes or purified B cells from normal mice [127]. It has also been reported that an extract from the deep layer of cultivated mycelia of the Cov-1 strain of *C versicolor* enhances the immune functions in old mice but not in young mice [149].

For some of the mushroom metabolites described, further research is needed to determine whether there are any in vivo benefits comparable to the in vitro effects reported. Although it is unlikely that high molecular weight polysaccharidse would be absorbed after oral administration, it is possible that it could exert a therapeutic effect by direct interaction with the mucosal immune system of the gastrointestinal tract. Thus, they could be developed as a preparation for use as a dietary supplement or pharmaceutical.

Some mushroom metabolites, such as the glucans lentinan and schizophyllan, or the polysaccharide-protein PSK, and the PSP, are used clinically for immune therapy [150, 151, 152, 153] and have been developed as pharmaceuticals in Japan and are now commercially available worldwide. PSK was commercialized by Kureha Chemicals, Japan. After extensive clinical trials, PSK was approved for use in Japan in 1977, and by 1985, it ranked 19th on the list of the world's commercially most successful drugs [154]. Annual Japanese sales of PSK in 1987 were worth US$357 million [154]. About 10 years after PSK, PSP appeared on the market. Both compounds have been isolated from *C versicolor*. In addition to clinically tested PSK and PSP, numerous other extract preparations of *C versicolor* are on the market as neutraceuticals and traditional medicines. Neutraceutical PSP preparations are sold worldwide in the form of capsules, ground biomass tablets, syrups, food additives, and teas [153].

Quality control of mushrooms poses significant challenges: small differences in genetics, soil, temperature, moisture, and time of harvesting can lead to significant differences in the concentration of important constituents. The cultivation of mushrooms to produce fruiting bodies is a long-term process requiring from one to several months for the first fruiting bodies to appear. Nowadays, more research is carried out in relation to submerged culture. Submerged culture has potential advantages for higher mycelial production in a compact space and for a shorter incubation time with a lesser chance of contamination. Further optimization of the culture medium composition and physicochemical conditions of growth allows regulation of fungal metabolism in order to obtain standardized nutriceutical substances in higher yield. Mycelia formed by growing pure cultures in submerged culture is the best technique for obtaining consistent and safe mushroom products [3, 12, 155]. Mushrooms are still far from being thoroughly studied.

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Activation of the alternative complement pathway by *Agaricus blazei* Murill

S. Shimizu[1], H. Kitada[1], H. Yokota[2], J. Yamakawa[3], T. Murayama[4], K. Sugiyama[5], H. Izumi[1], and N. Yamaguchi[1]

[1]Department of Serology & Microbiology, Kanazawa Medical University, Uchinada Kahoku-gun, Japan
[2]Department of Radiology,
[3]Department of General Medicine, Kanazawa Medical University, Uchinada Kahoku-gun, Japan
[4]Division of oersistent and oncobenic viruses, Center of chronic viral disease, Faculty of medicine, Kagoshima University, Kagoshima, Japan
[5]Department of Clinical Pharmacokinetics, School of Pharmacy, Hoshi University, Tokyo, Japan

Summary

Components of *Agaricus blazei* Murill have been demonstrated to have a wide range of immunopotentiating activities. The present study was designed to evaluate the effect of *A. blazei* Murill upon activation of the complement system in human serum *in vitro*. Additional studies were performed to determine the cytotoxic effect of complement-opsonized particles of *A. blazei* Murill against human tumor cells in culture. A fine particle of *A. blazei* Murill (ABP), prepared by mechanical disruption, was used throughout the experiments. ABP activated the human complement system via the alternative pathway in human serum. Activation of the alternative pathway was both time- and dose-dependent. When the particles from fruiting bodies of *A. blazei* Murill (ABP-F) were reacted with human serum, the formation of complement-opsonized ABP, iC3b-ABP-F complexes, and binding of the complexes to human peripheral blood monocytes, were demonstrated *in vitro* by immunofluorescence. Further, the resident human peripheral nucleated cells incubated in the presence of iC3b-ABP-F complexes inhibited the proliferation of human tumor cell line TPC-1 *in vitro*.

Key words: *Agaricus blazei* Murill, complement, macrophage, cytotoxicity, tumor cell

Introduction

Biological response modifiers (BRM) derived from various natural sources have been investigated extensively for their anti-tumor (Di Luzio et al., 1976; Mansell et al., 1975; Di Luzio et al., 1980; Kitamura et al., 1994) and anti-infective (Williams et al., 1978; Di Luzio, 1984) activities. Lentinan (Chihara et al., 1969, 1970) and schizophyllan (Komatsu et al., 1969; Sugawara et al., 1984) are the best-known polysaccharides and have been used clinically for tumor immunotherapy.

We have already demonstrated the immunopotentiating activity of preparations of *A. blazei* Murill in mitomycin C-treated mice: a potential to restore impaired-immunological functions such as leukopenia, atrophy of spleen, depressed response to antigenic stimulation and shortening of survival time in syngeneic mouse tumor models (manuscript in preparation). Fujiyama et al. (1998) isolated an acid-treated fraction from *A. blazei* Murill and found that administration of the fraction resulted in the infiltration of natural killer (NK) cells with marked tumoricidal activity, using the double-grafted tumor system in mice. Although other groups have also reported that polysaccharides isolated from *A. blazei* Murill demonstrated marked tumoricidal activity (Mizuno et al., 1990; Kawagishi et al., 1990; Ito et al., 1994), the exact mode of action causing the tumoricidal effect is still unknown.

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It is well known that the activation of complement via the alternative pathway is important in natural immunity to bacterial infections (Alper et al., 1970; Winkelstein et al., 1975). On the other hand, although there are a few reports concerning the relationship between complement-activating and tumor-regressing activity of glucan including lentinan, the positive correlation between the two activities was found by Okuda et al. (1972); however, the opposite result, no correlation, was found by Hamuro et al. (1978). Thus there is no consistent view about the correlation between the two activities.

In the present study, the role of *A. blazei* structures in the activation of the human complement system *in vitro* was evaluated, in correlation with the induction of cytotoxic activity of the human peripheral blood nucleated cells co-cultured with *A. blazei* structures, opsonized with human serum *in vitro*.

Materials and Methods

Agaricus blazei Murill

Agaricus blazei Murill was supplied by Japan Applied Microbiology Research Institute, Ltd., Yamanashi, Japan. For industrial production of the satisfactory preparation, inspection of the products has been placed under the control of both the company and the Japanese government office, the Public Health Center. In order to get fine particles of *A. blazei* Murill, the fruiting bodies of the mushroom and cultured mycelium were kept in a Model PC-30 A dryer (Kuroda Industry Co., Japan) at 60 °C for 8 hours and treated by mechanical disruption with a universal homogenizer (Nihon Seiki Seisakusho Co., Japan) for 30 min. The resulting powder was treated further in an agate mortar with a pestle and applied to mesh (150 μm width). The two kinds of particulate preparations obtained in this manner from the fruiting body and the mycelium will be referred to throughout the text as ABP-F and ABP-M, respectively, and were used as stimulants for complement throughout the experiments.

As a stimulant for the alternative complement pathway, dextran sulfate (DS; Sigma, USA) was also examined. The molecular weigh of the 5 kinds of DS used ranged from 5,000 to 500,000.

Tumor

The human thyroid carcinoma, TPC-1, was maintained by successive cultivation at 5 day-intervals in RPMI 1640/10% FBS.

Preparation of human nucleated cells from peripheral blood

Blood was collected from a healthy volunteer into a heparin-containing syringe (1/10 volume for the blood). Peripheral blood nucleated cells (PBN) were isolated using Mono-Poly Resolving Medium (Dainippon Pharmaceutical Co., Ltd., Japan) according to the manufacture's specifications. After three washes with RPMI 1640 (Sigma, USA), the cells were suspended in RPMI 1640/10% FBS (Cell Culture Laboratories, USA). These cells were used in the phagocytosis assay and the cytotoxicity assay.

Preparation of fresh human serum

Serum as the source of a complement was collected from the same healthy volunteer (37 years of age, male) throughout the experiments. The blood was allowed to clot at 0 °C for 2 h. The clotted blood was then centrifuged at 2,500 rpm for 7 min at 4 °C in an refrigerated centrifuge. The serum was removed and maintained at 0 °C until used (within 30 min). Serum was used fresh for all experiments.

In order to differentiate between the two different pathways of complement activation, ethylenediamine-N-N-N′-N′-tetraacetic acid, disodium salt, dihydrate- (EDTA; WAKO, Japan) and 0, 0′-bis(2-aminoethyl)ethyleneglycol-N,N,N′,N′-tetraacetic acid (EGTA; WAKO, Japan) -plasma was collected from blood treated with 1/10 volume of 0.1 M EDTA, pH 7.4, and 0.1 M EGTA, pH 7.4, respectively. It has been demonstrated that classical pathway function requires both Mg^{2+} and Ca^{2+}, whereas activation of the alternate pathway requires only Mg^{2+}. By using EDTA, which is a chelator of both Ca^{2+} and Mg^{2+}, it is possible to block both pathways, the classical and the alternate. On the other hand, EGTA was shown to selectively block the classical pathway by chelation of Ca^{2+} (Fine et al., 1972).

Complement assays

In preliminary experiments, ABP was tested at final concentrations ranging from 0.1 mg/ml to 10 mg/ml serum (total volume of 1 ml). Reaction time was also tested at 37 °C (ranging from 30 min to 4 h) to determine the optimal condition for complement activation. After incubation, the reaction mixtures were centrifuged and the sera thus obtained were used for analysis immediately or stored at 0 °C until assayed (usually with 1 to 4 h). The fragmentation of complement component was measured using immunoelectrophoretic determination of conversion of factor B to activated factor B and C3 to iC3b, utilizing specific antisera.

Immunoelectrophoresis

Goat anti-human C3 proactivator (factor B; OTNS, Germany), goat anti-human C3 (MELOY, USA), and goat anti-whole human serum (E.Y., USA) were used. Immunoelectrophoresis was performed in 1.2% Agarose-II (WAKO, Japan) gel on a glass plate (Ko-

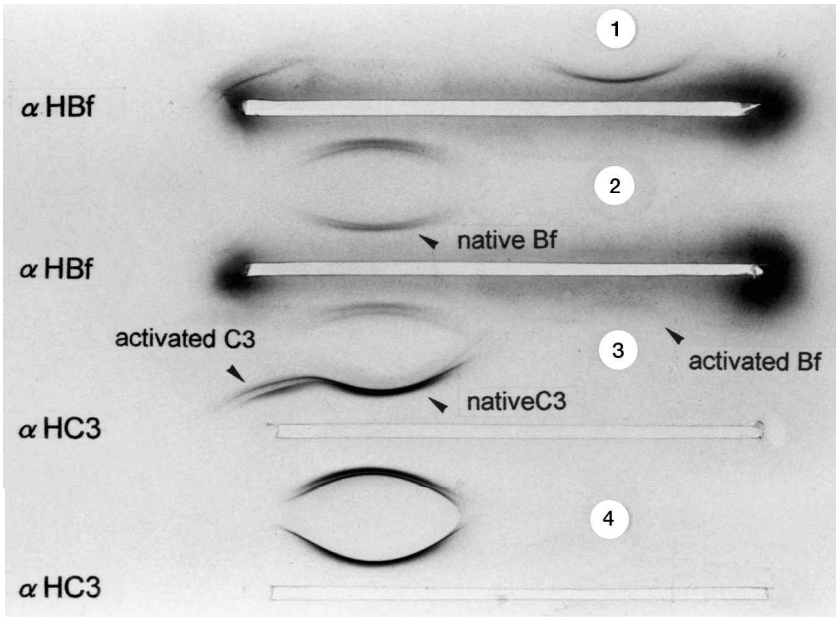

Fig. 1. Demonstration of the alternative pathway activation of the human complement system by ABP-M. Antigen wells contained: (1) 10 mg of ABP-M incubated for 4 h with normal serum, (3) EGTA-plasma and (2), (4) EDTA-plasma, respectively.

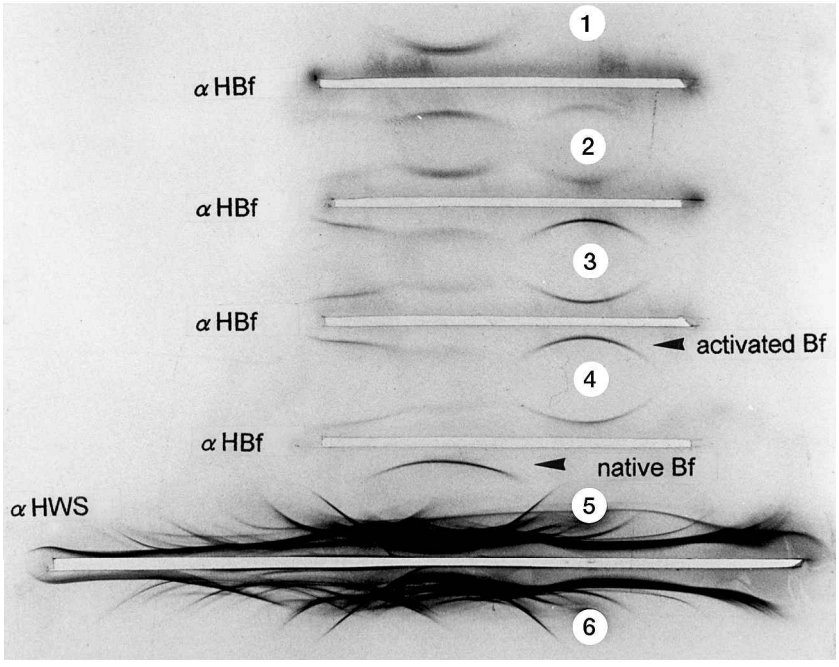

Fig. 2. Dose-dependent activation of the alternative pathways of the human complement system by ABP-M. Antigen-wells contained: (1) 1 ml of human serum incubated for 1 h with 0.1 mg, (2) 1 mg, (3) 5 mg, and (4) 10 mg ABP-M, respectively. Normal serum was placed in (5) and (6).

dak, 8.3 cm × 10.2 cm) using veronal-acetate HCl buffer, pH 8.6. Following electrophoresis, the precipitation reaction was performed using antisera described above for 48 h at room temperature.

Immunofluorescence

The detection of iC3b bound to the surface of ABP-F was accomplished using the direct immunofluorescence technique, employing fluorescein isothiocyanate (FITC)-conjugated goat anti-human C3c (MBL, Japan). The antiserum was absorbed by both PBN and ABP-F before use. One mg of ABP-F was reacted with 1 ml serum and the mixture was incubated at 37 °C for 2 h. After incubation, the reaction mixture was centrifuged, washed three times with Hank's balanced salt solution (HBSS, GIBCO-BRL, USA), and resuspended in 1 ml HBSS.

The aliquot of the opsonized ABP-F suspension thus obtained was spread on slide glasses and air-dried. The slide glasses were then stained with 1 ml solution con-

taining 15% FITC-goat anti-human C3c serum (GAHC3c) in HBSS for 1 h at 37 °C. Thereafter, the slide glasses were washed thoroughly with cold phosphate buffered saline (PBS), and the fluorescence associated with ABP-F was observed microscopically using an oil-immersion lens.

Phagocytosis of ABP-F with C3

The PBN (5×10^6 cells) suspended in 5 ml of RPMI 1640/10% FBS were layered onto glass cover-slips (22×22 mm) in tissue culture dishes (6 cm-diameter; Falcon, USA). The dishes were incubated for 2 h at 37 °C in a humidified CO_2 (5%) atmosphere. Nonadherent cells were removed by washing with a stream of HBSS. The cover-slips were then layered with 100 µl of iC3b-ABP-F complexes as described above or native ABP-F suspension (10 mg/ml). The dishes were then incubated at 37 °C for 4 h in a humidified CO^2 (5%) atmosphere (total volume, 5.0 ml). The cover-slips were washed with PBS to remove unbound targets, air-dried and fixed in 100% methanol. The slips were stained using a Diff-Quik stain (Kokusai-shiyaku Co., Ltd., Japan) and examined using a fluorescen microscope with an oil-immersion lens.

Cytotoxicity assay

RPMI 1640/10% FBS was used as culture medium. Isolated PBN (1×10^6 cells) and TPC-1 tumor cells (1×10^4 cells) were added into tissue culture dishes (6-cm diameter) at target: effector cell ratio of 1:100 (total volume, 5 ml). These dishes were incubated with 1 mg of iC3b-ABP-F complexes at 37 °C in a humidified CO_2 (5%) atmosphere. On days 3, 4, 5 and 6 after the incubation, TPC-1 cells were harvested from each dishes and cytotoxic activity was evaluated by measurement of the viable cell count of TPC-1 tumor cells in a hemocytometer employing the trypan blue dye (trypan blue 0.05%, w/v) exclusion method. All determinations were performed in duplicate.

Statistics

The results were evaluated statistically using the Student's *t* test.

Results

Activation of complement system

Fine particles from the fruiting body (ABP-F) or mycelium (ABP-M) of *A. blazei* Murill were each suspended to the desired concentration in human serum and the conversion of factor B to activated B was determined by immunoelectrophoretic patterns developed by anti-human factor B serum. As shown in Figures 1 and 4, factor B was converted to Bp fragments when normal human serum without chelating agents was incubated with both ABP-M and ABP-F. In order to confirm the activation pathway of C3 and factor B in human serum, experiments were carried out in the presence and absence of the chelating agent. The two preparations, ABP-M and ABP-F, were each incubated at a concentration of 10 mg/ml in human serum diluted 9/10

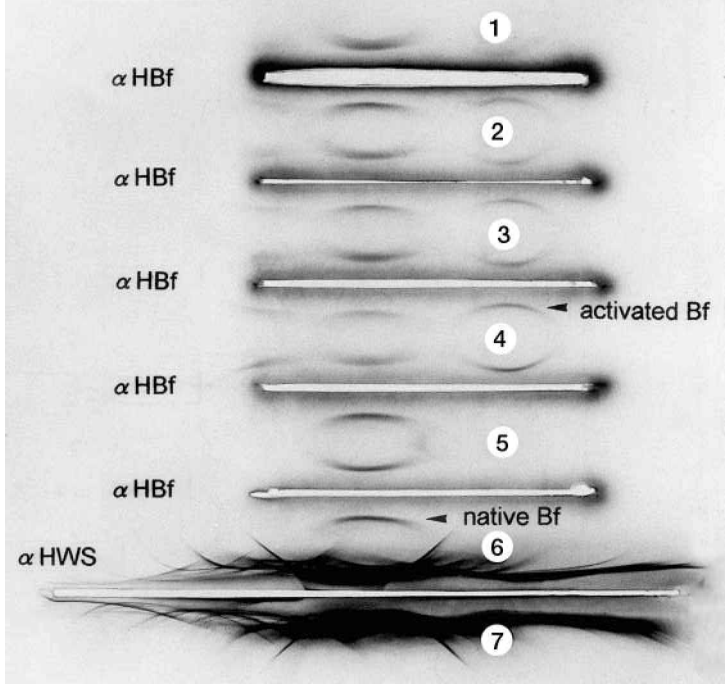

Fig. 3. Time-dependent manner of activation of the human complement system by ABP-M. Antigen well shows the activated product incubated with 1 mg ABP-M for (1) 30′, (2) 1 h, (3) 2 h and (4) 4 h, respectively; (5) EDTA-plasma were incubated for 2 h. Normal serum was placed in (6) and (7).
Note that the Bb fragment appeared at each incubation hour and became stronger according to the incubation hours. But the sample of EDTA-plasma did not show the Bb fragment.

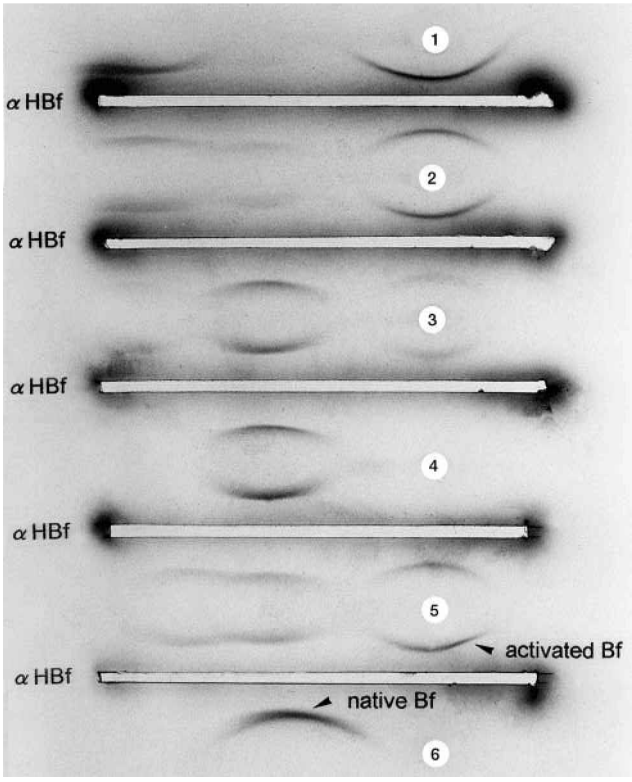

Fig. 4. The effect of ABP-F on the human complement system. Antigen wells in this figure contained: (1) 10 mg of ABP-F incubated for 2 hours with normal serum, (4) EDTA-plasma and (5) EGTA-plasma, respectively; (2) is equal to (1) except that reaction time was 30′; (3) is equal to (1) except for the low dose, 0.1 mg. Normal serum was placed in (6).

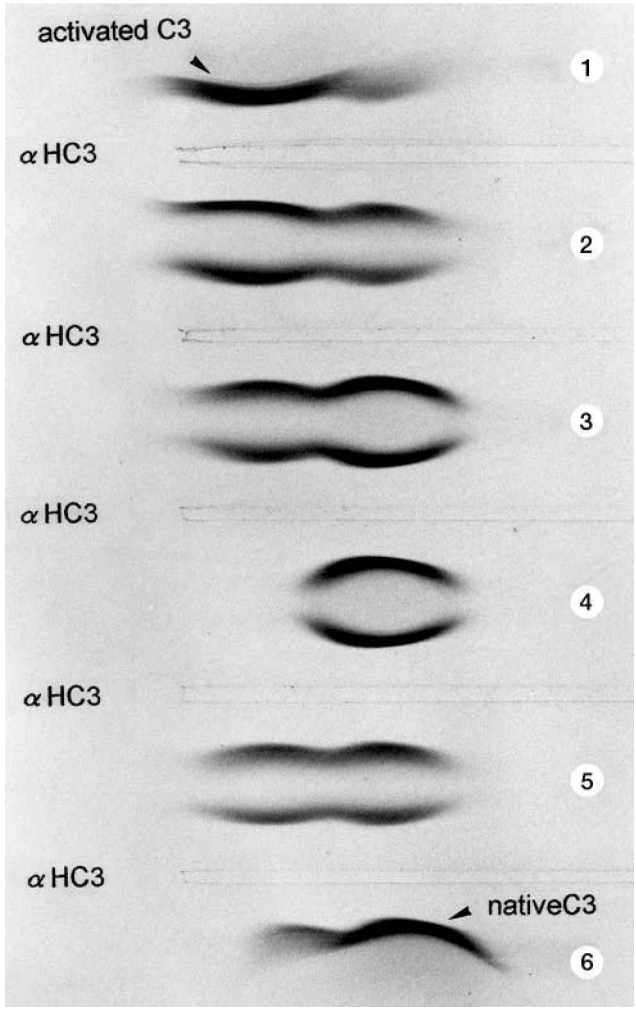

Fig. 5. The effect of ABP-F on the human complement system: Antigen wells contained: (1) 10 mg of ABP-F incubated for 2 hours with normal serum, (4) EDTA-plasma and (5) EGTA-plasma, respectively; (2) is equal to (1) except that reaction time was 30′; (3) is equal to (1) except for the low dose, 0.1 mg. Normal serum was placed in (6).

in a solution of EDTA or EGTA for 2 to 4 h at 37 °C. As shown in Figure 1, EGTA slightly fails to prevent activation of factor B in the presence of 10 mg/nl of ABP-M (well: 3). As shown in Figure 4, ABP-F with EGTA (well: 5) converted most of factor B to activated B at the concentration of 10 mg/ml. Under the conditions employed, ABP-F was more active on a weight basis than ABP-M in conversion of factor B in the presence of EGTA. On the other hand, by use of EDTA instead of EGTA in the above experiments, the conversion of both C3 and factor B were completely inhibited (in the case of ABP-F, see Figures 4–5 and in the case of ABP-M, see Figure 1). The dose responsibility of complement activation by ABP-M was measured by immunoelectrophoretic conversion of factor B to activated B. One ml of human serum was incubated at different concentrations ranging from 0.1 mg/ml to 10 mg/ml serum at 37 °C for 1 h. As shown in Figure 2, 0.1 mg of ABP-M/ml serum converted faintly factor B, and almost complete conversion of factor B was achieved by a dose of 10 mg of ABP-M/ml serum. The time course of the factor B conversion was measured. One mg of ABP-M/ml serum was incubated at 37 °C for definite time ranging from 30 min to 4 h. As shown in Figure 3, the precipitation line of Bb fragment migrated to the cathode appeared at each incubation time and became stronger according to the incubation time. But a sample of EDTA-plasma incubated with 1 mg of ABP-M at 37 °C for 4 h did not show Bb fragment (well: 5). Additionally, the dose- and time-dependency of the conversion of factors B (Figure 4) and C3 (Figure 5) by ABP-F was essentially identical to that of ABP-M.

As a stimulant besides *Agaricus* for the complement system, synthetic polysaccharide, dextran sulfate (DS) with different molecular weights ranging from 5,000 to

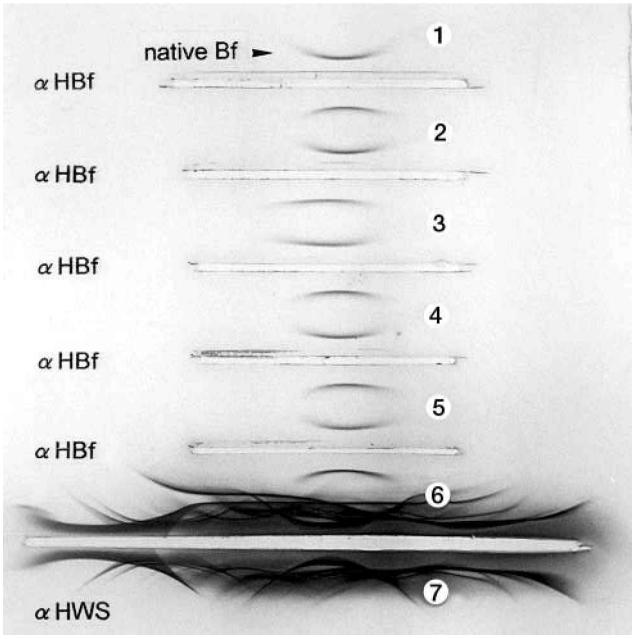

Fig. 6. The effect of dextran sulfate on the human complement system. Antigen wells contained: (1) 1 ml of human serum incubated for 2 hours with 5 mg dextran sulfate of molecular weight 500,000, (2) 40,000, (3) 10,000, (4) 8,000 and (5) 5,000, respectively. Normal serum was placed in (6) and (7).

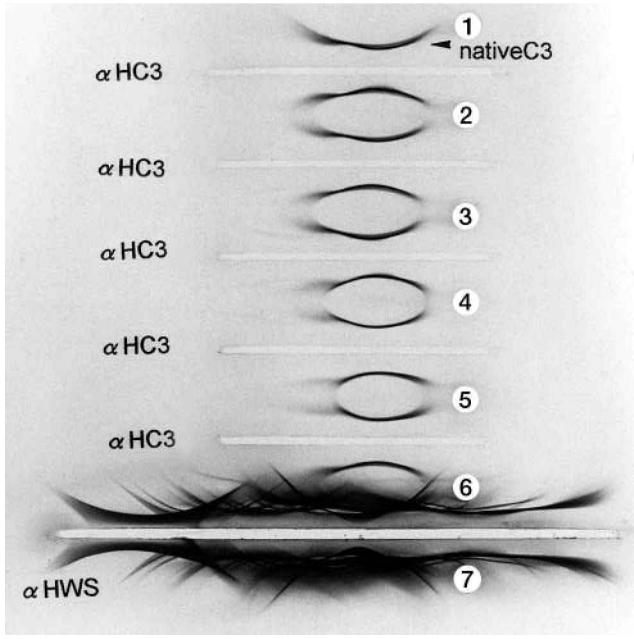

Fig. 7. The effect of dextran sulfate on the human complement system. The experimental conditions were the same as in Figure 6 except for using αHC3 as antibody.

500,000, was incubated with normal human serum (5 mg/ml) at 37 °C for 2 h. As shown in Figure 6, no DS examined cleaved human Bf. The result for the C3 component was essentially identical to that of Bf (Figure 7).

Binding of C3b to the surface of ABP-F

ABP-F incubated with normal human serum was reacted with FITC-labelled goat anti-human C3c. After washing, the reactants, iC3b-ABP-F complexes, were spreaded on slide glass and were observed under a fluorescence microscope. As shown in Figure 8, A, ABP-F coated with iC3b was generated by incubating ABP-F with normal human serum but not with HBSS (Figure 8, B).

Binding of C3-opsonized ABP-F to human peripheral monocytes

The binding of iC3b-ABP-F complexes to monocytes was examined by adding the complexes to monocyte cultures, after which the dishes were stained doubly with FITC-conjugated goat anti human C3c and Diff-Quik stain. As shown in Figure 9, A, iC3b-ABP-F complexes were illuminated well in the cytoplasm of cells morphologically compatible with monocyte-macrophage lineage, confirmed by the observation of their shape under the microscope (Figure 9, B).

Cytotoxicity of iC3b-opsonized ABP-F

In order to determine whether PBN which treated with iC3b-ABP-F complexes were capable of killing the TPC-1 cells, PBN and TPC-1 cells were co-cultured with or without the complexes, iC3b-ABP-F. At timed intervals, the number of viable tumor cells was counted using a haemocytometer and the dye exclusion method. As shown in Figure 10, the PBN co-cultured with iC3b-ABP-F complexes inhibited the proliferation of TPC-1 cells. The PBN co-cultured with native ABP-F did not cause such a marked inhibition of the proliferation. These results may indicate that the iC3b-ABP-F complexes triggered the cytotoxic activities of the resident PBN, possibly through binding of the complexes to the C3-receptor sites of the PBN.

Discussion

The present studies were performed in order to determine the efficiency of the *A. blazei* subcellular component, ABP-F from the fruiting body and ABP-M from the mycelium, in activation of the human complement

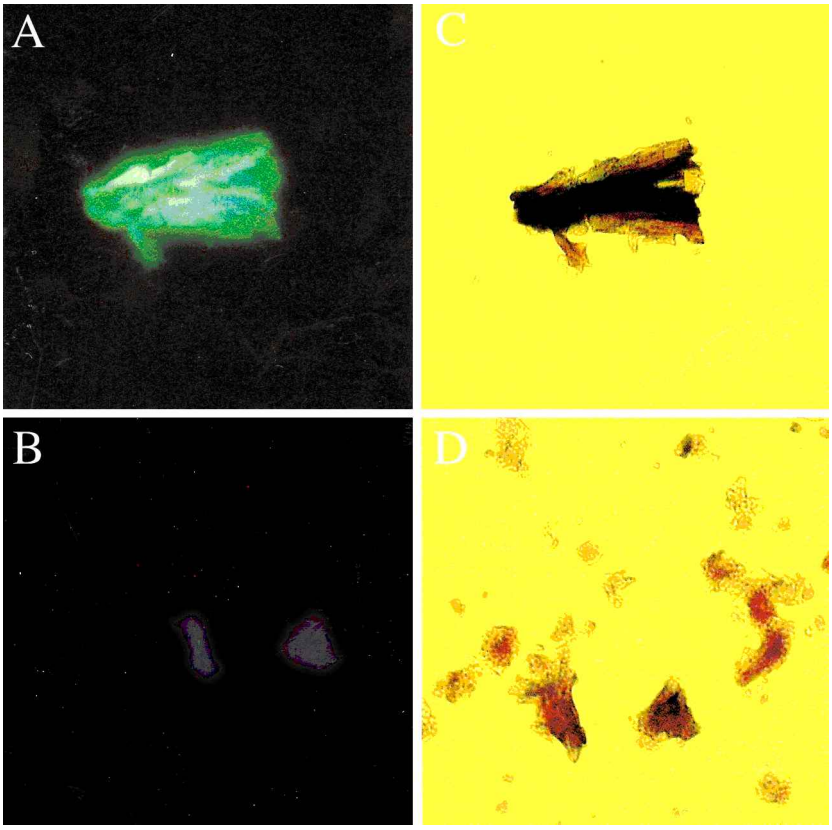

Fig. 8. Immunofluorescence with ABP-F bonded with activated human C3: (A) ABP-F were incubated with fresh human serum for 4 hours and then vigorously washed five times, after which FITC-labelled goat anti human C3c IgG was reacted for one hour at 37 °C. After five washings, iC3b-ABP-F complexes were spread on slide glass and were observed in UV spectrum by fluorescence microscope. (B) is the control experiment. ABP-F did not react with fresh human serum. Note that ABP-F bonded C3 showed illumination, but native ABP-F did not. (C) The photograph shows the same field as (A), but not taken UV. (D) The photograph shows the same field as (B), but not taken UV.

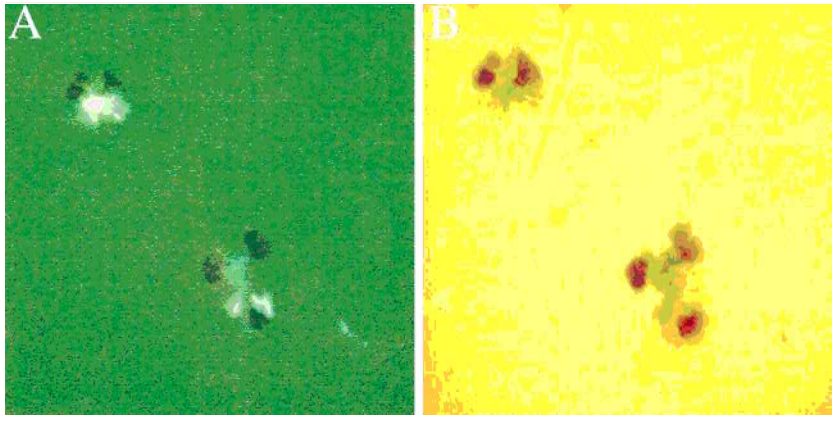

Fig. 9. Immunofluorescent analysis with iC3b-ABP-F complexes incorporated by human peripheral monocytes via C′ receptors: (A) Immunofluorescent iC3b-ABP-F complexes analyzed by FITC-labelled anti human C3c. Note iC3b-ABP-F complexes illuminated in monocytes. (B) The photograph shows the same field as (A), but not taken UV.

pathway. Both particles have been demonstrated to be potent activators of the complement system in human serum in a dose- and time-dependent manner (Figures 2–4).

Analysis of the conversion of the C3 and factor B precipitation line on immunoelectrophoresis of the serum treated with both ABP-F and ABP-M strongly suggested that these particles activated the complement system through the alternative pathway (Figures 1, 4, and 5). Although ABP-F was more active on a weight basis than ABP-M, it must be emphasized that our methods for isolating ABP-F and ABP-M are crude. The decreased ability of ABP-M, compared to that of ABP-F, in activation of the alternative pathway in the presence of EGTA, may be reflected by quantitative and qualitative differences of composition between the two preparations. Further studies are planned to identify and isolate the responsible moiety within the two preparations.

Activation of complement by either the classical or alternative pathway results in the generation of a wide spectrum of biological activities with the potential to

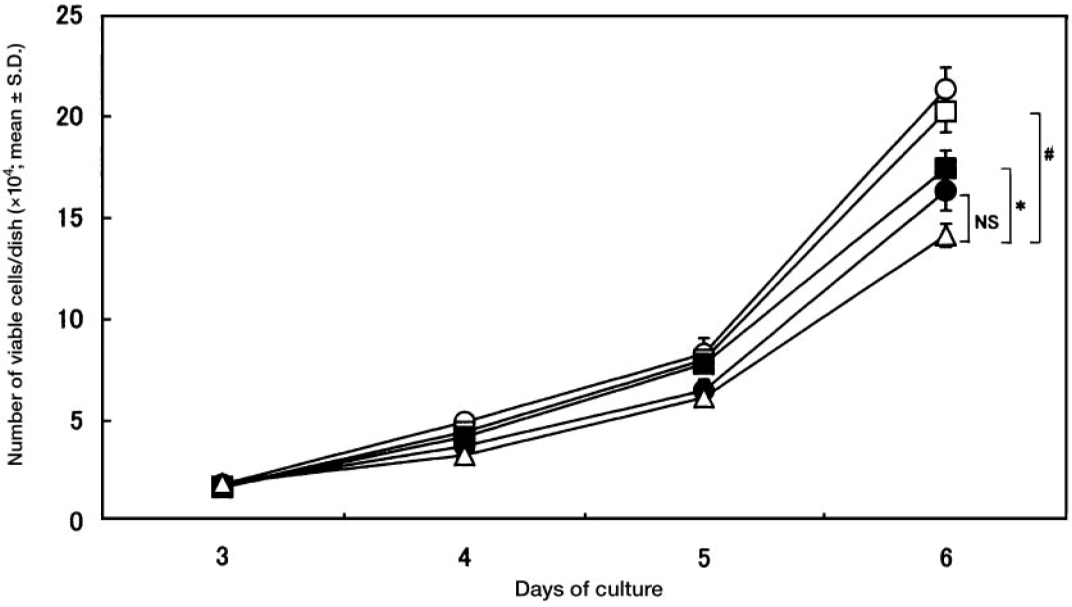

Fig. 10. Cytotoxicity of iC3b-ABP-F complexes *in vitro*. TPC-1 tumor cells were cultured with PBN in the presence of iC3b-ABP-F complexes or native ABP-F for 3–6 days. TPC-1 cells alone (○ – ○); TPC-1 cells + PBN (● – ●); TPC-1 cells + native ABP-F (□ – □); TPC-1 cells + PBN + native ABP-F (■ – ■); TPC-1 cells + PBN + iC3b-ABP-F (–). * $P < 0.05$ vs. the TPC-1 cells + PBN + native – *Agaricus* group; # $P < 0.05$ vs. the TPC-1 cells + native – *Agaricus*: NS: not significant. Bars indicate the SD.

modify immune response (Di Luzio, 1985; Ross et al., 1999). Okuda et al. (1972) reported on the complement-activating activity of a variety of anti-tumor polysaccharides. They observed a correlation between the ability to activate complement via the alternative pathway *in vitro* and inhibition of tumor growth *in vivo*. Somewhat similar observations were reported by Sadler et al. (1979) using a strain of *Corynebacterium parvum* that had been demonstrated to have antitumor activity. Hamuro et al. (1978), however, found just the opposite, i.e., ommitting the correlation in experiments with a variety of antitumor polysaccharides. The authors speculated that one of the reasons for the discrepancy might be due to differences in experimental conditions. Thus the contribution of glucan-induced complement activation via the alternative pathway to the demonstrated enhancement in antitumor resistance remains to be clarified. In the present study, our attention has been focused on the iC3b-ABP-F complexes, and whether or not the complexes co-cultured with PBN induce cytotoxicity against tumor cells. The reaction mixtures for the cytotoxicity assay are made up by washed-iC3b-ABP-F complexes as stimulants, PBN as effector and human tumor cells, TPC-1, as targets *in vitro*.

According to the interpretation of Ross et al. (1999), recent data concerning complement receptor type 3 (CR3) suggest that CR3 serves as the major receptor for β-glucan with human or mouse leukocytes (neutrophil, monocyte, macrophage, and NK cell), and may, therefore be responsible for all reported functions of β-glucan *in vitro* and *in vivo*. Thornton et al. (1996) and Vetvicka et al. (1996) reported that binding of particulate β-glucan to CR3 resulted in receptor priming for subsequent cytokine release triggered by ligation to an iC3b-opsonized target cell (EC3bi); however, EC3bi without β-glucan did not trigger NK cell cytokine release. Thus, after polysaccharide priming of CR3, ligation to an iC3b-target cell resulted in secretion of TNF-α, IFN-γ, IFN-α, and IL-6. In this context, the inhibition of TPC-1 cell proliferation observed in this study might be triggerred by dual ligation of these two CR3 binding sites, mediated by an iC3b fixed on the surface of ABP-F (Figure 8) for the protein site and a polysaccharide moiety distributed ont he same ABP-F surface for lectin site. Because the PBN tested in these experiments consist of various cell types, i.e., neutrophils, monocytes, and NK cells, it is impossible to specify effector cell(s) and/or factor(s) exhibiting the cytotoxicity at the present time. In addition, particulate β-glucan has been shown to be large enough to cross-link membrane CR3 of neutrophils and monocytes, triggering respiratory bursts, degranulation, and cytokine release (Ross et al., 1987; Vetvicka et al., 1996; Ljungman et al., 1998). In conformity with these views; the native-ABP-F showed a less than opsonized-ABP-F, but moderate, cytotoxic effect in our experiment (Figure 10). Schorlemmer et al. (1976)

reported that attachment of C3b to macrophages in culture results in a dose-dependent and time-dependent release of lysosomal enzymes with no loss of viability. At present it is unclear whether iC3b-ABP-F complex-mediated release of lysosomal enzyme could participate in TPC-1 inhibiting activity in our system.

In analogy with our study, Murayama et al. (1982) reported already that a Streptococcal preparation, OK-432, as an immunopotentiator, activated complement via the alternative pathway in mouse serum. The resident peritoneal exudate cells from mice co-cultured with C3-OK-432 complexes generated cytotoxic activity to the mouse mammary carcinoma, MM2, *in vitro*. This study has shown that *Agaricus blazei* structure activates the alternative pathway of complement, binds to macrophages, and is taken up by them. It also inhibits the growtn of human tumor cell, TPC-1, as a opsonized form, iC3b-ABP-F, in culture.

In our preliminary experiments, C3 components or its fragments which visualized by goat antimouse C3c IgG were detected in the liver of mouse treated with *Agaricus* orally by immunohistochemical technique (data nor shown), althouth the action of *Agaricus in vivo* after oral administration remains to be established.

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Address

Shoji Shimizu, Ph.D., Department of Serology, Kanazawa Medical University, Uchinada, Ishikawa 920-0293, Japan
Tel.: ++81-76-286-2211; Fax: ++81-76-286-2841;
e-mail: shi-shi@kanazawa-med.ac.jp

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Original Article

IL-12 Production Induced by *Agaricus blazei* Fraction H (ABH) Involves Toll-like Receptor (TLR)

H. Kasai[1], L. M. He[3], M. Kawamura[2], P. T. Yang[1], X. W. Deng[2], M. Munkanta[1], A. Yamashita[1], H. Terunuma[1], M. Hirama[3], I. Horiuchi[3], T. Natori[3], T. Koga[3], Y. Amano[1], N. Yamaguchi[4] and M. Ito[1]

[1]Interdiciplinary Graduate School of Medicine and Engineering and [2]Department of Alternative Medicine, University of Yamanashi Faculty of Medicine, Yamanashi, [3]Japan Applied Microbiology Research Institute Ltd, Tamaho, Yamanashi and [4]Department of Serology, Kanazawa Medical University, Uchinada, Japan

Agaricus blazei Murill is an edible fungus used in traditional medicine, which has various well-documented medicinal properties. In the present study, we investigated the effects of hemicellulase-derived mycelia extract (*Agaricus blazei* fraction H: ABH) on the immune system. First, we examined the cytokine-inducing activity of ABH on human peripheral mononuclear cells (PBMC). The results indicated that ABH induced expression of IL-12, a cytokine known to be a critical regulator of cellular immune responses. Flow cytometric analysis demonstrated the induction of IL-12 production by the CD14-positive cell population, consisting of monocytes/macrophages (Mo/Mϕ). Furthermore, the elimination of Mo/Mϕ attenuated IL-12 production in PBMC. ABH-induced IL-12 production was inhibited by anti-CD14 and anti-TLR4 antibodies but not by anti-TLR2 antibody. The activity of ABH was not inhibited by polymyxin B, while the activity of lipopolysaccharide used as a reference was inhibited. Oral administration of ABH enhanced natural killer (NK) activity in the spleen. These findings suggest that ABH activated Mo/Mϕ in a manner dependent on CD14/TLR4 and NK activity.

Keyword: Agaricus blazei Murill – IL-12, Toll-like Receptor – Monocyte/Macrophage

Introduction

Agaricus blazei Murill, an edible mushroom, shows immunomodulatory and antitumor activities (1–5). In Brazil, this fungus is used as a traditional medicine for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis and chronic hepatitis. The polysaccharides, proteoglycans, polysaccharide–protein complexes, glycoproteins and steroids from *Agaricus blazei* Murill mycelia and fruiting body extracts possess antitumor cytotoxic activities (3–10). However, the mechanisms of the antitumor functions of these mushroom components have yet to be determined. The bacterial lipopolysaccharide, mycoplasma lipopeptide (MALP-2), interacts with toll-like receptors (TLRs) and induces cytokine production. Similarly, Dectin-1, a fungal β-glucan receptor subunit, has been shown to interact with TLR2 and mediate cell activation (11,12). *Agaricus blazei* extract was shown to induce cytokine (IL-12) gene expression. Based on these observations, we hypothesized that *Agaricus blazei* Murill fraction H (ABH) may interact with TLRs and induce signals for IL-12 production. The specific objectives of this investigation were: (i) to identify the characteristics of the cell population in peripheral blood mononuclear cells (PBMC) stimulated by ABH; (ii) to determine the optimal concentration of ABH required to induce cytokine production *in vitro*; (iii) to identify the nature of TLR interacting with ABH on the cell surface; and (iv) to evaluate the influence of ABH on natural killer (NK) cell activity.

For reprints and all correspondence: Masahiko Ito, Interdiciplinary Graduate School of Medicine and Engineering, University of Yamanashi Faculty of Medicine, Tamaho, Yamanashi 409-3898, Japan. Tel: +81-55-273-9539; Fax: +81-55-273-6728; E-mail: mito@yamanashi.ac.jp

Subjects and Methods

Preparation of *A.blazei* Extracts

After 2 weeks in culture, *A.blazei* mycelia were digested with hemicellulase for 1 h at 45°C. Then, the enzymes were inactivated at 70°C and freeze-dried. This compound is similar to Agaricus Blazei Practical Compound (ABPC; Japan Applied

Microbiology Research Institute. Ltd, Yamanashi, Japan) and all ABPC products were made from this compound. Samples of 1.5 g of *A.blazei* compound were ground and mixed with distilled water to a final concentration of 0.1 g/ml (w/v). After centrifugation, the supernatant was collected and passed through a 0.45 μm filter (Millipore Co., Bedford, MA) for use in the experiments.

Preparation of PBMCs

Heparinized human peripheral blood was obtained from healthy donors. PBMCs were isolated using the Ficoll-Hypaque density-gradient method, as described previously (13). Peripheral blood was centrifuged at 2000 r.p.m. for 10 min to remove plasma. Blood cells were diluted with phosphate buffered saline (PBS), then overlaid onto Ficoll-Hypaque solution (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) and centrifuged at 2000 r.p.m. for 30 min. The PBMC layers were collected and washed twice with PBS. The cells were resuspended to a concentration of 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Reagents and Monoclonal Antibodies (mAb)

Anti-human (h) TLR4 mAb HTA125 (mouse IgG2a) and FITC-labelled HTA125 were purchased from BD Biosciences Clontech (San Jose, CA) and eBioscience (San Diego, CA). Anti-TLR2 mAb TL2.1 (mouse IgG2a) and FITC-labelled TL2.1 were purchased from eBioscience and Cascade Bioscience (Winchester, MA).

RNA Preparation Quantification and RNase Protection Assay

After stimulation of human PBMC, total RNA was extracted from the cells using TRIzol (Invitrogen Corp., Carlsbad, CA). RNase protection assay was performed using the hCK-2RiboQuant Multiprobe RNase Protection Assay system (BD Pharmingen, San Jose, CA) according to the manufacturer's instructions. Aliquots of 10 μg of RNA were used for each assay. Gels from three distinct experiments were analyzed using a BAS3000 and Image Gage (Fuji Film, Tokyo, Japan).

Quantification of IL-12p40 and p70 in Culture Supernatants

Isolated human PBMC were cultured in media containing ABH, 1 ml of 10% FCS/RPMI1640 with various concentrations of ABH, for 0–16 h. The levels of IL-12p40 in the supernatants were measured using an enzyme-linked immunosorbent assay (ELISA) kit, optEIA™ ELISA kit (BD Pharmingen) according to the manufacturer's protocol. The concentration of IL-12p40 and p70 was determined using the data analysis program Softmax (Molecular Devices, Menlo Park, CA).

NK Activity Assay

NK activities of human PBMC and murine spleen cells were determined by lactate dehydrogenase (LDH) assay. The NK-sensitive mouse cell line YAC-1 was used as target cells. Target and effector cells were mixed at the indicated effector/target (E/T) ratios at 0.2 ml/well using 96-well round-bottomed multi-well plates (BD Labware, Lincoln Park, NJ). After incubation for 4 h, cells were centrifuged at 250 *g* for 4 min, and then the cell-free supernatant was collected for LDH assay using CytoTox96 (Promega, Madison, WI). The percentage of specific LDH release was calculated by the following formula: % cytotoxicity = [(experimental LDH release) − (spontaneous LDH release by effector and target)/(maximum LDH release) × (spontaneous LDH release)] ×100. For the control experiments, the target cells were incubated either in culture medium alone to determine spontaneous release or in a mixture of 2% Triton X-100 to define the maximum LDH release. The spontaneous release was always <10% of the maximum release. All assays were performed in triplicate.

Results

Treatment of PBMC with ABH induced IL-12 Production

Expression of IL-12 messenger RNA (both p35 and p40 subunits) was determined in PBMC with or without ABH stimulation by RNase protection assay. As shown in Figure 1, IL-12 messenger RNA expression (p40 subunit) was induced within 4 h after ABH stimulation. In unstimulated controls, IL-12 messenger RNA was not induced until 16 h. We quantified the release of IL-12 p40 protein in human PBMC culture

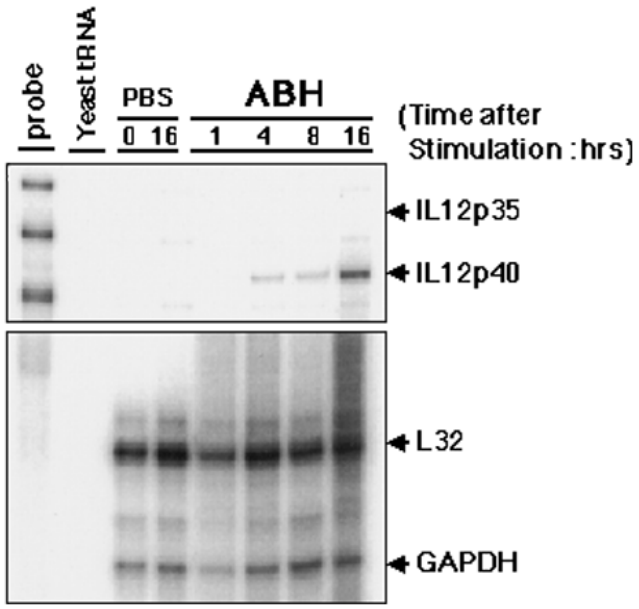

Figure 1. ABH induces IL-12 gene expression. Ficoll-isolated human PBMCs were stimulated with 40 ng/ml ABH for the indicated times. RNase protection assay was performed with total RNA from stimulated PBMC. Aliquots of 10 μg of RNA were used for each determination. Lane 1, free probe; lane 2, yeast tRNA; lanes 3 and 4, unstimulated cells (–); lanes 5–8, cells stimulated with *Agaricus blazei* extract for the times indicated at the top of the panel. The upper panel shows IL-12p35 and p40 mRNA, and the lower panel shows L32 and GAPDH as a control. Data shown are representative of three independent experiments.

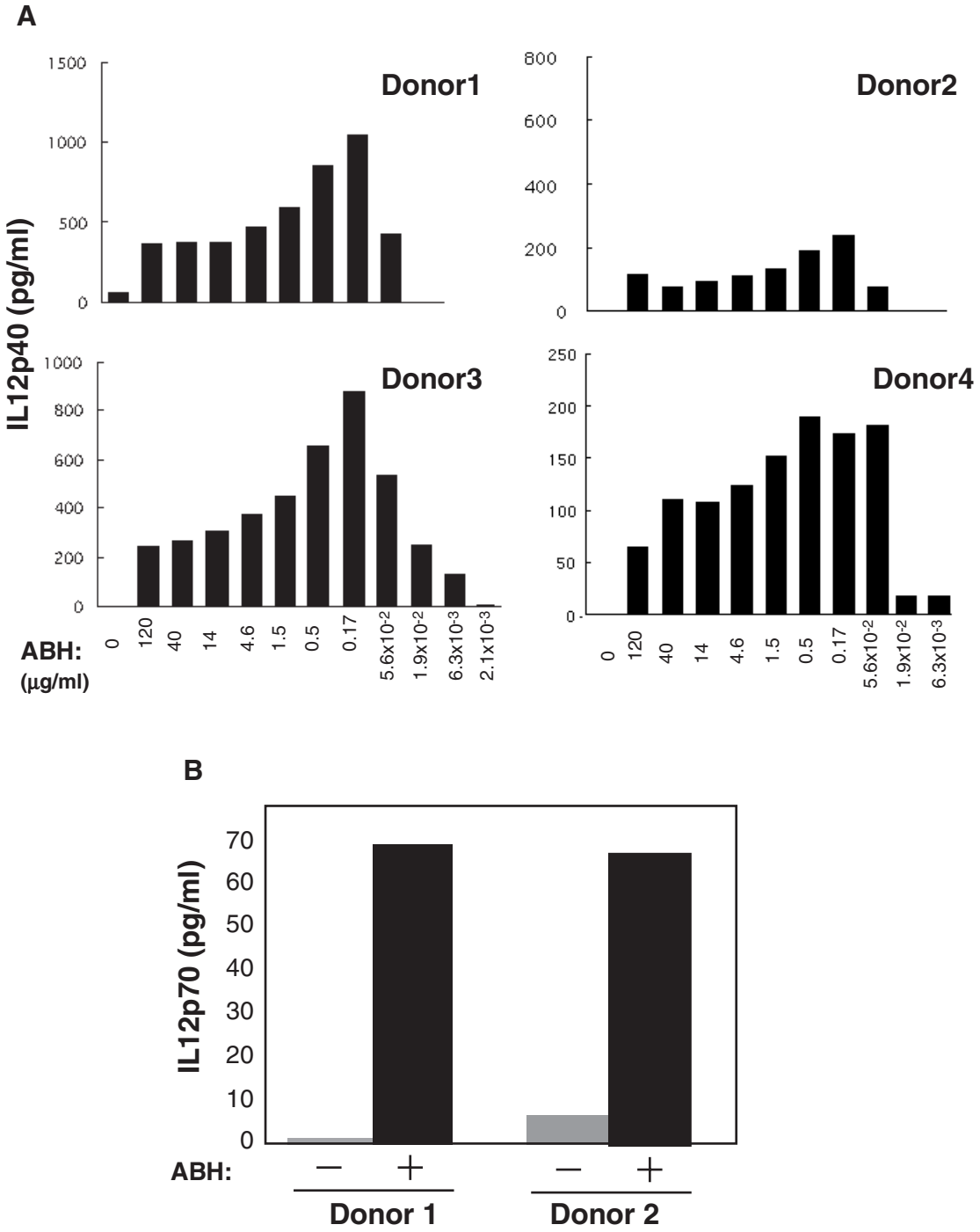

Figure 2. ABH induces IL-12p40 and p70. (**A**) Ficoll-isolated human PBMC (1×10^6/sample) were stimulated with ABH at various concentrations as indicated at the bottom of the panels. After incubation for 16 h, supernatants were collected and the amounts of IL-12 p40 were determined. (**B**) PBMCs were stimulated with 0.17 ng/ml ABH for 16 h. The supernatant was collected and the amounts of IL-12 p70 were determined. (A and B) IL-12 p40 and p70 in culture supernatants were measured by ELISA. Data of IL-12 p40 and p70 shown are from four and two different donors, respectively.

supernatants in relation to ABH dose. As shown in Figure 2A, IL-12 p40 was induced significantly in all donors. The levels of IL-12 production were reduced at concentrations of ABH in excess of 170 ng/ml. Figure 2B shows induction of IL-12 p70 (heterodimer of p40 and p35) by ABH. These observations suggest that ABH induces IL-12 production by PMBC.

CD14-positive Cells Produced IL-12 on Stimulation with ABH

To determine the target population of ABH, we analyzed IL-12-production levels of each population by intracellular cytokine staining method and flow cytometry. After 16-hour

culture, only CD14 positive population was induced IL-12p40 (Figure 3). Neither normal media (control) nor peripheral dendritic cells (lin-/CD11c and HLA-DR+) induced production of IL-12 p40 protein (data not shown).

We then added anti-CD3/CD19 and CD14 monoclonal antibody-conjugated Dynal beads to Ficoll PBMC fractions to deplete each fractions. IL-12 production was observed in PBMC from which CD3 and CD19 were depleted.

However, depletion of CD14 caused a 20% reduction in the level of IL-12p40 production (Figure 4).

Involvement of CD14 and TLR4 in IL-12 Induction by ABH

Human TLRs have been shown to transduce intracellular signals by some mycobacterial ligands. These reports suggest that TLRs initiate human innate immune responses and pattern recognition by TLRs regulates the nature of not only innate but also adaptive immune responses (14–16).

Using neutralising antibodies (anti-hTLR4:HTA125, anti-hTLR2:TL2.1 and anti-CD14), we examined whether TLR2, TLR4 and CD14 are involved in the induction of IL-12 expression by ABPC. Preincubation of PBMC with anti-hTLR4 mAb (HTA125) or anti-CD14 mAb (61D3) clearly inhibited IL-12 production in a dose-dependent manner. PBMC from three different donors were used, and the same results were observed in all cases. In contrast, pre-treatment with anti-hTLR2 (TL2.1) showed no effect (Fig. 5A).

Furthermore, flow cytometry was performed to quantify the IL-12-producing cells. In PBMC pre-treated with anti-hTLR4 and anti-hCD14 monoclonal antibodies, the levels of IL-12 production by CD14-positive cells were reduced to 1.33% and to 0.19% in samples treated with anti-hTLR4 and anti-hCD14 mAb, respectively. No effect was observed in samples treated with control antibody. There were no differences in the ratio of CD14-positive cells in PBMC before and after antibody treatment (Fig. 5B). These results suggested that anti-hTLR4 and anti-hCD14 mAbs blocked signal transduction via TLR4 and CD14.

Induction of IL-12 by ABH was Not Inhibited by Polymyxin B

CD14 and TLR4 have been reported as subunits of the lipopolysaccharide (LPS) receptor complex. The CD14-TLR4-MD2 heterotrimer recognises the LPS/LBP-complex (17–20). Our results indicated that CD14 and TLR4 were essential components for induction of IL-12 by ABH. To exclude the possibility that cellular activation by ABH was a result of endotoxin contamination in the extracts, the abilities of ABH and reference LPS to induce IL-12 production by human PBMC were examined in the presence and absence of polymyxin B. Polymyxin B showed little effect on IL-12 induction by ABH,

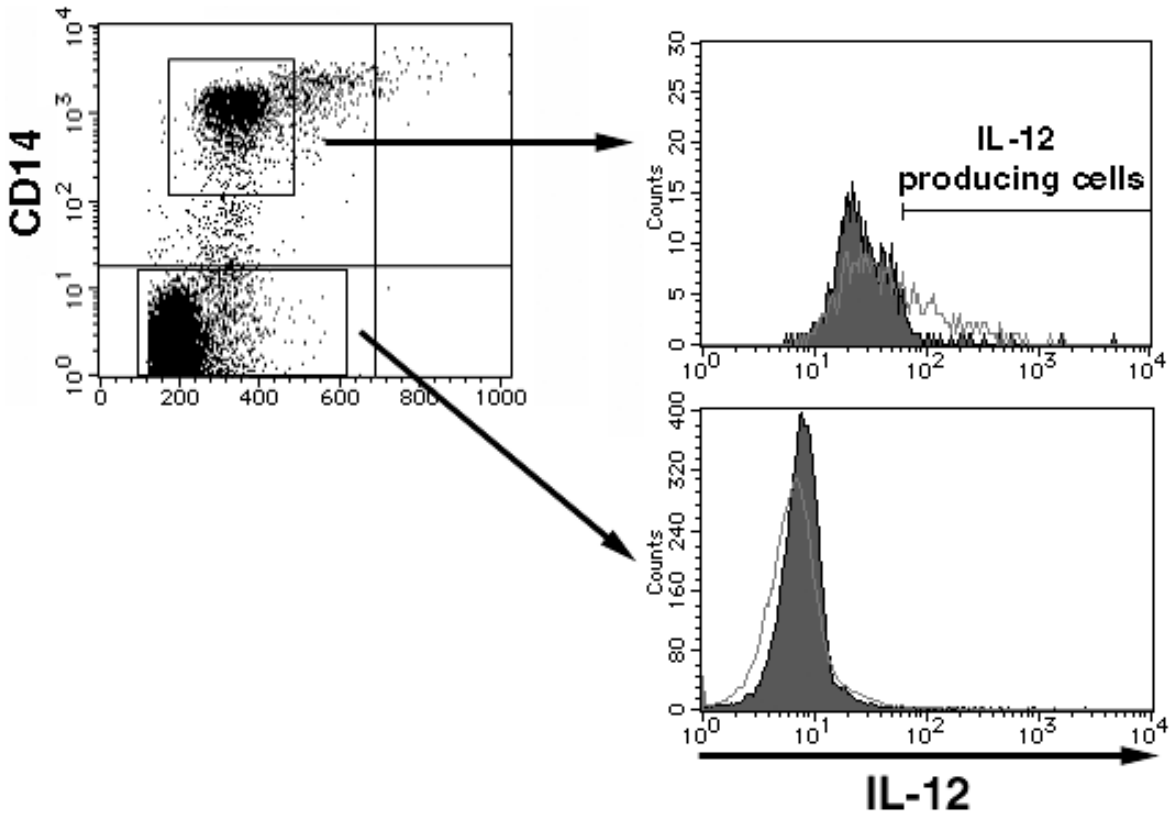

Figure 3. CD14-positive cells showed induction of IL-12. Human PBMC was stimulated with 0.17 ng/ml ABH for 16 h. IL-12 production was quantified by flow cytometry (right panels). The flow cytometric data in the right panels represent three gated populations of CD14-positive and negative cells. Results of ABH stimulated and unstimulated samples shown as open and closed histograms respectively. The region indicated by the bar represents IL-12-producing cells (shown in left panels). Data shown are representative of three independent experiments.

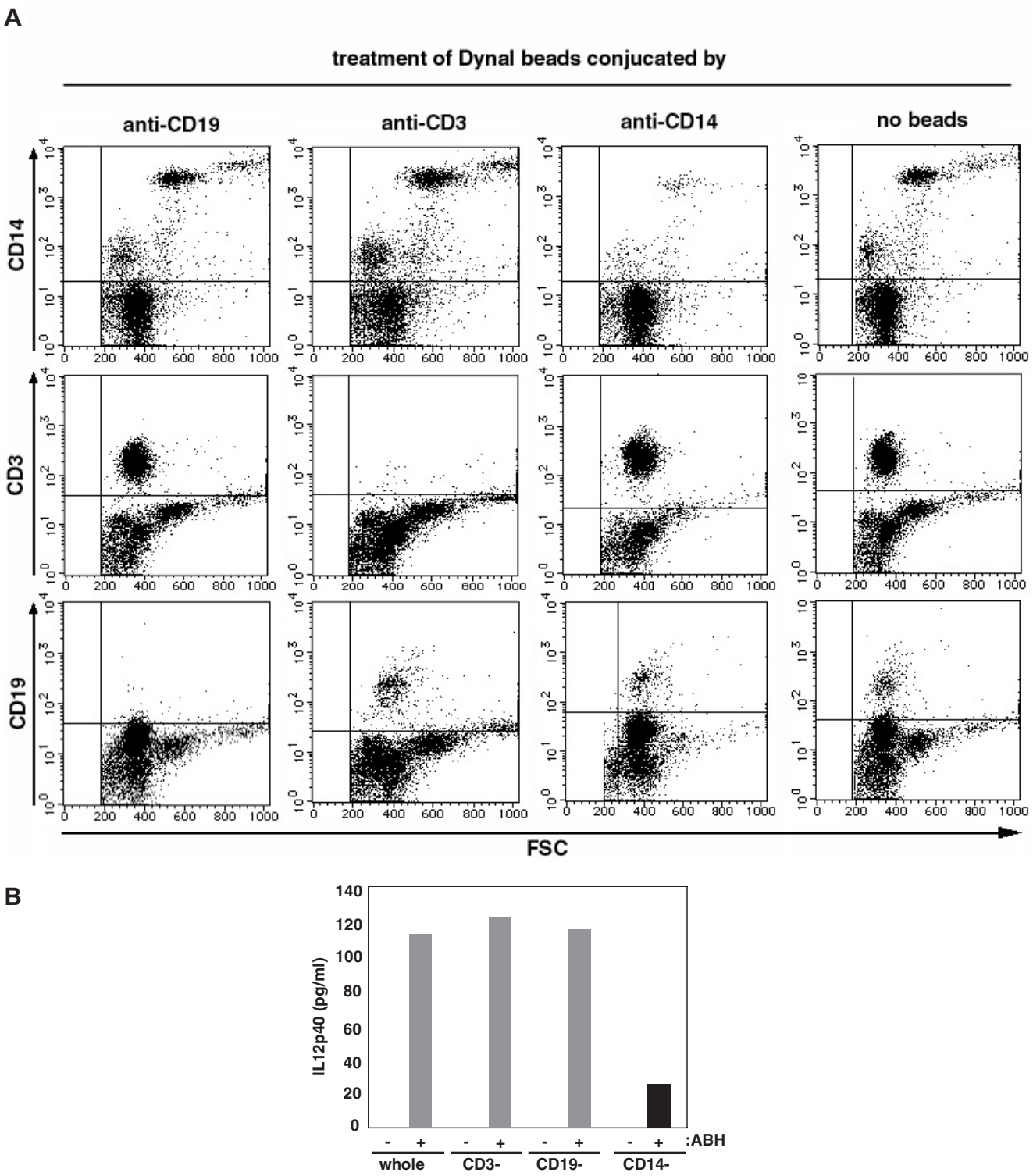

Figure 4. Depletion of CD14-positive cells reduced IL-12 production. (**A**) CD14-, CD3- or CD19-positive cells were depleted using beads conjugated with monoclonal antibodies specific for each surface marker, as indicated at the top of the panels. After depletion, CD14-, CD3- and CD19-positive cells were quantified by flow cytometry. (**B**) After treatment, PBMCs were stimulated with 0.17 ng/ml ABH for 16 h. Supernatants were examined by ELISA. Data shown are representative of three independent experiments.

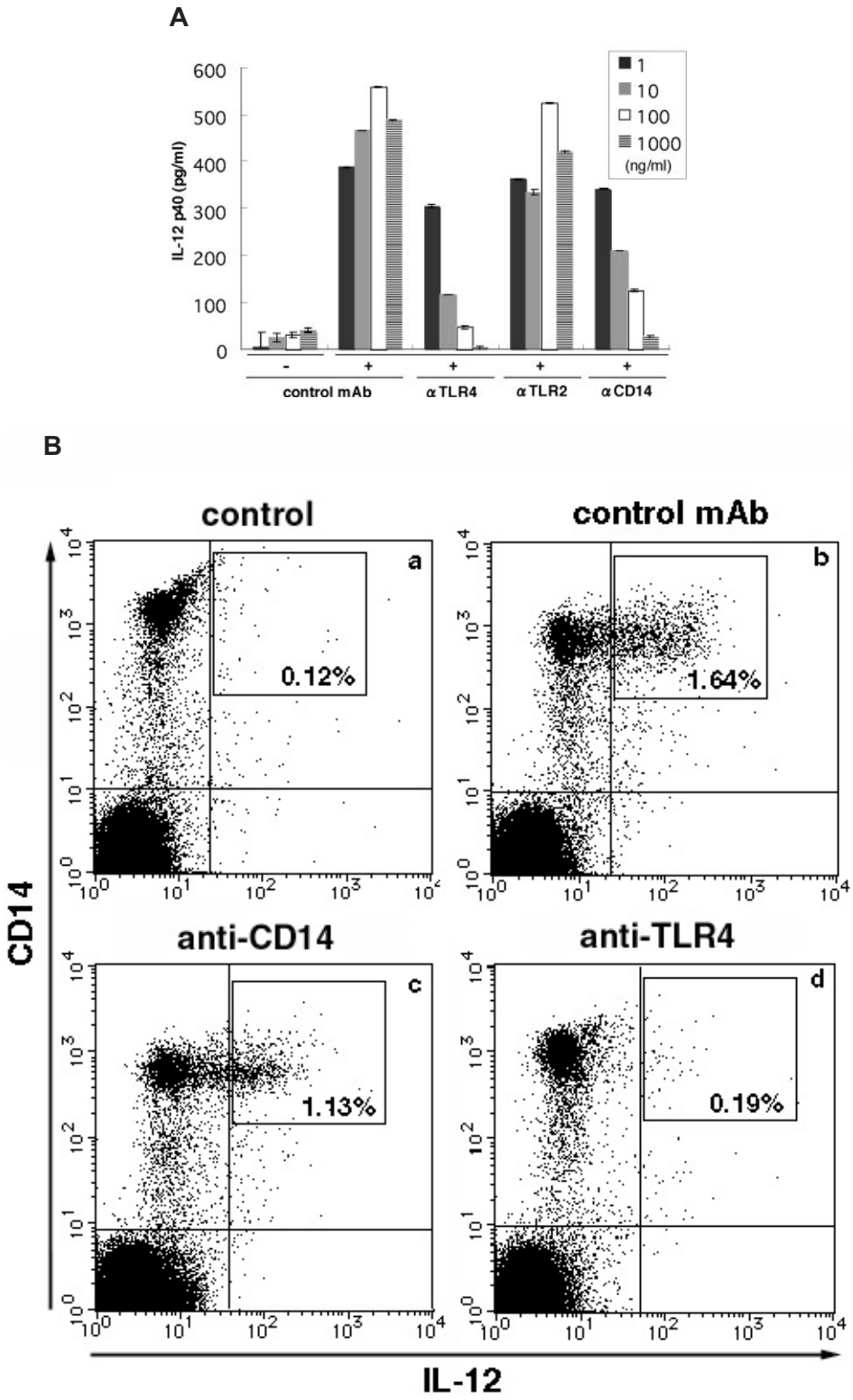

Figure 5. Anti-CD14 and anti-TLR4 monoclonal antibodies neutralized the IL-12-inducing effect of ABH. (**A**) PBMCs were pre-incubated with anti-CD14, anti-TLR4, anti-TLR2 or control monoclonal antibody at four different doses (1000, 100, 10, 1 ng). *Agaricus blazei* extract was added after 4 h and incubation was continued for 16 h. IL-12 p40 in supernatants was quantified by ELISA. Data from three independent donors are shown. (**B**) Cells pretreated with 1 μg of control monoclonal antibody (b), 1 μg of anti-CD14 antibody (c) or 1 μg of anti-TLR4 antibody (d) were stimulated with ABH for 16 h (b–d). Then, IL-12-producing cells were quantified by flow cytometry. Unstimulated PBMCs (a) were included as controls. Data shown are representative of three independent experiments.

whereas that of LPS was inhibited completely (Fig. 6). We measured the amount of endotoxin in the ABH extract using an Endospecy kit for endotoxin quantification (Seikagaku Corp., Tokyo, Japan). The ABH extract used in the present study was essentially free from endotoxin contamination (data not shown).

ABH Extract Enhanced NK Activity

IL-12 is a critical factor in immune responses against pathogens and tumors as it is the most potent promoter of type 1 responses in CD4 T cells (Th1 responses). Th1 responses regulate

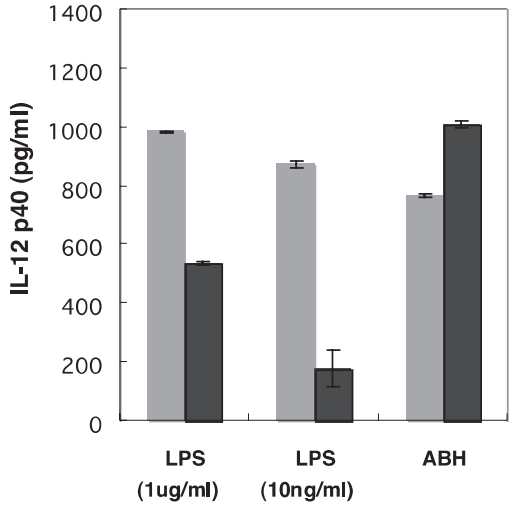

Figure 6. Influence of polymyxin B on IL-12-inducing activity of ABH. ABH (0.17 ng/ml) and reference LPS (10 ng/ml) were pre-treated with 5 μg of polymyxin B for 30 min, and then used to stimulate PBMC for 16 h. The IL-12 secreted into the supernatant was measured by ELISA.

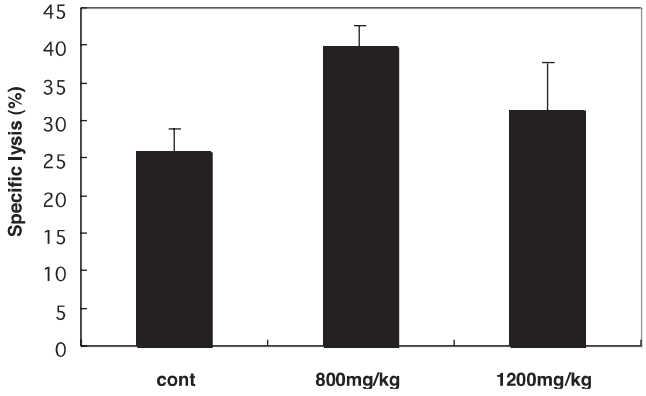

Figure 7. Oral administration of ABH enhanced NK cytotoxic activity in the spleen. The indicated amounts of ABH were administered for 21 consecutive days. Control mice were administered water. NK activity of spleen cells was quantified using CytoTox96 reagent (Promega). Results are expressed as means ± SEM, $n = 6$.

proliferation, interferon-γ (IFN-γ) production and cytotoxic activities by T cells and natural killer cells (21–23). To evaluate its effects *in vivo*, we examined the effect of oral administration of ABH on NK cytotoxic activity of murine spleen cells.

ABH-treated mice showed significantly higher levels of NK cytotoxic activity than controls; the ABH-treated group showed 40% cytolysis, while the control group showed 25% cytolysis (Fig. 7). This result suggested that oral administration of ABH can induce NK activity *in vivo*.

Discussion

Hemicellulase-digested *A.blazei* extract showed a very high degree of water solubility. Almost 95% by dry weight was extracted in water, in contrast to non-digested samples, which showed only about 20% extraction (data not shown). Various methods were employed to extract the components from *A.blazei*, including treatment with acid and organic solvent. These methods for extraction were not suitable for evaluation and analysis of total activity of all components of *A.blazei*. The high degree of water solubility obtained by hemicellulase digestion is a major advantage for analysis of the effects of *Agaricus*. In the present study, we were able to analyze the total effect of *Agaricus* extract as we could extract almost all of the components.

As the first step to determine the effects of ABH, we attempted to identify inducible or suppressible cytokines. We found the IL-12-inducing activity in the water extract. The optimum concentration of ABH for induction of IL-12 was from 170 to 510 ng/ml, and higher concentrations showed lower levels of induction.

Agaricus blazei extract was reported previously to show a suppressive effect against proliferation and cytokine expression using PBMC (24). The effects of elements with the ability to suppress cell activity may appear at high doses. Suppressive elements did not have sufficient effects to suppress the induction of IL-12 to $<$170 ng/ml.

The results of intracellular cytokine staining and population-specific elimination showed CD14 positive cells, monocytes/macrophages (Mo/Mϕ) to be the target population of ABH. We detected no IL-12 production by peripheral dendritic cells (DC). Peripheral circulating Mo circulate for 1–3 days before entering tissues, where they differentiate into mature resident Mϕ or DCs (25). Our results suggested that ABH possessed the potential to activate immature Mo and promote the Th1 response in tissue. As a result of this activity, ABH maintains the Th1 response level and cellular immunity. ABH-treated mice have been reported to show higher levels of NK activity in the spleen than untreated controls. The ability of DCs to produce cytokines differs among DC subsets depending on the tissue to which they belong (26,27). Peripheral Mo heterogeneity was reported previously (28–30). Thus, we speculate that ABH may have different effects on different subsets of Mo and DC. Therefore, the effects of ABH should be analyzed from various viewpoints.

Various models to explain the mechanism of the immunomodulatory effects of many mycobacterial extracts and components have been reported. These include cell activation by intracellular signals transduced by TLRs. The results of the present study indicate that the activity of ABH to induce IL-12 production is dependent on CD14 and TLR4 (Fig. 5). LPS causes activation of Mo/Mϕ via the CD14/TLR4/MD2 receptor complex (31). The *Limulus* activity of ABH was 80 pg/ml, equivalent to *E.coli* LPS (data not shown). Therefore, the endotoxin content in the ABH used in the present study was $<$1 pg/ml, which was completely inactive in our system. On the other hand, polymyxin B-treated ABH also induced IL-12 production at almost the same level as untreated ABH (Fig. 6). This excluded the possibility that the activation of Mo/Mϕ was due to response to contamination of the ABH extract by LPS.

TLR2 was also reported to be a signal transducer for components of fungi (11,12,32). The anti-TLR2 mAb TL2.1 did not inhibit induction of IL-12 production by ABH.

Agaricus blazei is rich in polysaccharides that can stimulate immune cells. One of these, β-1,6-D-glucan, has been reported to be the main component responsible for this activity. Ohno *et al*. demonstrated that the functional centre of β-1,6-D-glucan is a region rich in β-1,3 links (33–35). β-Glucans activate cells via TLR2 (32). The mannan fraction from *Candida albicans* and *Saccharomyces cerevisiae* induced TNF-α production in a manner dependent on CD14 and TLR4 (36). TLR4 is also involved in pattern recognition of soluble branched β-(1,4)-glucans from Acetobacter AC-1 (37).

We speculate on two possible explanations for this observation: β-Glucan in ABH may be recognised by a different receptor complex, or other components, such as mannan, may be mainly responsible for the IL-12-inducing activity of ABH. As ABH is a mixture of various components, it is necessary to identify and isolate the elements responsible for the action of ABH.

Some studies of the anti-tumor effects of *A.blazei* have demonstrated direct cytotoxic effects (1). The chloroform/methanol extracts of *A.blazei* were shown to have antitumor activity against solid tumors. Ergosterol in this extract inhibits angiogenesis and causes the death of tumor cells by preventing neovascularisation (5). When the particles from fruiting bodies of *A.blazei* Murill (ABP-F) were reacted with human serum, the formation of complement-opsonised ABP, iC3b-ABP-F complexes, and binding of the complexes to human PBMCs were observed. In addition, PBMCs incubated in the presence of iC3b-ABP-F complexes inhibited the proliferation of a human tumor cell line *in vitro* (38).

These reports indicated that components of *A.blazei* have various activities. It was reported that *A.blazei* extract acts mainly through modulation of the immune system, activating macrophages, neutrophils and lymphocytes (3,4,8). On the other hand, ethanol extracts of *A.blazei* mycelia were shown to reduce the cytopathic effects of the western equine encephalitis (WEE) virus (39). Several fungi incubated with immune cells showed activation of some cells, especially antigen-presenting cells, and induced cytokine production (40–42,44–48). The ethanol extracts of *A.blazei* stimulated macrophages and induced expression of the cytokines IL-8 and TNF (49).

This report indicates that the extract of *A.blazei* mycelia induces IL-12 production. IL-12 exerts multiple biological activities, which include activation of CD8+ CTLs, differentiation of CD4+ T lymphocytes, induction of nitorogen oxide production by macrophages, induction of type 1 responses by helper T lymphocytes and NK cell activation (50,51). IL-12 has also been shown to possess potent antitumor activity in a wide variety of murine tumor models (49,52–55). Recent reports have demonstrated that the *in vivo* antitumor capacity of IL-12 is mediated via NK and/or NKT cells (56,57). The findings of the present study will help in furthering our understanding of the immunomodulatory and anti-viral effects of *A.blazei*. IL-12 promotes and sustains the cellular immune system. Oral administration of ABH was shown to enhance the NK activity of mouse spleen cells (Fig. 7). It is notable that ABH modulates immune responses not only *in vitro* but also *in vivo*. In the context of *A.blazei* administration as a functional food, the induction of IL-12 and enhancement of cellular immunity are important for various biological activities.

In conclusion, we have demonstrated that hemicellulase-derived ABH induces IL-12 production by human peritoneal monocytes via TLR4-CD14 and enhances cytotoxic activity against tumor cells *in vivo*. These responses may be involved in the biological activities of ABH, such as its anti-tumor, anti-infection and anti-allergic effects. ABH is a non-proliferative and non-pathogenic microbial component that can trigger an immune response that is useful in the fight against infectious diseases and cancer. The accumulation of such data concerning the effects of components and clarification of mechanisms of their action are essential to understand features of mushroom such as the biological response modifier (BRM).

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Review

The Medicinal Mushroom *Agaricus blazei* Murrill: Review of Literature and Pharmaco-Toxicological Problems

F. Firenzuoli[1], L. Gori[1] and G. Lombardo[2]

[1]Center of Natural Medicine and [2]Department of Internal Medicine, S. Giuseppe Hospital, Az USL 11, Empoli, Italy

Agaricus blazei Murrill (ABM) popularly known as '*Cogumelo do Sol*' in Brazil, or '*Himematsutake*' in Japan, is a mushroom native to Brazil, and widely cultivated in Japan for its medicinal uses, so it is now considered as one of the most important edible and culinary-medicinal biotechnological species. It was traditionally used to treat many common diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis and cancer. *In vitro* and *in vivo* ABM has shown immunomodulatory and antimutagenic properties, although the biological pathways and chemical substances involved in its pharmacological activities are still not clear. The polysaccharides phytocomplex is thought to be responsible for its immunostimulant and antitumor properties, probably through an opsonizing biochemical pathway. Clinical studies are positive confirmations, but we are still at the beginning, and there are perplexing concerns especially relative to the content of agaritine. Argantine is a well-known carcinogenic and toxic substance in animals, that must be completely and fully evaluated.

Keywords: *Agaricus blazei* Murrill (ABM) – cancer prevention – immune response – agartine – medicinal mushroom

Introduction

Mushrooms and primarily basidiomycetous fungi are a popular and valuable food, low in calories and high in minerals, essential amino acids, vitamins and fibers (1); some of them produce substances having potential medical effects, and are called medicinal mushrooms. *Agaricus blazei* Murrill (ABM) is known in Brazil as *Cogumelo do sol* or *medicinel*, in Japan as *Himematsutake, Agarikusutake* or *Kawarihiratake* and in China as *Ji Song Rong*. It was brought to Japan due to alleged health effects and is widely used today in Oriental 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 cancer. In accordance with Brazilian tradition, it would be useful against a variety of diseases like diabetes, atherosclerosis, hepatitis, hypercholesterolemia, heart disease and so on. In Japan, researchers demonstrated immunostimulant and anticancer effects of ABM extracts experimentally, and due to the improving consumption of this mushroom in recent years, a considerable effort investigated the putative effects with interesting, but still insufficient clinical studies. Experimental studies increased commercial interest for ABM because of many requests as popular remedy especially in Japan, stimulating not only the production, but also the registration of new names and brands with new popular names. This makes it difficult for the public to identify pure ABM strains.

For reprints and all Correspondence: Dr Fabio Firenzuoli, Center of Natural Medicine, S. Giuseppe Hospital, via Paladini 40, 50053 Empoli, Italy. Tel: +39-0571-702661; Fax: +39-0571-702639.
E-mail: f.firenzuoli@usl11.toscana.it

History and Ethnopharmacology

Agaricus blazei Murrill is a mushroom originally native to a small village, name Piedade, in the highland areas of Atlantic forest, near Tauape, in the province of

Sao Paolo, Brazil. It was discovered in 1960 by Takatoshi Furumoto a grower and researcher who sent it to Japan in 1965 for investigation. It was identified as ABM by the Belgian botanist Heinemann in 1967 (2). Later it was given the common name of *Himematsutake* in Japan, while in Brazil it was named *Cogumelo Piedade*. The mushroom is traditionally believed to fight physical and emotional stress, stimulate immune system, improve the quality of life in diabetics, reduce cholesterol, prevent osteoporosis and peptic ulcer, treat circulatory and digestive problems and fight cancer (2). All traditional and not-proved beliefs, as often happens, are intentionally used and publicized on the web and mass media for commercial purposes often without any real scientifically demonstrated clinical benefit for patients (3). Over the last decade, the mushroom has been studied as a novel functional food in Japan, Korea, China and Taiwan. The fruiting bodies are still quite expensive to grow, so a relatively cheap and stable source for commercial purpose is still sought. Medicinal mushrooms have an established history of use in traditional oriental therapies: historically, hot-water soluble fractions from medicinal mushroom were used as medicine in the Far East from where this knowledge and practice seem to have been originated. The first historical description about the use of mushroom of *Agaricus* genus for medicinal purposes is probably described by Byzantine medical treatises in the Mediterranean area, from the 4th century AD to the 15th century AD by Orivasios and Apuleius for treating malignant ulcers of the larynx (4).

Botanics

Agaricus L.: Fr. Emend Karst. is the type genus of the family *Agariaceae* in the order *Agariales* (5), and is generally described as having small to large fruit bodies with white, yellow or brown pileus; free lamellae that are pallid or pinkish when young, later becoming chocolate-brown; and also dark-brown, smooth basidiospores (6). This highly diverse genus was divided into three subgenera by Heinemann (7): *Agaricus, Conioagaricus* and *Lanagaricus*. In this subdivision, the subgenus *Agaricus* contains the most typical species.

Agaricus spp. are saprophytes widely distributed over geographical areas from the tropics to the boreal regions, inhabitating a variety of habitats from alpine meadows, to salty and sandy seashores, to deciduous and conifer woodlands (6) The most economically important species is *A. bisporus* Imbach that is the most widely cultivated edible mushroom, accounting for 32% of the more than million metric tons of mushrooms produced worldwide in 1997 (8). ABM (Fig. 1) is a large *Agaricus* species with a brownish-gold cap (7–2 cm broad), convex, fleshy, the stem short and hard, with chocolate brown basidiospores ($5 \times 4\,\mu m$) and is closely related to *A. subrufescens* (9,10).

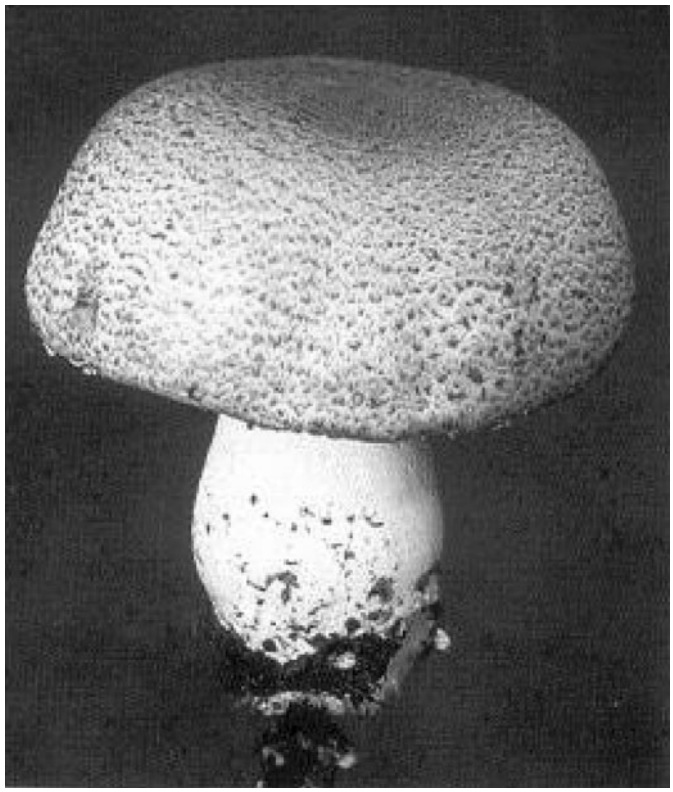

Figure 1. *Agaricus blazei* Murill mushroom.

The mushroom grows with a stalk length and a cap diameter that are about equal (campestroid type). As a litter-decomposing fungus, it naturally grows well in soils rich in lignicolous debris, in mixed woods, along forest edges and manures. Nowadays main cultivation centers are established in Japan, China and Brazil, where the fungus is cultured in enriched composts or pasteurized substrates supplemented with nitrogenous additives (10). New data indicate that the medicinal mushroom from Brazil and Japan could be biologically and phylogenetically the same species as *A. subrufescens* Peck from North America, although a search on the web and a review of diverse commercial product literature indicate that association of the name ABM with the Brazilian mushroom is attributed to P. Heinemann (11). So there would be an interfertility between North American *A. subrufescens* and the 'medicinal *Agaricus*'; the presence in hybrids of genetic materials from two progenitors and novel phenotypes indicate that members of these geographically distant mushroom populations might constitute a single 'biological species' (11). This could be confirmed by the paper of Colauto (12) showing little genetic variability among commercialized strains based on results of RAPD analysis data of 20 primers from fungi cultivated in malt-agar medium for DNA extraction. The paper showed that some commercialized *A. blazei* spawns in Brazil have identical genotypes, and are probably clones having the same origin, which could be Japan (12). Nevertheless, recently Wasser has

published (13) an historical-botanical analysis of the mushroom concluding that ABM differs from *A. blazei* ss. Heinemann in (i) size, shape of fruit bodies and pileal surface; (ii) type of pileal covering; (iii) presence of cheilocystidia; and (iv) spore size. That is, North American endemic species *A. blazei* ss. Murrill and the widely cultivated medicinal *A. blazei* ss. Heinemann would be two different species; and *A. blazei* ss. Heinemann should be considered a new species: *A. brasiliensis* (13,14). This problem is probably to be considered still open until an official international consensus statement will end this botanical dispute.

Phytochemical Constituents

Chemical Composition

In general, the gross composition of mushrooms is water (90%), protein (2–40%), fat (2–8%), carbohydrates (1–55%), fiber (3–32%) and ash (8–10%) (ash is mainly composed of salts, metals and so forth). Active metabolites can be isolated from fruiting bodies, pure culture *mycelia* and culture filtrate, and nowadays many attempts are being made to obtain active metabolites from *mycelia* through submerged fermentation culture to obtain cheaper preparations. Kawagishi was the first to separate an active anticancer compounds purified from the sodium hydroxide extract of the fruit body of ABM (15). The author detected polysaccharides with apparent antitumor activity, the major fraction being FIII-2-b, which comprised a protein complex composed of 43.4% protein and 50.2% carbohydrates (15). The FIII-2-b fraction contained simple (1-6)-β-D-glucopyranosyl chains. A significant contribution to the anticancer activity of the protein moiety of FIII-2-b was also speculated following the complete loss of antitumor activity after formolysis. ABM fruiting bodies in different stages of maturity contain α-glucans and β-glucans: the yield and structural diversity of glucans increase as the fruiting bodies mature; so the time of the harvest and conservation is of great importance, to obtain the best extract, data that almost invariably are not reported in scientific articles.

The α and β-glucan Structure

ABM glucans are side branches of a (1-6)-β-backbone as found by Dong and Ohno, who described that active fraction of β-glucans of ABM fruiting bodies had a (1-6)-β-backbone structure (or functional center) with (1-3)-β-side branches in the ratio of 1 : 2 (16); while the linear (1,6)-β-glucan seems to be inactive (17) (Fig. 2). The biochemical importance of (1-3)-β-side branches has been confirmed and has shown the enhancement of the immunomodulatory activity of polysaccharides (18); and Mizuno (19) reported an important antitumor

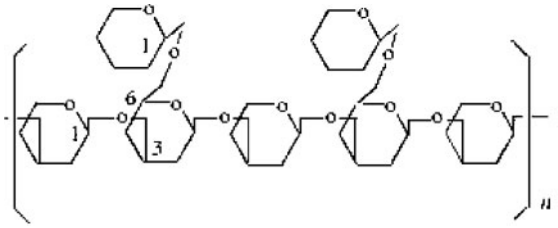

Figure 2. (1–6)-β-backbone structure (or functional center) with (1–3)-β-side branches.

activity linked to the water-soluble (1-6)-(1-3)-β-D-glucan. However, a significant increase of water-soluble (1-4)-α-glucan with apparent antitumor activity occurs during maturation (19); so probably cap-opened, more fragile mature fruiting bodies of ABM should be selected over immature ones for the production of nutraceuticals because they contain the most useful glucans (20). In addition, an α-1,6 and α-1,4 glucan complex (21) and a glucomannan with a main chain of β-1,2-linked D-mannopyranosyl residues have been isolated from this mushroom and found to inhibit tumorigenesis (22).

Mechanisms of Tumorigenesis and Carcinogens

These results suggest that whole-mushroom extracts contain compounds that may modulate tumorigenesis and carcinogenesis at different stages and/or may act at the same stage through different mechanisms. Responses to such highly different polysaccharides are likely to be mediated by different cell-surface receptors, which may be present only on specific subsets of cells, and may trigger distinct downstream responses. A combination of such responses involving different cell subsets could conceivably provide greater tumor inhibition than could be induced by a single polysaccharide. Nevertheless, a very important problem is the wide number of different and only partially homogeneous ABM extracts used to study the pharmacological activities of its constituents representing a difficult challenge to establish the best extract and active substances. Thus all these similar constituents could potentially provide additive, or even synergistic, effects in the prevention and treatment of cancer. Moreover they could interfere with other substances or healthy physiological functions. This has been shown by an *in vitro* study in which increasing fractionations of an ABM extract enhanced some biological activities but abolished others (23).

Immunologic Intervention

The specific mechanisms that contribute to an enhanced state of immunity remain partially understood. Recent insights in two rapidly expanding fields, the cytokine-mediated homeostasis of mature lymphocytes by cytokines, such as interleukins and autoreactive T cells by

$CD4^+CD25^+$ regulatory T cells, provide the foundation for what might be occurring.

Recent advances in immunology have demonstrated the importance of local interactions between antigen-presenting cells and effector cells such as natural killer cells and T-lymphocytes for an effective immune reaction against tumors (24). Interferon stimulate such interactions, while interleukins play a central role in the activation of NK cells and T-lymphocytes. Interferons were investigated as potential anticancer agents because of their antiproliferative and cytotoxic effects, their ability to activate specific components of the immune system and their relatively modest toxicities. Increasing biological evidence supports the hypothesis that tumor-generated chemokines provide more than simply angiogenic signals. Tumor-derived chemokines may potentially act as inhibitors of anti-tumor immune responses as well as autocrine growth factors for the tumor. All these chemokines activating activities of *A. blazei* Miller remain to be completely evaluated both in animal model and in real clinical practice.

However, immunologically active glucans are (1-3)-β-D-linked glucose polymers, which occur as a primary component in the cell walls of bacteria and fungi or are secreted extracellularly by various fungi, and actually seem the most important active substance.

Studies *in vitro*

Biological Activities on the Immune System

The immunostimulant and immunomodulatory activity of both mycelial and fruiting bodies of ABM using water and ethanol extracts have been demonstrated in many *in vitro* experiments, although not always the results are concordant, but sometimes contradictory. Water extracts of the mycelial culture and fruiting bodies such as fractions B-4, B-5 obtained from ethanol precipitation (respectively 44% and 50%) of fruiting bodies, markedly induced TNF production and IL-8 of macrophages derived from rat bone-marrow (23). Fraction B-5 induced a significant increase in nitric oxide production, (23). In another paper, the same group using ethanolic fractions obtained from *mycelia* inhibited the occurrence of the viral cytopathic effect induced by Western equine encephalitis and *herpes simplex* (25). Other extracts containing lignin-based derivatives have shown the induction of TNF-γ, IL-8 and nitric oxide secretion by macrophages (26), anti-viral activity of different viruses (27), and direct anticancer activity (28); so lignin derivatives apparently have more different and important pharmacological activities. While, on the contrary, a down-regulation of IL-2, IL-4 and INF-γ in human peripheral blood mononuclear cells has also been documented (27). A hemicellulase-treated ABM fraction derived from *mycelia* composed of 63.3% carbohydrates, 30.9% proteins, 0.3% lipids and other minor components has shown to stimulate immature dendritic cell obtained from mice bone-marrow and up-regulate the expression of costimulatory molecules and MHC antigen, although did not increase the production of inflammatory inducible cytokines (30). ABM pre-treated dendritic cells inhibited some bacteria-mediated dendritic cells' responses, ABM pre-treated macrophages reduced LPS-induced NF-κB activity, while ABM-mediated dendritic cells enhanced the Th1 response in allogenic mixed lymphocyte reaction. These antithetical effects may probably help to maintain immunological homeostasis, so the question is: can they be clinically effective in cancer patients? (30). Fine particles of ABM fruiting body and *mycelium*, respectively, prepared by mechanical disruption, activated the human complement system via the alternative pathway in human serum, is another proof of its activity in enhancing natural immunity in bacterial infections (31). From these studies it is clear that the ABM fractions act on many different biological receptors of the immune system but can also have antithetical pharmacological activity; so further studies are warranted to completely identify the real importance of this mushroom as an immunostimulant and/or immunomodulator.

Anticancer Activity

Anticancer Activity of Different Extracts

The ABM aqueous extract demonstrated no clastogenic activity whilst to have anticlastogenic properties with a 100% reduction of chromatid and 144.4% reduction of isochromatid breaks, is apparently really important for cancer prevention in humans since it is usually consumed in its natural form as tea or as food (32). Nevertheless, we emphasize that if in the same study methanolic and hexanic extracts were anticlastogenic, *n*-butanolic extract were both anticlastogenic and clastogenic (32); and in another article different hexane extracts of the fruiting body in culture of mammalian cells were at different concentrations genotoxic, cytotoxic and anticlastogenic; so these findings clearly suggest further studies are needed (33). Oliveira (34) studied ABM aqueous extracts, by simultaneous and pre-incubation administration, and demonstrated a strong protective effect based on the cytokinesis-blockmicronucleus (CBMN) assay under both conditions, suggesting a desmutagenic activity, and Menoli (35) observed a protective effect against CBMN induced by methyl methanesulfonate, when cells were treated with an aqueous extract of ABM strains mixture; and in the comet assay, the same authors also observed antigenotoxic potential. Moreover, resident human peripheral nucleated cells incubated in the presence of complement-opsonized complexes of fruiting bodies

inhibited the proliferation of the human thyroid carcinoma cell line TPC-1 (31). Recently it has been shown on human gastric epithelial AGS cells that an aqueous extract can activate apoptosis through induction of caspase-3 and related cell cycle arrest at the G2/M phase (36).

Different Anticancer Activity of ABM Strains

ABM extracts have not always shown a protective effect against cancer. Delmanto (37) using the micronuclei test against genotoxicity induced by cyclophosphamide, found a decrease in the frequency of micronuclei after treating mice of mixed lineages with teas pre-treatment, but there was no lower micronuclei frequency with the isolated lineage AB 99/26. Luiz (38) did not find any antimutagenic activity in ABM aqueous extracts against methyl methanesulfonate in V79 cells, using the CBMN and comet assays. While using the comet assay, Guterrez (39) found no protective effect for ABM aqueous extracts in V79 cells, suggesting that differences in the cultivation, storage and extracts preparation could influence the effectiveness of preparations. In testing ABM aqueous extracts of three different origins (Botucatu-SP, Londrina-PR and Piedade-SP), Guterrez (39) observed any genotoxic potential, while an antigenotoxic activity only for ABM from Piedade-SP with pre-, post- and simultaneous treatments, and for ABM from Londrina-PR only following simultaneous treatment.

All these data implicate that lineages and pre-treatment types influence the pharmacological anticancer activity of ABM extracts and as confirmed by Manzi and Pizzoferrato (40) beta glucans, apparently the most important constituent, in mushrooms are distributed variably both in the soluble and in the insoluble dietary fraction. Luiz (41) has demonstrated that ethanol and chloroform/methanol extracts have anticlastogenic activity, although without a dose-response correlation, and the author suggests that because of a deficient repair of CHO-xrs5 cells, an activity as modulators of DNA replication and repair, and that probably fatty acids contained in the extract (especially linoleic and eicosapentanoic acid) could have a role in the antimutagenic activity of the mushroom.

In vitro Studies on Cytokines Stimulation of ABM

Examination of the cytokine-inducing activity of hemicellulase-derived *mycelia* extract on human peripheral mononuclear cells have shown induced expression of IL-12, a critical regulator of immune response against pathogens and tumors as it is the most potent promoter of type 1 responses in CD4 T cells; confirmed in the same article that oral administration to mice showed significant higher levels of NK cytotoxic activity of murine spleen cells (42). Stimulation of NK-cells is obtained by higher production of IFN-γ through hydroalcoholic extracts of fruiting body fractions, although the activity was significantly reduced after heat treatment of 2 h at 120°C in murine spleen cell (43). In an experimental research using a 9% solution of an aqueous ABM, extracts containing 300 $\mu g\,ml^{-1}$ of β-glucans were examined to determine changes of gene expression caused by the extract on a human monocyte cell line, and drastic effects on gene expression were found: genes related to immune function were selectively up-regulated, particularly pro-inflammatory genes such as the interleukins IL-1β and IL-8. Although most genes induced by ABM were also induced by LPS, ABM produced a unique profile, e.g. as to a particular increase in mRNA for the chemokine ligands 1, 2 and 3, IL-1A, as well as prostaglandine-endoperoxidase synthase 2 (cyclooxygenase2); and gave rise to 63% inhibition of DNA synthesis and 30% inhibition of protein synthesis (44) (Table 1).

Table 1. Chemokine-stimulated secretion

TNF-α secretion by macrophages (23)
IL-8 (23), 12 (42), 1 (44) and 2B (44),10 (44)
Nitric oxide production by macrophages (23)
Stimulation of immature dendritic cells (30)
Enhanced Th1 response (30)
Activated the human complement system via the alternative pathway (39)
Stimulation of NK cytotoxic activity (42)
Higher production of IFN-γ (43)
mRNA increase for chemokine ligand 1, 2 and 3 (44)
mRNA increase for cyclossigenase 2 (44)
mRNA increase for regulator of G-protein signaling 1 (44)
Induction of caspase 3 (36)

ABM extracts act mainly through modulation of the immune system activating macrophages, neutrophils and lymphocytes (21,45,46,47) confirming the possible anticancer activity by an enhanced immunostimulation. About protection against atherosclerotic vascular diseases, ABM has shown interesting antioxidant activity. In one study, ethanolic extract had a remarkable antioxidative substance effective in the auto-oxidation of linoleic acid (48); and as confirmed by an *in vitro* study through the trx1Δtrx2Δ mutant method, ABM has demonstrated to contain thermostable potent antioxidant substances, since the extract was prepared by boiling the mushroom in water for 120 min and autoclaving (49); and a wine produced by ABM contains 0.68% β-D-glucan and 8% alcohol showing fibrinolytic activity on artificial bovine thrombus, that, a preventive effect on thrombosis may pose the problem of administration in hypocoagulable defeated patients of more concentrated extracts (50).

On the basis of these discrepancies and the lack of homogeneity in the type of extracts used for experiments, it is difficult to now obtain reliable and definitive data about anticancer activity *in vitro* of ABM extracts, such as the immunological effects; so these studies are still in their infancy and probably a better definition of active

principles is needed regarding the action mechanism in cell and interactions with cell physiological processes.

Anticancer Activity of β-Glucans

Early reports showed that β-glucans functioned by stimulating host defense mechanisms and were not toxic for tumors, but in following years β-1,3;1,6-glucans from fungi (e.g. mushrooms) and yeast became a new biological entity, so-called biologic response modifiers that function as immunostimulants against infectious diseases and showing a possible tumoricidal activity (51). Unlike most other natural products, purified β-1,3-glucans retain their bioactivity, and this has permitted the characterization of how β-1,3-glucans can work on a cellular and molecular level, showing that they function through stimulation of granulocytes (neutrophils and eosinophils), monocytes, macrophages and NK-cells (52). Certain data also suggested that β-glucans could promote T cell-specific responses, perhaps, through triggering the secretion of IFN-γ, IL-6, IL-8 and IL-12 from macrophages, neutrophils and NK-cells, and a role for T cells in β-glucan function was also proposed because of absent tumoricidal activity in nude or T-cell-depleted mice (53).

Anticancer Activity and β-Glucan Conformation

Polysaccharide antitumoral activity has been evaluated most often against allogenic sarcoma 180 in CD-I mice, a tumor sensitive to immunomodulating compounds. Of the polysaccharides with immunomodulating capacity, only those which consist of a (1→3)-linked β-glucan backbone with (l→6)-linked β-D-glucopyranosyl units as branches produce complete inhibition of tumor growth. (1→3)-β-glucans from fungi commonly have a tumor inhibition percentage of 99–l00%, while other polysaccharides exhibit l0–40% inhibition (54). Contradictory data exist on the influence of molecular weight, degree of branching, conformation and intermolecular associations of β-glucans on antitumor activity and on the mechanism(s) of their action (55). Most of the (1→3)-linked β-glucans with biological response modifier activity have been isolated from Basidiomycetes; a few with pronounced antitumor activity have come from Ascomycetes and Oomycetes (56).

Evidence suggests that the activity of these polysaccharides is also dependent on their size, with high molecular weight (100 000–200 000) fractions being most active, while fractions from the same source with molecular weights of 500–10 000 show no activity (55). The fact that there are polysaccharides with different chemical structures, but all of which have immunomodulating activity (56), suggests that the immune response is in part non-specific, determined by size rather than by chemical structure.

β-Glucan and Human Receptors

At least four receptors have subsequently been identified: complement receptor 3, lactosylceramide, scavenger receptors and Dectin-1. In addition to an iC3b binding site, complement receptor 3 possesses a lectin site for β-glucans that, in combination with iC3b, enhances phagocytic and cytotoxic responses (57). β-glucans can also prime the receptor for subsequent iC3b-mediated cytotoxic responses, including the iC3b-restricted antitumor activity (57). Lactosylceramide, a major glycosphingolipid of polymorphonuclear leukocytes, and selected scavenger receptors have also been identified as receptors for β-glucans, although their role in β-glucan-mediated responses is less clear (57).

Willment (56) has recently shown that the human receptor is widely expressed, functions as a pattern recognition receptor for β-glucans, and can also recognize T-lymphocytes (57). In contrast to the mouse receptor, the human receptor is alternatively spliced and splicing appears to be regulated in different cell types and various receptor isoforms generated by alternative splicing that differ in their ability to recognize β-glucans.

Although the two predominant isoforms are both expressed in multiple tissues they are expressed differently in various cell types, suggesting that the alternative splicing of these two isoforms can be regulated (57). While the significance of this is unclear, the presence or absence of a stalk does not seem to have significant effects on the ability of this receptor to recognize β-glucans. The other isoforms represent a minor population of the splice variants, and they may serve regulatory roles, a phenomenon described for other cell surface receptors such as CD40 (59) and scavenger receptor type A (60).

In addition to its ability to recognize glucans, the human β-glucan receptor also recognizes a subset of T cells. Given the similarity of the β-glucan receptors to those of the NK-cell-like C-type lectin-like domains (53,61) which normally recognize specific major histocompatibility complex class I molecules on target cells, it is possible that the ligands on T cells are major histocompatibility complex class I molecules. The ability of the human β-glucan receptor to recognize only one of the four T cell lines tested suggests that the ligand is restricted to a subset of T cells and we are currently exploring this possibility further (57). While the biological function of this interaction is unknown at present, it poses an intriguing role for this receptor in the recognition of self and non-self ligands (58).

Gastrointestinal Absorption of β-Glucans

There are also reports that some mushroom β-1,3;1,6-glucans could mediate tumor regression when given orally (62,63). In more recent studies using human

tumor xenografts, orally administered soluble barley β-1,3;1,4-glucan or i.v. antitumor monoclonal antibodies were ineffective as single agents, but when combined, elicited a substantial antitumor effect (64,65). However, the mechanism by which large β-1,3-glucans could be taken up orally by the gastrointestinal tract and function to prime leukocyte CR3 was unknown.

Opsonizing Activity of β-1,3-Glucan

An important investigation showed that these large β-1,3-glucans were taken up by gastrointestinal macrophages and shuttled to reticuloendothelial tissues and bone marrow. Within the marrow, the macrophages degraded the β-1,3-glucan and secreted small soluble biologically active fragments that bound to CR3 of mature bone marrow granulocytes. Once recruited from the bone marrow by an inflammatory stimulus, these granulocytes with β-1,3-glucan-primed CR3 could kill iC3b-coated tumor cells. As had been found earlier with i.v. soluble yeast β-1,3;1,6-glucan therapy, oral β-1,3-glucan-mediated tumor regression required the presence of iC3b on tumors and CR3 on granulocytes, and therefore failed in mice deficient in C3 or CR3.

Yan (65) showed that the tumoricidal activity of soluble CR3-binding polysaccharides such as β-glucan was specific for neoplastic cells that had been opsonized with C3 through the action of naturally occurring tumor-reactive antibodies. Tumors that bore a sufficient density of C3 for recognition by the CR3 of circulating leukocytes responded to therapy with β-glucans, whereas tumors that were not opsonized with C3 did not respond to therapy (66). It is well known that β-glucan responses occur only in certain strains of mice bearing specific tumors, so this report (66) suggests that reports of the sensitivity or resistance of specific tumors to β-glucan corresponds to the presence or absence of antibodies capable of opsonizing the tumor with iC3b, opening new possible therapeutic options for treatment of immune-based therapies for human cancer.

β-glucans can potentially be used to generate a novel cell-mediated effector mechanism for tumor vaccines and antibodies to tumor antigens that otherwise rely mostly on the direct cytotoxic action of chemotherapy. This therapy appears to have the greatest applicability to metastatic tumors that have lost MHC class I and thus have escaped recognition cytotoxic lymphocytes (66,67). Such metastatic tumors frequently express polysaccharide or ganglioside tumor antigens for which there is an array of available vaccines and antibodies.

Studies in Animals

ABM Extracts as Anticancer Agents

Aqueous extracts of ABM given in the drinking water to rats and mice before chemical cancer induction exhibited antimutagenic effects, but were ineffective when administered in the post-induction period demonstrating protection only in the initiation step of liver carcinogenesis (37,68,69). These studies are of particular interest because the extraction consisted in preparing a 'crude aqueous extract' leaving a powdered dry fruiting body in water at room temperature for 2 h, which is the way ABM is popularly prepared (69).

Interestingly, rats fed with a dry powdered form of ABM using two strains (99/26 and 99/29) and prepared from two different moments of harvest (opened or closed basidiocarp) at 10% of the diet, on the basis of the strain and harvest (the more effective was strain Ab 26 closed basidiocarp) exhibited different significant antimutagenic activity, more evident when considering the reduction of both size and number of the preneoplastic lesions, even when given in the post-initiation period (70). Moreover, not only polysaccharides but also the lipid fraction of ABM was found to contain a compound with antitumor activity, subsequently identified as ergosterol (a precursor of ergocalciferol), that inhibited tumor-induced neovascularization in sarcoma 180-bearing mice by oral administration for 20 days without side effects, though the extract had no cytotoxic effect *in vitro* (71). Kimura (72) has identified from the lipid fraction sodium pyroglutamate, which has shown not only to have antiangiogenic (inhibition of von Willebrand Factor expression in tumors) and antitumor activity in Lewis lung carcinoma-bearing mice but also an inhibitory effect on the cancer-induced reduction of immune functions.

Ethanol fractions obtained from hot-water extract of *mycelium* or dried fruiting induced TNF-α and IL-8 secretion in rat bone-marrow macrophages. Further fractionation with increasing ethanol concentrations resulted in the reduction of this cytokine-inducing ability in mycelial extracts, but enhanced it in fruiting body extracts. Whereas mycelial fractions did not induce nitric oxide production, fractions obtained by precipitation of fruiting-body extract with high ethanol concentrations stimulated macrophages to produce significantly higher levels of nitric oxide than controls (23).

In a mouse model of peritonitis induced by i.p. injection of fecal stem solutions the pre-challenge oral administration of an aqueous ABM extract protected mice against lethal septicemia after fecal peritonitis as demonstrated by a reduction in bacteremia and increase in survival rate, which was comparable with the survival of a *verum* group

to which were administered *per os* metronidazole and doxycicline (73); and confirming previous results of the same group in mice given i.p. infection with the virulent *Streptococcus pneumoniae* serotype 6B (74).

ABM Extracts as Immunostimulants

In an interesting article (75), experimenting intradermal injections of four different ABM extracts (ethanol, water, oxalate soluble and insoluble fractions) in a bilateral Meth-A tumor model, mice administered with the oxalate soluble fraction were tumor free after 21 days. In the same article, oral *ad lib* administration of the same fraction had no antitumor effect but enhanced that of the intratumorally injected fraction ($P<0.01$ versus injection alone) (75). Significant macrophage chemotactic factor, but not neutrophil chemotactic factor activity, was detected, while serum levels of immunosuppressive chemotactic factor (a marker protein of activated macrophages and neutrophils in response to biological response modifier) increased dramatically suggesting an immunopotentiating activity besides a direct cytotoxic action on tumor cells (75).

The oxalate soluble fraction consisted of a large amount of carbohydrates (exclusively glucose as determined by HPLC) and small amounts of proteins; carbohydrates containing (1-4)-β-D-glucan and (1-6)-β-D-glucan in the ratio of approximately 1 : 2 (45,75).

Treatment with hot-water extracts of ABM fruiting bodies increased NK activity of spleen cells in naive BALB/c mice (75). In Meth A-bearing BALB/c mice, the same extracts enhanced the induction of antigen-specific cytotoxic T-lymphocytes and IFN-γ production. Up-regulation of NK and TC activity was triggered by IL-12 dependent activation (76) although it is not yet clear whether oral administration of ABM extract enhances IL-12 production *in vivo* (77).

These data are confirmed by a study of Itoh (78) showing moderate antiblastic activity in mice Meth-A tumor model but only by i.p. administration of FIII-2-b fraction, confirmed in another study where a new polysaccharide–protein complex (called ATOM: antitumor organic substance Mie) administered p.o. and i.p. appeared highly active against in mice sarcoma 180, Ehrlich ascites carcinoma, Shionogi carcinoma 42 and Meth A fibrosarcoma models, trough activation of immunostimulatory activity mediated by macrophage and complement (79).

Ehrlich carcinoma-bearing mice treated *per os* with the *n*-hexane (mainly unsaturated fatty acids), dichloromethane (sugar and amino acids), or methanol (mainly unidentified polymers) extracts from ABM fruiting bodies were able to maintain the NK activity of spleen cells during the first 10 days after tumor implantation (80). NK activity in these groups was similar to that of normal controls and higher than that of tumor-bearing mice treated with water. After 30 days, animals treated with *n*-hexane extract showed lower tumor growth than the other groups, but mice assuming dichloromethane extract presented signs of presumed toxicity (necrotic lesions in the extremity of the tail probably due to residues of organic solvents). The dichloromethane and methanol groups produced a more intense humoral response than the *n*-hexane and ethanol extract groups.

The results of NK activity on the 30th day after the injection of tumor cells suggest that none of the three extracts was able to maintain the lytic activity against Yac-1 target cells. And 30 days later, the Ehrlich carcinoma cells were enough to decrease the NK activity, perhaps, by the production of soluble factors like prostaglandins, TGF-β, or IL-10 (62).

Table 2. Main active anticancerogenic substances

Ergosterol	Antiangiogenic (71)
Pyroglutamate	Antiangiogenic, antiblastic, (immunostimulant?) (72)
Polysaccharides(1-6)-β-backbone	Immunostimulant, (antiblastic?) (81–83)
Lignin derivatives	Immunostimulant (TNF,IL8) (antiblastic?) (27,29)
Polysaccharide–protein complex	immunostimulant, (antiblastic?) (15,78,79)

Tumoricidal Acivity of β-Glucan

In a mouse model, for the first time in 2005, Kobayashi (81) has demonstrated that daily oral supplementation of β-glucan by a hydrochloric acid fraction (16.6% proteins, 90% glucose composed by 1-4-α-D-glucan and 1-6-β-D-glucan in the ratio 1 : 2) (82) seems the only orally active preparation in mice with respect to aqueous ammonium oxalate-soluble and ethanol-insoluble derivatives of ABM that are active only if administered intratumorally (83), probably due to a direct effect on tumor invasion and metastasis through a direct modulation of signalling cascades: inhibition of thymidine incorporation in a dose-dependent fashion in ovarian cancer cells *in vitro* although not in Lewis lung cancer cells. Data *in vitro* have been confirmed in a mouse model of peritoneally disseminated metastasis of human ovarian cancer by intraperitoneal injection and confirmed by oral administration, without influence on mean body weight or food consumption and the average number of formation of pulmonary nodules

was lower on experimental lung metastasis of Lewis lung cancer (81). These actions are probably due to the suppression of cell proliferation, apoptosis and inhibition of urokinase-type plasminogen activator through promotion of p38 MAPK activation (81).

In reference to other popular supposed pharmacological activities, the only experimental data are those from a model (84) of rats with a streptozocin induced diabetes treated by oral administration of a dried fruiting body hot-water extracts showed anti-hyperglycemic, anti-hypertriglyceridemic, anti-hypercholesterolemic and anti-arteriosclerosis activity indicating overall anti-diabetic activity in diabetic rats, but oligosaccharides obtained by hydrolization of β-glucans by means of *Bacillus megaterium* showed higher activity than β-glucans.

So, many studies show in animals the potential activity of ABM extracts, and more than a direct antiblastic activity. Moreover on the basis of *in vitro* studies it is possible to assume that the most promising pharmacological activities are immunostimulatory and antiangiogenic probably by means of different extracts (Table 2)

Clinical Studies

ABM in Cancer Patients

According to reports, 100 000–300 000 kg of the dried body of ABM is produced every year in Japan, and about 300 000–500 000 persons for the prevention or treatment of cancer assume the 3–5 g three times a day by a typical hot-water extract (71). In a small survey on the use of complementary therapies by patients affected by urological cancer in Japan, on a total of 293 patients surveyed, 52 were assuming ABM extracts representing a percentage of 31% and being the most commonly used 'health food' (85). Ahn (86) investigated the beneficial effects of the oral daily assumption of an extract of *Agaricus blazei* Murrill Kyowa (in the exact content and quantity of substances assumed by patients, and drop out for any cause were not described in the study) on immunological status and qualities of life in cancer patients undergoing chemotherapy. They observed that NK-cell cytotoxic activity, was significantly higher after a 6 weeks period compared with placebo, although there was no difference in white blood cells decrease in patients upon chemotherapy (carboplatin, etoposide and taxol). However, chemotherapy-associated side effects such as appetite, alopecia, emotional stability and general weakness were all improved on the base of the QLQ-30 Scoring Manual 2nd edition of EORTC modified by authors (86).

Table 3. Clinical studies

Gynecological patients (86)	Improvement of quality of life and immunitary status
Hypertension (87)	Lowering of diastolic and systolic blood pressure
Hypercholesterolemia and obesity (88)	Hypocholesterolemic and antiobesity activity
C-type hepatitis (89)	Lowering of γ-GTP

Clinical Sudies in the Treatment of Hypertension, Hypercholesterolemia and Hepatic diseases

Administration of γ-aminobutyric acid (GABA)-enriched *A. blazei* (AG-GABA) to mild hypertensive human subjects, in an open test and double blind cross-over test, showed that during AG-GABA intake period, both systolic and diastolic blood pressure values decreased to statistically significant levels, if compared with those of the pretest period or placebo intake period. No significant difference was observed, neither in the values of cholesterol nor of hepatic transaminases and γ-GTP (87).

The effects of protein-bound polysaccharides (A-PBP and L-PBP) that were extracted from the *mycelia* of *A. blazei* on serum cholesterol and body weight were investigated in 90 female volunteers for 8 weeks: the weight-reduction effect (11.8%) and hypocholesterolemic effect (11.0%) was most significant, indicating their synergistic action. These data suggested that the weight-controlling and hypolipidemic effect of L-PBP and A-PBP protein-bound polysaccharides were involved, at least in part, in absorption of cholesterol as their role of dietary fiber, as well as cholesterol metabolism (88).

A study evaluated the clinical effects and safety on human volunteers with elevated γ-GTP activity of *A. blazei* Condensed Liquid (*Agaricus* Mushroom Extract; ABCL) in the treatment of C-hepatitis. A total of 20 patients (50% of men) with chronic C-type hepatitis received the ABCL orally, twice a day, for 8 weeks. Decreasing effect for serum γ-GTP activity was found in 80% of the patients in both sexes; without any toxicological findings and other side effects (89). These initial clinical data (Table 3) are interesting, but we think it is soon to establish definitely a real benefit from the assumption of ABM extracts although it is not known exactly which are the active substances, although actually only β-glucans can be considered as the more active substance.

Toxicity

The Problem of Heavy Metals and Radioactive Substances

An important clinical-toxicological concern represented by mushrooms, especially wild ones, is the possible contamination with substantial levels of toxic metals such as arsenic, lead, cadmium and mercury as well as 137Cs, because many mushrooms species have the ability to accumulate radioactive substances such as relatively high concentrations of metals (90). So high levels of toxic compounds may offset whatever health benefits a diet rich in mushrooms or their extract could potentially confer (88,91,92,93).

Pharmacological Interferences

ABM extract can also down-regulate the expression of cytochrome P4501A and can be useful in reducing the production of metabolically activated procarcinogen from xenobiotics. It can consequently prolong the duration and intensity of drugs' activity, and could give rise to unpredictable side effects or adverse drug reactions (94).

Toxicity in Animals

In a study to evaluate 90-day subchronic toxicity of an aqueous extract in F344 rats, there were no consistent treatment-related changes in clinical signs, body weight and food consumption at the dose of $2654\,mg\,kg^{-1}$ b.w. day^{-1} for male and $2965\,mg\,kg^{-1}$ b.w. day^{-1} for female rats, although there was an increase of blood urea nitrogen in males that was considered unlikely to be of toxicological significance (contemporary decrease of creatinine and no histopathological changes reported) (95). Although it has not been established the direct quantity toxic or cancerogenic for humans, a main problem for the administration of ABM remains the problem of aromatic hydrazines (i.e. agaritine and its derivatives) whose cancerogenicity and chronic systemic effects are well known in animals for many years (96); probably due to metabolized toxic intermediates capable of damaging cellular macromolecules and stimulating proteolysis giving rise to hydrazine-mediated DNA strand scissions (97). Toth demonstrated that the administration of hydrazine analogs administered s.c. in Swiss mice induced fibrosarcoma in 24% of males, and in both sexes soft tissue tumors (98); while in another article agaritine administered in drinking water at 0.062 and 0.031% did not give rise to cancer although a substantial number of animals (Swiss mice) developed convulsive seizures (99); while hydrazine analogs (from *A. bisporus* and *Gyromitra esculenta*) administered in drinking water in Swiss mice and Syrian hamsters gave rise to liver neoplasms (benign hepatomas, liver cell carcinomas, angiomas, angiosarcomas) and adenomas and adenocarcinomas of lungs (100).

Hydrazines in general have also been found potent irreversible inactivators of some hemoproteins (101). Although the stability of the molecule was examined and that agaritine degrades within 48 h in tap water and that degradation appeared to be oxygen-dependent (102), the presence in plasma from agaritine-administration in mice or rats as its definite toxicity remains unclear (103). The concentration of agaritine in methanol extracts of food was $112–1836\,\mu g\,g^{-1}$ dry weight, in a commercial product of ABM arrived at $1791\,g\,g^{-1}$ dry weight and the calculated one-day intake of the product was estimated to be 8955 g according to the label (104). For this reason the Ministry of Health, Labour and Welfare of Japan demanded a cessation of sales and voluntary recall of the product from K-Company after a request to the Food Safety Commission to assess the safety of products containing ABM. Nevertheless, in a clinical test placebo-controlled to verify human toxicity of 'Freezing dryness *A. blazei* (Iwade strain 101) Himematsutake', after 16 weeks, there were no clinical problems in the blood examination, urinalysis, physical examination and history taking (105).

Toxicity in Humans

Three cases of severe hepatic dysfunction in cancer patients have been reported recently. These are preliminary data, although one patient underwent rechallenge with the same extract that resulted in deterioration of liver function again (106). Nevertheless, other causes cannot be ruled out: there is an apparent probable relationship between ABM extract and liver damage that deserves full attention due to the large assumption of the mushroom as OTC remedy.

Conclusions

Careful clinical studies comparing the activity of isolated compounds, whole mushroom extracts and epidemiological data are still necessary to determine whether ABM provide real clinical benefits. Dose-response studies and isolation, as well as chemical identification and quantification of specific compounds responsible for the potential benefit from ABM mushroom ingestion should be fully developed, although there seems to be clear evidence that ABM extracts are rich in β-glucans that presumably contribute to the observed immunostimulatory activity.

Other substances are probably involved as well, the immunostimulation following ingestion of polysaccharides is possible and probably useful in cancer patients if it does not give rise to pharmacological interferences. A main safety concern is represented by the toxicity and cancerogenicity of agaritine and its derivatives that should be completely evaluated; and probably would be useful for these mushrooms like other herbal remedies, to completely define the problem of heavy metal contents. Due to the large consumption of ABM in popular medicine, probably more data are needed on action mechanisms of its component and safety before counseling the assumption for prevention and treatment of cancer and immunodepressive disorders.

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Original Article

Immunomodulating Activity of *Agaricus brasiliensis* KA21 in Mice and in Human Volunteers

Ying Liu[1], Yasushi Fukuwatari[1], Ko Okumura[2], Kazuyoshi Takeda[2], Ken-ichi Ishibashi[3], Mai Furukawa[3], Naohito Ohno[3], Kazu Mori[4], Ming Gao[4] and Masuro Motoi[5]

[1]Mibyou Medical Research Center, Institute of Preventive Medicine, Tokyo, Japan, [2]Department of Immunology, School of Medicine, Juntendo University School of Medicine, Tokyo, Japan, [3]Laboratory for Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan, [4]Department of Acupuncture and Moxibustion, Suzuka University of Medical Science and Mie, Japan, and [5]Toei Pharmaceutical Co., Ltd., Tokyo, Japan

We performed studies on murine models and human volunteers to examine the immunoenhancing effects of the naturally outdoor-cultivated fruit body of *Agaricus brasiliensis* KA21 (i.e. *Agaricus blazei*). Antitumor, leukocyte-enhancing, hepatopathy-alleviating and endotoxin shock-alleviating effects were found in mice. In the human study, percentage body fat, percentage visceral fat, blood cholesterol level and blood glucose level were decreased, and natural killer cell activity was increased. Taken together, the results strongly suggest that the *A. brasiliensis* fruit body is useful as a health-promoting food.

Keywords: *A. brasiliensis* – clinical research – cold water extract – NK activity – outdoor-cultivated – safety

Alternative medicine is the general term for 'medicine and treatment that have not been verified scientifically or applied clinically in modern Western medicine' (1–12). The range of alternative medicine varies widely to include traditional medicine and folk remedies as well as new therapies that are not covered by health insurance. Considering the current world population, the percentage of people utilizing modern Western medicine is surprisingly low, with the World Health Organization (WHO) indicating that 65–80% of health management is by traditional medicine. 'Mibyou' is a recently established term that means a half-sick person having clinical laboratory data that borders healthy individuals and patients. Education of the mibyou population about eating habits is also significantly important for maintaining public health by the government.

For reprints and all correspondence: Naohito Ohno, Professor, Tokyo University of Pharmacy and Life Science, School of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Tel: +81-426-76-5570; Fax: +81-426-76-5570; e-mail: ohnonao@ps.toyaku.ac.jp

In Japan, an increasing number of people are turning to alternative medicine mainly in the form of health foods such as amino acids, lipids, carbohydrates, plants, seaweeds, insects, bacteria, yeasts and mushrooms. Such mushrooms as *Lentinula edodes*, *Ganoderma lucidum* and *Grifola frondosa* are commercially available. *Agaricus brasiliensis* (*A. blazei ss. Heinemann*) is a health food that has received recent attention. *A. brasiliensis* has been reported to improve symptoms of lifestyle-related diseases including obesity, hypertension and diabetes, and to have anti-inflammatory, antitumor, cancer inhibitory and immuno-enhancing effects (13–18). However, many reports were either animal studies or clinical studies with few cases.

Many mushrooms, also called as macrofungi, are classified as higher-order microorganisms, Basidiomycota. To discuss the functions of Basidiomycota, it is important to compare them under the same conditions, including not only the species but also the strain, as well as methods of cultivation and processing. Basidiomycota products involve mycelia, spores and fruit bodies in each

species. The fruit body and the mycelium are distributed widely in foods. To maintain the manufacturing process, the mycelium is superior to the fruit body; however, its components are known to be quite different. There are many ways to obtain the fruit body, e.g. collecting naturally grown mushrooms from hills and fields, and outdoor or indoor cultivation.

Agaricus brasiliensis KA21 used in this study is a fruit body cultivated outdoors in Brazil. Fruit bodies were air dried by a ventilator with a blowing temperature lower than 60°C to maintain their enzyme activities. We have recently examined the structure and antitumor activity of polysaccharide fractions of the fruit body and concluded significant contribution of the highly branched 1,3-β-glucan moiety on the activity. We also prepared the cold and the hot water extracts (AgCWE and AgHWE) and examined on a murine diabetic model C57Bl Ksj-db/db, and found that AgCWE showed much stronger pharmacological activity to this model. These facts strongly suggested that pharmacological action of cold water extract differ from that of hot water extract. We have also shown that the cold water extract contains enzymes such as polyphenol oxidase and peroxidase (19–25). Table 1 shows the general constituents of *A. brasiliensis* KA21. KA21 has high protein and fiber content. It also has high levels of vitamins B1, B2, B6, niacin, pantothenic acid, folic acid and biotin. It contains many minerals including large amounts of iron, potassium, phosphorus, magnesium, zinc and copper, and certain amounts of manganese and selenium. In addition, it contains detectable concentrations of vitamin D as it is cultivated under the sunlight.

To successfully achieve and maintain food safety for citizens, laws related to foods have become strictly controlled. Recently, medical doctors in National Cancer Center Hospital East in Japan reported three cases of severe hepatic damage, taking *A. blazei* extract (26). They mentioned it is necessary to evaluate many modes of complementary and alternative medicines, including the *A. blazei* extract, in rigorous, scientifically designed and peer-reviewed clinical trials. Very recently we have experienced evacuation of one health food originated from *A. brazei*, because of inducing genotoxicity in experimental animals. Ministry of Health, Labor and Welfare reported it is only the case of one product and the molecular mechanisms are under investigation. It is also simultaneously reported that other related products did not show such toxicity. Agaritine is a well known toxic metabolite of agaricaceae, such as *Agaricus bisporus*, and the relationship between agaritine content and the toxicity has attracted attention. In any case, function as well as safety of products originated from macrofungi, especially agaricaceae should be precisely examined as much as possible.

Thus, to safely and effectively use alternative medicine including *A. brasiliensis*, analysis at the molecular level by basic research and proving their effects by clinical research are important. In a human safety study, we found that long-term intake of the fruit bodies of *A. brasiliensis* KA21 cultivated outdoors had no adverse effects (22). In the present study, we demonstrated the immunomodulating effect of *A. brasiliensis* KA21 both by animal and human studies. As described earlier, the fruit body contained many enzymes even after the drying process, and cold and hot water extracts were prepared and administered orally to examine

Table 1. Composition of *A. brasiliensis* KA21

Energy	288.00 kcal
Protein	38.50 g
Fat	2.60 g
Carbohydrate	27.70 g
β-glucan	12.4 g
Fiber	20.60 g
Sodium	8.40 mg
Calcium	22.50 mg
Iron	10.10 mg
Potassium	2920.00 mg
Phosphorus	952.00 mg
Magnesium	96.50 mg
Zinc	7.87 mg
Copper	7.67 mg
Manganese	0.825 mg
Iodine	0
Selenium	88.00 μg
Arsenicum	0 .48 ppm
Cadmium	2.01 ppm
Plumbum	0.13 ppm
Hydrargyrum	0.18 ppm
Total chromium	0 μg
Vitamin in A (total caronene)	0
Vitamin B (total caronene)	
Vitamin B1 (Thiamin)	0.63 mg
Vitamin B2 (Riboflavin)	3.04 mg
Vitamin B6	0.54 mg
Vitamin B12	0 μg
Niacin	33.50 mg
Pantothenic acid	22.90 mg
Folic acid	230.00 μg
Biotin	123.00 μg
Total vitamin C (Total c acid)	0 mg
Vitamin D	56.7 μg
Vitamin E (Total tocopherol)	0
Vitamin K1 loquinone)	0
Agaritine	15.3 ppm

Note: In 100 g dry weight, measured by Japan Food Research laboratories.
Agaritine was measured by MASIS laboratories by HPLC method.

immunomodulation in mouse models. Drinking such cold water extracts of *A. brasiliensis* is a traditional custom in Brazil. In the clinical study, we determined the weight, body mass index (BMI), percentage body fat, percentage visceral fat and blood biochemical levels [total protein, blood glucose, cholesterol, neutral fat, glutamate oxaloacetate transaminase (GOT), glutamate pyrvic transaminase (GPT) and glutamyl transferase (γ-GTP)], and natural killer (NK) cell activity before and after administration of *A. brasiliensis* KA21. Analysis of the data from the viewpoint of mibyou is also included.

Methods

Agaricus brasiliensis Fruit Bodies

Strain KA21 was cultivated outdoors in Brazil, and its fruit bodies were washed and dried using hot air at 60°C or lower.

Measurement of Ingredients

All ingredients except for agaritine were measured by Japan Food Research Laboratories, Shibuya, Tokyo using the standard protocols recommended by the Resources Council, the Science and Technology Agency of Japan. The concentration of agaritine was measured by HPLC/MS/MS by MASIS Inc, Minamitusgaru, Aomori.

Preparation of Hot Water Extract (AgHWE) and Cold Water Extract (AgCWE) of A. brasiliensis

The fruit bodies of KA21 (100 g each) were ground using a domestic coffee mill, suspended in 0.1 g/ml physiological saline (Otsuka Pharmaceutical Co., Ltd), and extracted in an autoclave (120°C, 20 min) or with cold water (4°C, 1 day). The supernatant after centrifugation was designated as AgHWE or AgCWE. The extracts were kept frozen at −20°C until use.

Oral Administration to Mice

AgHWE and AgCWE prepared by the earlier-described method were administered to mice orally for 2 weeks, and cell count and cell population were determined.

Murine Tumor Model

Solid form tumor: Sarcoma 180 cells (1×10^6/mouse) were subcutaneously administered to the groin of ICR mice on day 0. AgHWE or AgCWE was orally administered (p.o.) daily for 35 days. Standard β-glucan, sonifilan (SPG) was administered intraperitoneally on days 7, 9 and 11. After 35 days, the mice were sacrificed and the weight of the solid tumor was measured.

Inflammatory Cytokine Production in Primed Mice

Balb/c mice were primed with a standard β-glucan, SCG (200 μg/mouse) from *Sparassis crispa* on day 0, and AgHWE or AgCWE was orally administered daily for 1 week. One week later, bacterial lipopolysaccharide (LPS, 10 μg/mouse) was administered intravenously, serum was collected 90 min after the LPS administration, and serum TNF-α and IL-6 expression levels were measured with ELISA. Antibodies and standards were purchased from Pharmingen Ltd.

Concanavalin A-Induced Hepatic Injury in Mice

AgHWE or AgCWE were orally administered for several days in mice. One day after the final administration, Concanavalin A (Con A) was intravenously administered to induce liver injury. Interleukin 6 levels in sera were measured 3 h after Con A administration. GOT and GPT were measured 24 h after Con A administration.

Clinical Research in Humans

Research was performed on 31 healthy subjects who were not taking any medication prior to or at the time of the study. We explained the study to them in writing, and obtained informed consent to use the test results. The subjects were divided into three groups, group 2 and group 3 (total 20 subjects) were administered the normal dose, and group 1 (11 subjects) were administered a 3-fold higher dose (safety clinical study group) of *A. brasiliensis*.

Group 1. For 6 months from May 31 to November 26, 2004, the 11 subjects (mean age 43.6 ± 12.6 years, male 6, female 5) were asked to take 30 tablets/day (divided into three administrations; each tablet contained 300 mg of *A. brasiliensis*), which is three times the normal dose. Then, we measured and analyzed the subjective changes in their condition, liver function (GOT, GPT, γ-GTP), renal function [blood urea nitrogen (BUN), creatinine] and nutritional status (total protein).

Group 2. For 3 months from April 12 to July 8, 2005, 12 subjects (mean age 45.3 ± 8.1 years, male 9, female 3) were asked to take the normal dose of 10 tablets/day (divided into two administrations; each tablet contained 300 mg of *A. brasiliensis*). Then, we measured body weight, BMI, percentage body fat, percentage visceral fat and blood biochemical levels (total protein, blood glucose, cholesterol, neutral fat, GOT, GPT and γ-GTP).

Group 3. For 3 months from May to August, 2005, 8 subjects (mean age 22.3 ± 0.5 years, male 6, female 2) were asked to take the normal dose, and immune function (NK cell count, NK cell activity) was measured. In the measurement of immune function, we divided the eight subjects into two groups in a double-blind manner, *A. brasiliensis* group and placebo group, administered

10 tablets/day (divided into two administrations; each tablet contained 300 mg of *A. brasiliensis*) for 7 days, and determined NK cell count and NK cell activity in peripheral blood. After two-month drug withdrawal, the same study was conducted with the tablets exchanged (crossover). We analyzed the cell fraction in peripheral blood and regarded mononuclear cells with $CD3^{-}CD16^{+}CD56^{+}$ as NK cells. Following the usual method, we measured NK cell activity by 4 h ^{51}Cr-release assay using K562 tumor cells as targets, at an effector/target ratio (E/T) = 20 or 10 (the mixing ratio of mononuclear cells and K562 cells is 20 or 10).

Statistical Analysis

Paired *t*-test was used to evaluate statistical significance. $P < 0.05$ was considered significant in all analyses.

Results

Chemical Analysis of *A. brasiliensis* KA21 for Safety Assessment

Before starting animal and human experiments, the chemical composition and additives were screened. The chemical composition and nutrients are shown in Table 1. Recently, a major toxic compound of agaricaceae 'agaritine' has attracted attention by showing tumor-promoting activity in rats. The agaritine content of *A. brasiliensis* KA21 was measured and it was as low as 15.3 ppm. Heavy metals, such as lead and mercury were lower than the detection limit. Three hundred types of pesticides were measured and none was detected (data not shown).

β-glucan content of *A. brasiliensis* KA21 was $12.4\,g\,100\,g^{-1}$ measured by Japan food research laboratories. We have already precisely examined the structure of polysaccharide fractions of KA21, and the major structure of β-glucan showing immunomodulating activity was determined to be β-1,6-linked glucan with highly branched β-1,3-segment (20).

Vitamin D is a well known vitamin of macrofungi and KA21 contained $56.7\,\mu g\,100\,g^{-1}$ (= ca. $2250\,IU\,100\,g^{-1}$). Same strain, cultured inside the house did not contain detectable concentration of vitamin D (data not shown). It is well known that concentration of vitamin D is strongly dependent on sunlight exposure. Vitamin D content of KA21 well reflected the culture condition of outdoor and under the sunlight.

From these data, *A. brasiliensis* KA21 was found to be chemically and analytically safe for animal and human studies.

Parameters and Effects on Experimental Animals

Effect on Normal Inbred Strains of Mice

For the animal experiments, AgCWE, AgHWE were prepared and examined. When AgCWE or AgHWE was administered orally at the dose of 20 mg/mouse to healthy mice (C3H/HeN) for 2 weeks, cell count in the thymus was not changed (data not shown), but that in the spleen was increased in the AgCWE group (Fig. 1).

Cells were doubly stained with CD4/CD8α, αβ/γδ, or CD3/B220, and the ratios of cell populations were calculated after measurement with a flow cytometer. No notable changes were seen in the thymus (data not shown), whereas the ratio of $CD4^{+}$ in the spleen was increased significantly in the AgHWE group (Fig. 1).

Antitumor Activity of Orally Administered AgCWE and AgHWE in Sarcoma 180 Transplanted Mice

We evaluated the antitumor effect of *A. brasiliensis* on Sarcoma 180 solid tumor, which is the standard system to measure antitumor effects in mice. Sonifilan (SPG) was used as standard material. Oral administration of

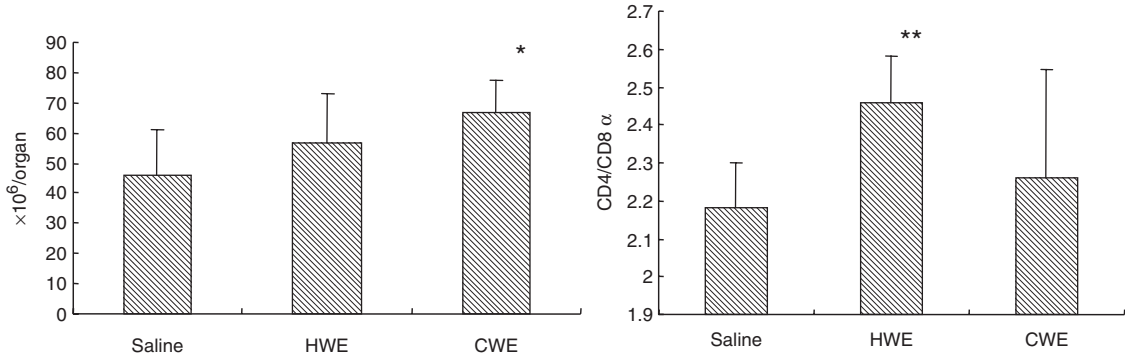

Figure 1. Cell number and population of splenocytes from AgHWE or CWE p.o. mice. AgHWE, CWE or saline (200 µl/mouse, 1 day, 1 shot), was p.o. administered to C3H/HeN mice for 14 days. The splenocytes were collected from each group of mice on day 14. Total cell number was counted with a hemocytometer (left). CD4/CD8α were measured by flow cytometory (right). The results represent the means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control by Student's *t*-test.

AgCWE or AgHWE for 35 days led to the suppression of tumor growth (Table 2).

Protection against Concanavalin A-Induced Liver Injury by Orally Administered AgCWE and AgHWE in Mice

The intravenous administration of Con A, a plant lectin, triggers acute hepatopathy in mice. We administered oral AgCWE or AgHWE as pretreatment, and then assessed the effects of Con A on hepatopathy. When 200 µl of AgCWE or AgHWE was administered for 7 days as pretreatment, GOT was found to decrease significantly in the AgCWE group. A similar trend was seen in the AgHWE group. When the dose was increased to 600 µl and administration was continued for 7 days, the effect became more notable (Fig. 2). GPT was decreased in a similar manner (data not shown). Similar studies were performed using different forms of administration and several mouse lines, and all cases showed a decreasing trend. Together, the results show that *A. brasiliensis* KA21 protects mice from hepatic injury.

Table 2. Antitumor effect of *A. brasiliensis* extracts on solid form of Sarcoma 180 in ICR mice

Name	Dose (mg)	Times	Route	CR/n	Tumor weight mean/SD (g)	% Inhibition	*t*-test
Control				0/12	8.6 ± 4.3	0.0	
SPG	0.1	3	i.p.	7/11	0.4 ± 1.1	95	<0.001
Control				0/10	15.0 ± 6.5	0	
AgCWE	2	35	p.o.	0/10	9.6 ± 6.5	36	<0.05
AgHWE	2	35	p.o.	0/10	7.9 ± 2.5	47	<0.01

Note: Dose, per mouse; times, day 7, 9, 11; CR/n, Number of tumor free mice/total mouse. SPG, Standard β-glucan as positive control.

Protection of Multiple Organ Failure Induced by Lipopolysaccharide by Oral Administration of A. brasiliensis *KA21*

Next, we investigated cytokine production induced by the administration of bacterial endotoxin, LPS, an agent that induces multiple organ failure in severe infections, to determine the hepatocellular protective effect of AgCWE and AgHWE. The levels of TNF-α and IL-6 generated by LPS administration were decreased in both groups (Fig. 3), indicating that *A. brasiliensis* controls the level of cytokine production to protect organs.

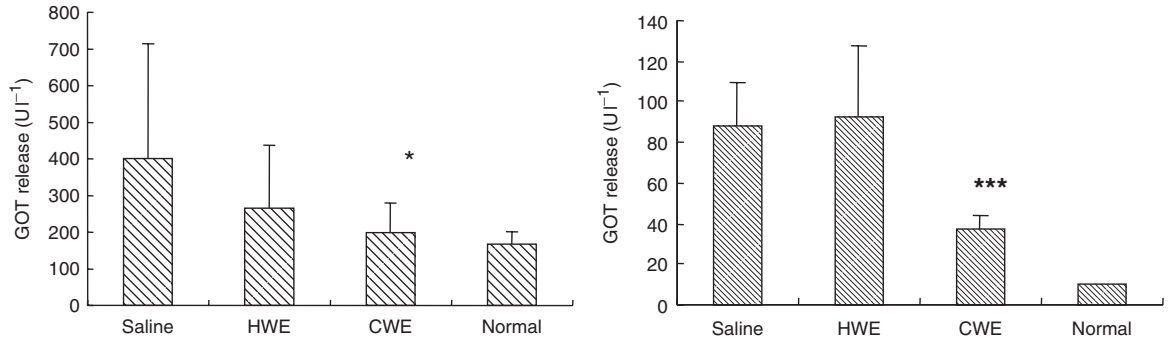

Figure 2. Effect of AgCWE or HWE p.o. on Con A-Induced liver injury. (Left) AgHWE or CWE (200 µl/mouse) was p.o. administered to Balb/c mice for 7 days. Con A (20 mg kg^{-1}) was iv administered on day 7 and the sera were prepared 24 h later from each group of mice. Results are expressed as the mean ± SD $^{*}P<0.05$ compared with control by Student's *t*-test. ($N=7$). (right) AgHWE or CWE (600 µl/mouse) was p.o. administered to Balb/c mice for 7 days. Con A (20 mg kg^{-1}) was iv administered on day 7 and the sera were prepared 24 h later from each group of mice. Results are expressed as the mean ± SD $^{***}P<0.001$ compared with control by Student's *t*-test. ($N=3$).

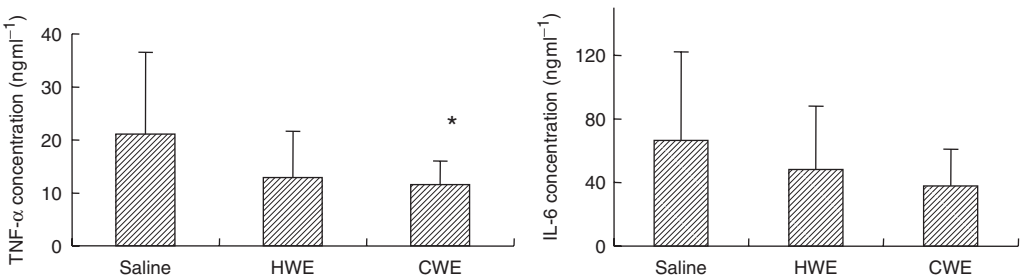

Figure 3. Effect of oral *A. brasiliensis* on LPS-induced cytokine production. β-Glucan (SCG, 200 µg/mouse) was i.p. administered to Balb/c mice on day 0. AgHWE or CWE was p.o. administered to these mice for 7 days. LPS (10 µg/mouse) was iv administered as a triggering reagent on day 7 and the sera were prepared 1.5 h later from each group of mice. IL-6 and TNF-α was measured by ELISA. Results are expressed as the mean ± SD $^{*}P<0.05$ compared with control by Student's *t*-test. (left) TNF-α, (right) IL-6.

Clinical Research

Safety of A. brasiliensis

Before determining the safety of *A. brasiliensis* KA21, a normal dose was administered for 3 months to 13 subjects as a preliminary experiment and measured changes of general clinical parameters. Mean body weight (71.2 → 70.9 kg), size of waist (85.4 → 83.5 cm), percentage body fat (34.4–33.0%) and BMI (27.8–27.6) did not show any clinical sign of illness by taking it. Thus to precisely determine the safety of *A. brasiliensis* KA21, a dose of three times higher than the normal dose was administered for 6 months to 11 subjects (group 1, see 'Methods'), and subjective changes in conditions, liver function, renal function and nutritional conditions were measured and analyzed. After measuring the biochemical parameters, we confirmed no statistically significant difference before and after administration, and no side effects caused by long-term administration (Table 3).

Effect of A. brasiliensis *on Biochemical Parameters related to Adiposis and Diabetes*

In order to evaluate the effect of *A. brasiliensis* KA21 on lifestyle-related diseases, the normal dose was administered to 12 subjects (group 2, see 'Methods') for 3 months and comparison of clinical biochemical data was made. The results are as follows: (i) Significant decreases were seen in body weight and BMI ($P<0.01$ each) after administration (Figs 4 and 5). (ii) Significant decreases were observed in percentage body fat ($P<0.01$) and percentage visceral fat ($P<0.01$) after administration (Figs 6 and 7). (iii) Significant increase was found in total protein level ($P<0.03$) after administration (Fig. 8). (iv) Significant reduction was seen in blood glucose level ($P<0.02$) after administration (Fig. 9).

Table 3. Safety of *A. brasiliensis* KA21 in human volunteers

Biochemical parameters	Before (mean ± SD)	After (mean ± SD)	Statistics (*P*-value)
Total protein ($g\,dl^{-1}$)	7.50 ± 0.16	7.41 ± 0.25	0.31
BUN ($mg\,dl^{-1}$)	15.81 ± 5.93	13.45 ± 2.25	0.12
Creatinine ($mg\,dl^{-1}$)	0.92 ± 0.21	0.90 ± 0.20	0.19
GOT (μl^{-1})	18.8 ± 4.75	19.8 ± 4.40	0.10
GPT (μl^{-1})	15.7 ± 6.90	16.3 ± 4.90	0.52
γ-GTP (μl^{-1})	35.4 ± 29.6GTP	35.9 ± 30.1	0.89

($N=11$).

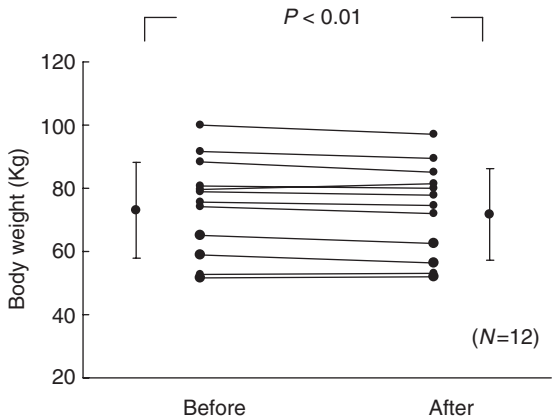

Figure 4. Effect of *A. brasiliensis* on body weight. Experimental protocol was shown in 'Methods'.

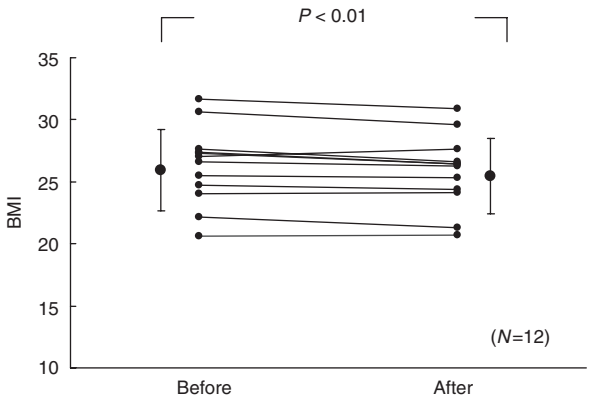

Figure 5. Effect of *A. brasiliensis* on BMI. Experimental protocol was shown in 'Methods'.

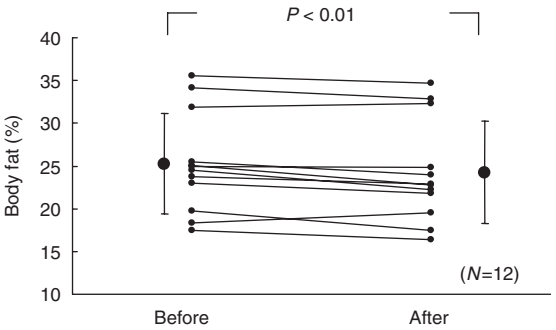

Figure 6. Effect of *A. brasiliensis* on percentage body fat. Experimental protocol was shown in 'Methods'.

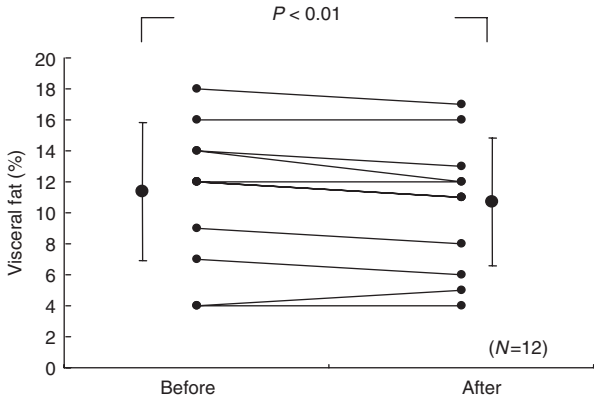

Figure 7. Effect of *A. brasiliensis* on percentage visceral fat. Experimental protocol was shown in 'Methods'.

In order to analyze the data more precisely, the subjects were divided according to total cholesterol level into a normal value group (T-CHO < 200 mg/dl) and a mibyou (slightly sick) value group (T-CHO ≥ 200 mg/dl) for comparison. No change was observed in the T-CHO < 200 mg/dl group before and after administration, whereas a decrease was seen in the T-CHO ≥ 200 mg/dl group after administration (Fig. 10).

The subjects were divided according to blood neutral fat level into a normal value group (TG < 120 mg/dl) and a mibyou value group (TG ≥ 120 mg/dl) for comparison. No change was observed in the former, whereas a decrease was observed in the latter after administration (Fig. 11).

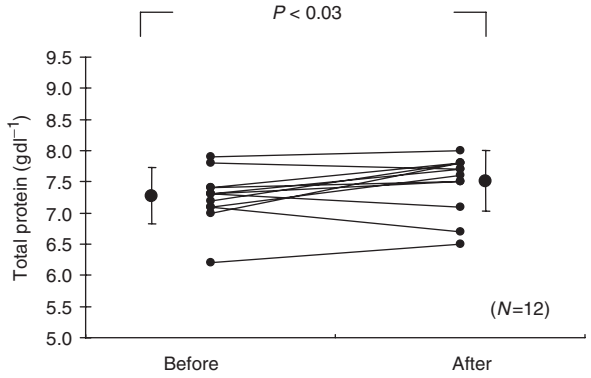

Figure 8. Effect of *A. brasiliensis* on total protein level. Experimental protocol was shown in 'Methods'.

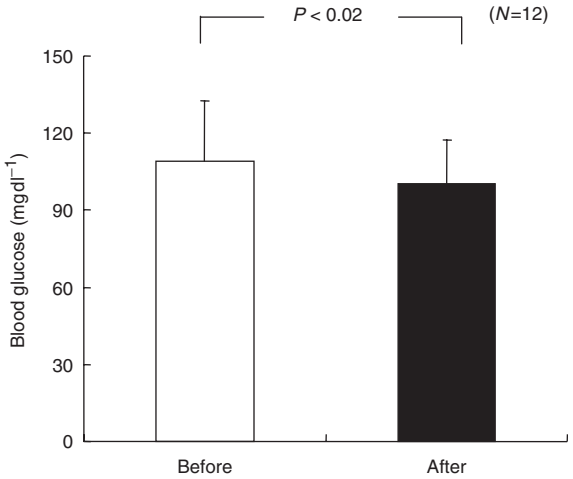

Figure 9. Effect of *A. brasiliensis* on blood glucose level. Experimental protocol was shown in 'Methods'.

Improvement of Liver Function by A. brasiliensis

To determine liver function, we compared GOT, GPT and γ-GTP values of the earlier mentioned subjects shown in the previous section. When comparison was made among all 12 subjects, no differences were seen before and after administration (Fig. 12). By contrast, after the subjects were divided into normal and mibyou according to GOT level, the average value of GOT in the normal value group (GOT < 25 IU l^{-1}) was found to increase slightly after administration, whereas that in the mibyou value group (GOT ≥ 25 IU l^{-1}) was found to decrease after administration, although the difference was not statistically significant (Fig. 13). The average value of GPT was increased in the normal value group (GPT < 25 IU l^{-1}) after administration, whereas that in the mibyou value group (GPT ≥ 25 IU l^{-1}) was decreased slightly after administration, the difference being not statistically significant (Fig. 14). The average value of γ-GTP was decreased slightly in the normal value group (γ-GTP < 30 IU l^{-1}) after administration, whereas that in the mibyou value group (γ-GTP ≥ 30 IU l^{-1}) was almost unchanged (Fig. 15).

Taken together, we determined that both lipid and blood glucose levels showed a decreasing trend for

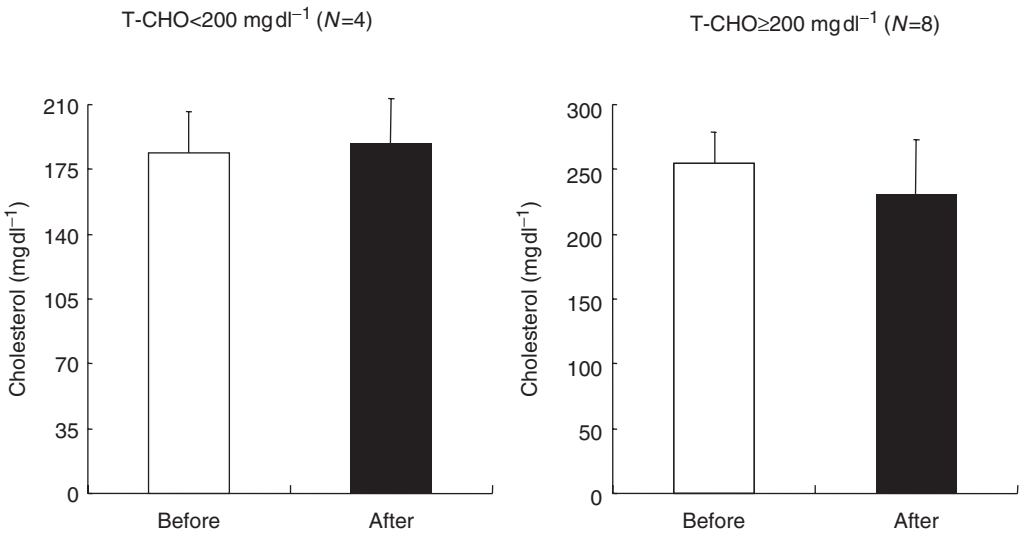

Figure 10. Effect of *A. brasiliensis* on blood cholesterol level from the viewpoint of Mibyou. Experimental protocol was shown in 'Methods'.

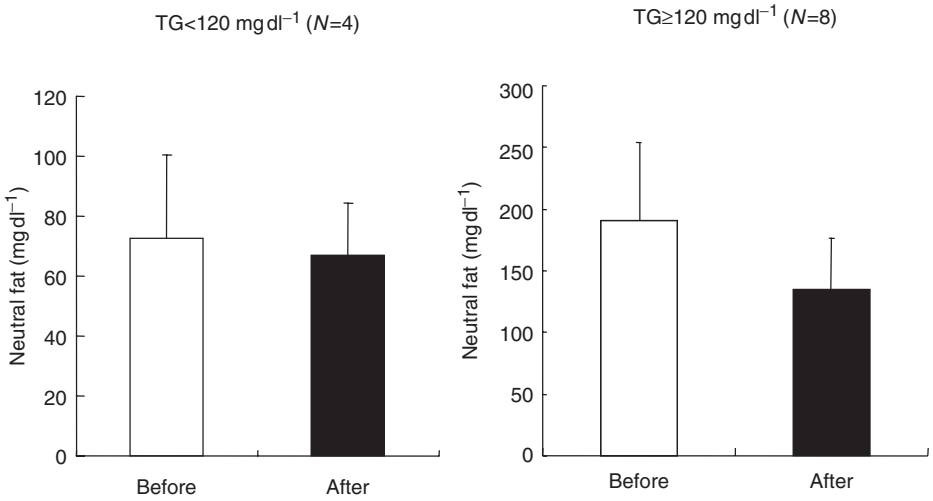

Figure 11. Effect of *A. brasiliensis* on neutral fat level from the viewpoint of Mibyou. Experimental protocol was shown in 'Methods'.

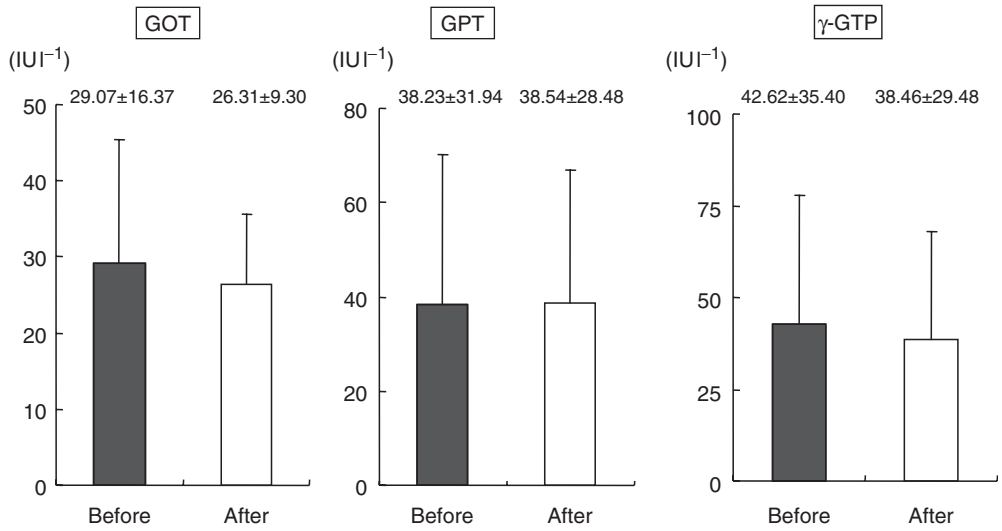

Figure 12. Effect of *A. brasiliensis* on liver function. Experimental protocol was shown in 'Methods'.

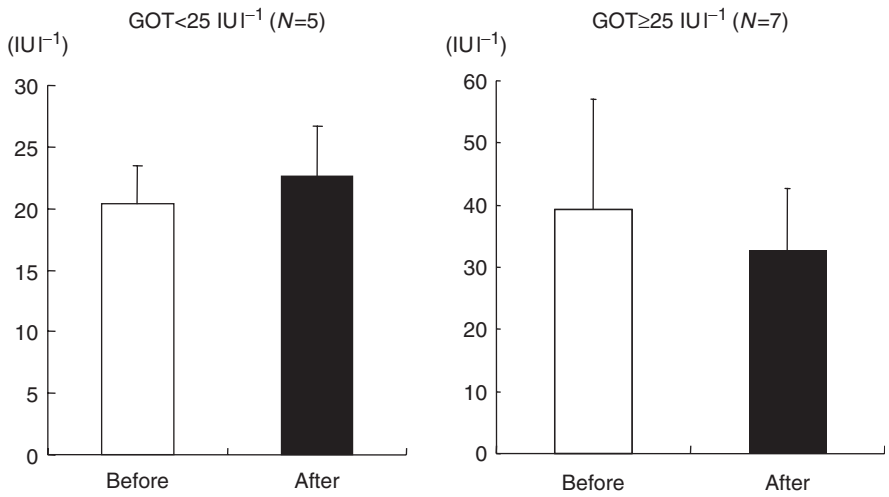

Figure 13. Effect of *A. brasiliensis* on liver function (GOT Value) from the viewpoint of mibyou. Experimental protocol was shown in 'Methods'.

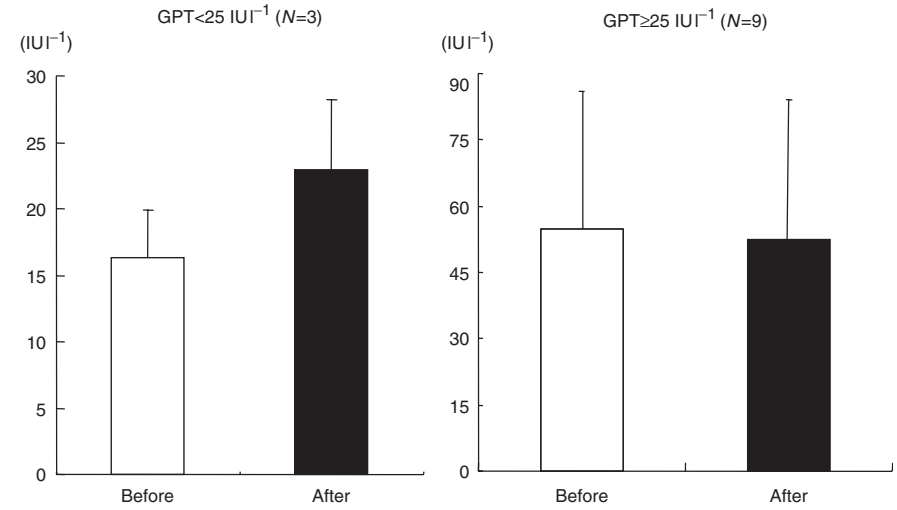

Figure 14. Effect of *A. brasiliensis* on liver function (GPT Value) from the viewpoint of mibyou. Experimental protocol was shown in 'Methods'.

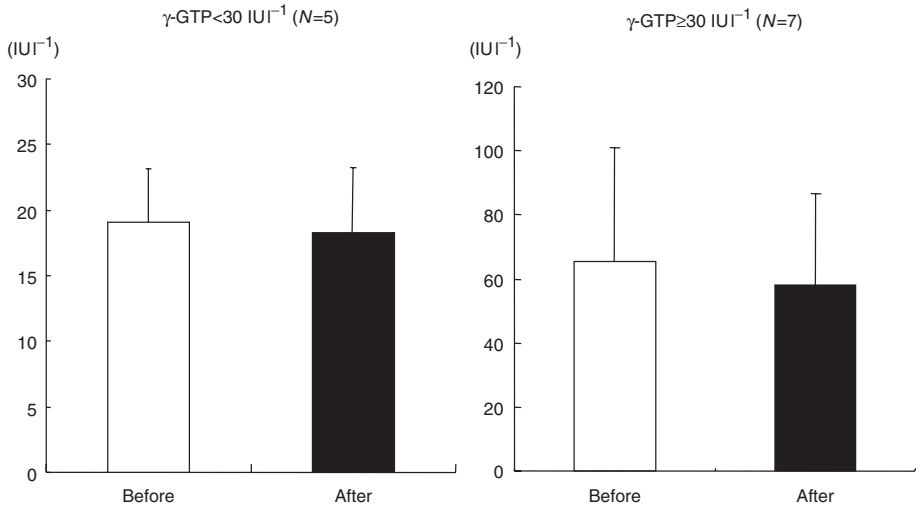

Figure 15. Effect of *A. brasiliensis* on liver function (γ-GTP Value) from the viewpoint of mibyou. Experimental protocol was shown in 'Methods'.

lifestyle-related diseases. In addition, an improvement in liver function was noted.

Modulation of Natural Killer Cell by A. brasiliensis

In order to evaluate the effect of *A. brasiliensis* KA21 on immune function, NK cell number and function were examined by eight subjects in a double-blinded experimental protocol shown in 'Methods' (group 3, see 'Methods'). The normal dose or placebo was administered to eight subjects for 7 days and NK cell number and activity in peripheral blood was compared as follows.

Effect of A. brasiliensis *on NK Cell Count*

Comparison of NK cell count before and after administration, and comparison between the *A. brasiliensis* group and placebo group were made, and no statistically significant differences were observed (Fig. 16).

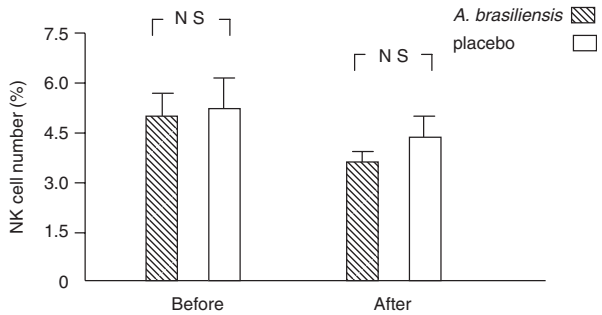

Figure 16. Comparison of NK cell count between groups before and after administration of *A. brasiliensis*. Experimental protocol was shown in 'Methods'.

Augmentation of NK Cell Activity by A. brasiliensis *KA21*

Before administration, no significant differences were observed between *A. brasiliensis* group and placebo group

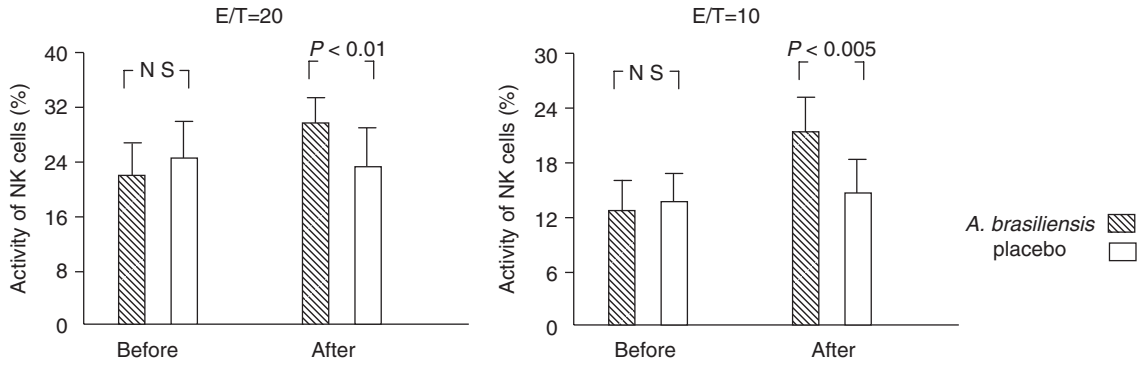

Figure 17. Effect of *A. brasiliensis* on NK cell activity. (Comparison between *A. blazei* group and placebo group). Experimental protocol was shown in 'Methods'.

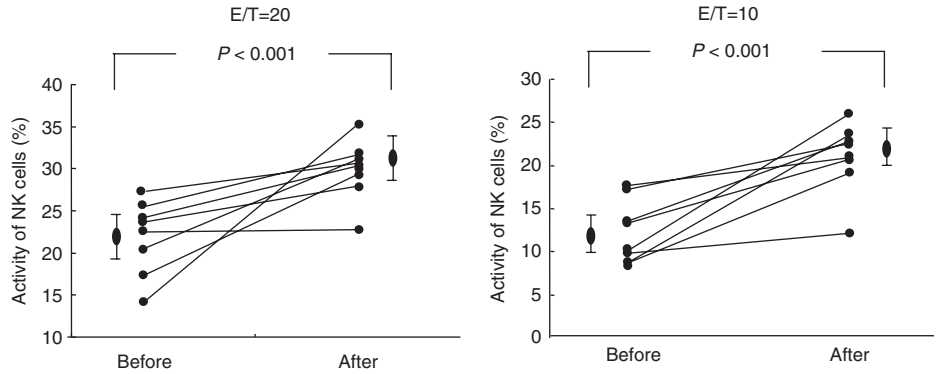

Figure 18. Comparison of NK cell activity before and after administration of *A. brasiliensis*. Experimental protocol was shown in 'Methods'.

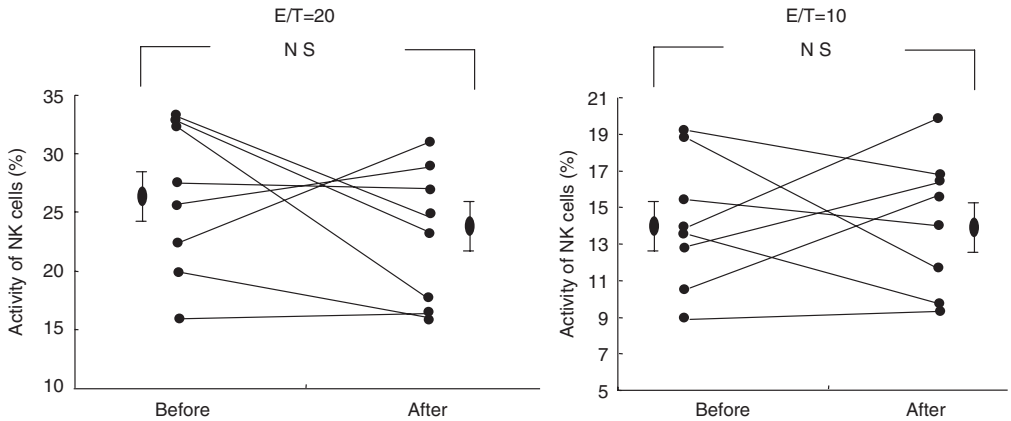

Figure 19. Comparison of NK cell activity before and after administration of placebo. Experimental protocol was shown in 'Methods'.

(Fig. 17 left). After administration, there were significant differences between the two groups, with $P<0.01$ for the E/T = 20% group and $P<0.001$ for the E/T = 10% group (Fig. 17 right). Figs 18 and 19 show individual changes in NK cell activity after administration of *A. brasiliensis* (Fig. 18) and placebo (Fig. 19) groups. NK cell activity was increased significantly in *A. brasiliensis* groups, with $P<0.001$ for the E/T = 20% group and $P<0.001$ for the E/T = 10% group. Meanwhile, NK cell activity was not increased significantly in the placebo group after administration.

Discussion

Japan is rapidly becoming a super-aging society, and such issues as decreased workforce, consumption and tax revenues, and increased international competition

among neighboring Asian nations are emerging. As a dramatic increase in the number of elderly patients is inevitable, the social security system is expected to become financially strained, and patient and consumer awareness of their rights will be enhanced because of the increased financial burden levied on them. Whereas genetic disposition is said to be involved in the development of lifestyle-related conditions and diseases, such as diabetes, hyperlipidemia and cancer, several other factors also determine their development; therefore, lifestyle is closely related to the development of such conditions and diseases. On the other hand, there is a need to reduce the significantly elevated medical expenses in the future. There are discussions as to whether we should pay medical expenses to aid people who do not practice a healthy lifestyle. The number of people who are not sick yet not healthy, that is, 'in poor health' or 'mibyou', is increasing at an accelerated pace (27,28). It is difficult to maintain regular eating habits in stress-laden daily life. Improvement of diet by consuming functional foods seems to contribute to the health improvement of people with poor health, as well as to the prevention of the development of lifestyle-related diseases.

There are many functional foods in Japan and they are expensive for customers, thus accurate information is needed to select the best food for each customer. All the parameters of safety, cost performance, evidence of function, as well as taste are important to disclose.

Mushrooms have been a part of oriental medicine for hundreds of years as being beneficial for health. Most traditional knowledge about the medicinal properties of mushrooms comes from the Far East, Japan, China, Korea and Russia. The most striking evidence is that lentinan from *L. edodes*, sonifilan from *Schizophyllum commune* and krestin from *Coriorus versicolor* have been approved for anticancer drugs mediated by immune stimulation. A great many mushroom products are on the market as health promoting foods, and basic and clinical researches of these products have been performed continuously (29–41).

Currently, there are 80 000 known fungal species in the world. It is surmised that 1 500 000 species exist, including undiscovered species. These fungi are classified by kingdom, phylum/division, class, genus and species. Many fungi are classified into Basidiomycota or Ascomycota, whereas others are also classified into the kingdom Protozoa or kingdom Chromista. Fungi include mushrooms, molds and yeasts, which have significantly different appearance and sizes. As mushrooms are too large to be considered microorganisms, they are referred to as macrofungi. Lichens of which two or more microorganisms live in a symbiotic relationship are also included. Fungi exhibit both the sexual form (for example, morphology of mushroom) and the asexual form for regeneration (for example, morphology of mycelium) and either form is used depending on surrounding environmental changes; however, the existence of both forms (holomorph) is not known for all fungi. Their nomenclature is also characteristic. The background of the discovery of a fungus is reflected in its name and different names may be given depending on whether the fungus exhibits the sexual form (teleomorph) or the asexual form (anamorph) of regeneration. Fungi, particularly mushrooms, are 'cultivated' and distributed products, and detailed analysis of their components has been performed. In the Standard Tables of Food Composition in Japan (Fifth Edition), 36 foods are classified as 'mushrooms'. The representative nutritional composition of mushrooms includes fiber, glucose and sugar alcohols, organic acids, fatty acids, inorganic substances, vitamins, free amino acids, bitter and pungent components, flavor components, enzymes, biophylactic substances, pharmacologically active substances and toxic components. Moreover, molds and yeasts are related to some fermented foods. A variety of foods including sake (rice wine), miso (bean paste), soy sauce, cheese and katsuobushi (dried bonito) are manufactured with the help of eukaryotic microorganisms. Fungus produces many secondary metabolites that are used as drugs or raw material for drugs, an example of which is penicillin.

As regards edible mushrooms, some are consumed raw, and cultivated hypha and culture broth are distributed as supplements after processing. Although they are from the same fungus, there is no proof that they contain the same components as the cultivated fruit bodies. In the early 1980s, we performed animal studies to compare the macromolecular components of *G. frondosa* fruit bodies, mycelia and fermented products. That the quantities and quality of components contained in each extract differed considerably was also reflected in the activity (29–32). *Grifola frondosa* has been well studied in Japan and in other countries. Interestingly, the major active component differs depending on the study group (33–37). Comparing mushrooms and mycelia at the product level, it was found that live fungus differs from dried products. From the viewpoint of stable supply, the dried product is desirable, but its components change according to the drying method. It is likely that the components differ if the 'fungal strain' differs. Thus, one type of mushroom may vary greatly when processed as food or other products. When we want to discuss or evaluate components and pharmacologic action, we need to conduct comparisons under detailed conditions, especially if we perform animal experiments.

Agaritine (N-[γ-L-(+)-glutamyl]-4-hydroxymethylphenylhydrazine) was identified in fruit bodies of cultivated mushrooms belonging to the genus *Agaricus*, including commerce *A. bisporus* and closely related species (42–46). 4-(hydroxymethyl) benzenediazonium ion that had mutagenicity is believed to be formed when agaritine is metabolized. Agaritine is most prevalent, usually occurring in quantities between 200 and 400 $\mu g\,g^{-1}$ as

fresh weight, 1000–2500 $\mu g\,g^{-1}$ as dry weight in cultivated mushroom. Recently, agaritine in *A. brasiliensis* (*A. blazei*) sample and products was measured. These samples contained 112–1791 $\mu g\,g^{-1}$ of agaritine as dry weight (47). In the present study, we have detected only low concentrations of agaritine (15.3 ppm; 15.3 $\mu g\,g^{-1}$) in the preparation made of *A. brasiliensis* KA21. This value was <1/100 of the quantity of average values of *A. bisporus*. Agaritine content is known to be significantly varied depending on processing. Household processing (e.g. boiling, frying, microwave heating or drying) will reduce the agaritine content in *A. bisporus* by up to 50% or even more (48). Also, agaritine has recently been shown to be degraded oxygen dependent in water (42,43). There have been long discussing the toxicity and carcinogenicity of agaritine (44,45). However, the conclusion is still controversial. Toth and co-workers (46,49–51) undertook the work to assess the possible carcinogenic activity of the phenylhydrazines and related compounds in *A. bisporus*. Their studies indicated that most of phenylhydrazine and related compounds in the mushroom are carcinogenic in Swiss albino mice. The only compound that was tested negative was agaritine, a finding that significantly muddled the interpretation of the carcinogenicity data. Also, these studies were the conservative risk model. In the absence of epidemiological data, no evaluation of carcinogenicity of agaritine to humans could be made.

We have analyzed *A. brasiliensis* KA21 from various aspects and reported the β-glucan, the enzymes of polyphenol oxidase, peroxidase and β-1,3-Glucanase. β-glucan content of *A. brasiliensis* KA21 was 12.4 g 100 g^{-1} measured by Japan food research laboratories. We have already precisely examined the structure of polysaccharide fractions of KA21, and the major structure of β-glucan showing immunomodulating activity was determined to be β-1,6-linked glucan with highly branched β-1,3-segment (20). During that study we have prepared hot water extract, cold alkaline extract, and hot alkaline extracts and analyzed polysaccharide structure of all these fractions. Of much interest, all the fraction showed quite similar structural features that major linkage is β-1,6-linked glucan. From these data, major polysaccharide component in *A. brasiliensis* is β-1, 6-linked glucan, and it is consistent with the previous study. However, we have mentioned that antitumor activity needs β-1,3-linkages in addition to β-1,6-linkage based on the results of the limited chemical degradation study. However, this conclusion is still temporal and structural activity relations needed human studies.

This study showed that the fungus is rich in vitamins; as it is cultured outdoors, it contains detectable concentrations of vitamin D. Vitamin D is a well-known vitamin of macrofungi and KA21 contained 56.7 $\mu g\,100\,g^{-1}$ dry weight. In the parallel experiments, vitamin D was contained lower than the detection limit (0.7 $\mu g\,100\,g^{-1}$) in the mycelium of this fungi cultured in the liquid medium and the fruit body of *A. blazei* imported from China. Much differences of vitamin D in these products well reflected the culture condition of outdoors and under the sunlight. Relationship between vitamin D content and sunlight exposure has been demonstrated in various macrofungi (52). Based on the definition in the manual of Health Food Regulation in Japan, the food containing more than 1.5 $\mu g\,100\,g^{-1}$ (= 60 IU 100 g^{-1}) of vitamin D is defined as the food containing high vitamin D content. Considering the rule, KA21 is the food containing high concentration of vitamin D. Micronutrients such as vitamins and minerals promote the metabolism of waste products, carbohydrates and lipids via cellular activation, and improved insulin resistance by decreasing blood glucose. Fiber and unsaturated fatty acids decrease blood pressure and promote decholesterolization. KA21 also contained other micronutrients, thus it is good for health for variety of reasons.

Meanwhile, in an analysis of the active components in bupleurum root, a crude drug, we found that polyphenols polymerized by enzymes have a strong immunoenhancing effect (53–55). *A. brasiliensis* also has a number of enzymes related to the polymerization of polyphenols (23,24). Polyphenols polymerized by these enzymes may be active components in this fungus. In our clinical research, decreases in body weight, BMI, percentage body fat, percentage visceral fat and blood glucose level were noted and a tendency to decrease blood cholesterol level, blood neutral fat level, GOT, GPT and γ-GTP was observed in the mibyou value group. On the basis of the earlier results, among the components of this fungus, all the polysaccharides, enzymes, vitamins and minerals may be involved in the normalization of biochemical test results.

This study measured immune function in mice. When we compared the number and population of immunocompetent cells after administration of AgCWE or AgHWE to healthy mice orally for 2 weeks, it was found that the percentage of spleen $CD4^+$T cells was increased in the AgHWE group and the number of spleen cells was increased in the AgCWE group. Furthermore, both AgCWE and AgHWE showed antitumor effects and AgCWE prevented Con A-induced hepatopathy and suppressed cytokine production induced by LPS. $CD4^+$T cells are divided into type 1 helper T cells (Th1) and type 2 helper T cells (Th2) based on T-cell antigen stimulation, and Th1 is considered to be a more important contributor to the antitumor effect. Th1 is thought to infiltrate local sites well, demonstrate strong cytotoxicity and cytokine production ability, and induce complete tumor regression by locally inducing CTL, which has the ability to produce IFN-γ (56–58). It is likely that the antitumor effect of *A. brasiliensis* is closely related to the increase in $CD4^+$T cell count.

As changes in immunocytes were demonstrated by the oral administration of *A. brasiliensis* in healthy mice, it is expected that the daily intake of *A. brasiliensis* may have preventive effects on immunoregulation failure.

Agaricus brasiliensis suppressed organ dysfunction accompanied by blood with excessively high cytokine levels, which is related to multiple organ failure. It is desirable that cytokines be produced at certain levels as needed. In these models, such as LPS-elicited cytokine production, *A. brasiliensis* controlled excessive cytokine production (Fig. 3). *A. brasiliensis* can not only promote but also control immunity, which is considered a desirable effect.

Among the effects of *A. brasiliensis* on immune function, we examined changes in the ratio of NK cells to peripheral mononuclear cells and NK cell activity in humans. Both the *A. brasiliensis* group and the placebo group showed no significant changes in the ratio and number of NK cells to peripheral mononuclear cells after 1-week administration. On the other hand, comparing the *A. brasiliensis* and placebo groups, NK cell activity was significantly enhanced by the administration of *A. brasiliensis*. When individual cases were examined, almost all cases showed increasing NK cell activity with the administration of *A. brasiliensis*, although there were differences in the degree of increase (Fig. 18).

The measurement of NK cell activity has been most widely used in both animal and human experiments, because NK cells play a critical role in natural immunology, and measurement of cytotoxicity is reliable for evaluation with good reproducibility (5). The immune function is affected by NK cells as well as various lymphocyte and humoral factors including antibodies, complement and cytokines. There have been several publications demonstrating products of macrofungi enhanced NK activity (59–63).

The effect of *A. brasiliensis* on the degree of NK cell activity enhancement varied significantly among individuals. It was recently clarified that effectiveness as well as the appearance of side effects with each medication were significantly different in each individual. This is explained partly by polymorphism and the linkage of CYP-related genes, a drug-metabolizing enzyme group (64,65). On the other hand, many causative genes have been discovered in immunity-related diseases, some of which are polymorphic. It is possible that polymorphism may be related to individual differences observed in the effects of *A. brasiliensis*. Research into receptors for mushroom components is not extensive. Dectin-1 was recently determined to be the receptor for cell wall β-glucan, a major component of mushrooms (66–68). The relationship between polymorphism of the receptor for pathogens and disease has been elucidated (69,70). The effects of *A. brasiliensis* and receptor gene polymorphism may be related. Further analysis is necessary in the future.

Through basic and clinical research, we confirmed that *A. brasiliensis* can help to improve symptoms of lifestyle-related diseases because of its anti-inflammatory, antitumor and immunoenhancing effects, and that *A. brasiliensis* is a useful health food to treat mibyou (primary prevention).

Very recently we have experienced recall of one health food originated from *A. brazei*, because of inducing genotoxicity in experimental animals. Ministry of Health, Labor and Welfare reported it is only the case of one product and the molecular mechanisms are under investigation. Based on the clinical examination shown in this study, KA21 is very safe for human health. Any adverse effect could not be detected in our study. We have also stated that content as well as pharmacological action is significantly influenced by culture conditions even in the same fungi, such as vitamin D content. In addition, proteins may be decomposed during processing. Much restricted regulation for each of the health foods might be needed for increasing human health. In any case, agaricaceae contained many species for functional foods, thus, much study should be needed continuously. This study helped to understand the mushrooms of agaricaceae are very safe and useful for human health.

Conclusion

(i) In basic research using a mouse model, we determined that *A. brasiliensis* has antitumor, anti-inflammatory and hepatocellular protective effects. It was suggested that the increase in the number of helper T cells and the enhancement of NK cell activity are related to these effects.

(ii) In clinical research on human volunteers, we found that *A. brasiliensis* decreased body weight, BMI, percentage body fat, percentage visceral fat and blood glucose level significantly, and reduced obesity. It also decreased blood cholesterol level and neutral fat level, normalized liver function and activated the immune function in mibyou patients (people with poor health).

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AGARICUS BLAZEI MURRILL—A NEW GOURMET AND MEDICINAL MUSHROOM FROM BRAZIL

Tjakko Stijve
Sentier de Clies no 12, 1806 St.-Légier, Switzerland.
Maria Angela L. De A. Amazonas
Centro Nacional de Pesquisa de Florestas, Embrapa Florestas, Colombo, Paraná, Brazil.

T. Stijve & M.A.L. De A. Amanonas (2002). *Agaricus blazei* Murrill—A new gourmet and medicinal mushroom from Brazil. *Australasian Mycologist* 21 (1): 29–33.

According to Paul Stamets, the well-known American mushroom grower, we soon shall see a brand new cultivated mushroom species on the markets on both sides of the Atlantic. It is called *Agaricus blazei* Murrill, an agaric that is already popular in Brazil, in Japan and in China. Moreover, it is likely to conquer the United States under the name of 'Almond Portobello'. This robust mushroom resembles The Prince (*Agaricus augustus*), and it is also an excellent edible mushroom. Indeed, *A. blazei* not only has an agreeable almond flavour, but also a texture that is much better than that of other edible agarics. In addition, *A. blazei* is already well-known for its medicinal properties, because both its mycelium and fruitbodies contain up to 12 per cent of beta glucans, immuno-potentiating polysaccharides which also inhibit the growth of malignant tumors.

Some history

In 1945 the American mycologist W.A. Murrill discovered an unknown representative of the genus *Agaricus* on the lawn of his friend R.W. Blaze, who lived in Gainsville, Florida. In honour of his friend, he described the new species as *Agaricus blazei* in a rather obscure scientific journal. For years, this new mushroom_which is unknown in Europe and far from common in North America_remains in the dark until it is rediscovered in the 1960s by Japanese coffee growers working in Brazil. It is told that one of them, the scientist Takatoshi Furumoto is intrigued by the observation that the inhabitants of the Piedade/Ibiuna district suffer far less from geriatric afflictions than the rest of the Brazilean population. When investigating the causes of this phenomenon, he discovers that the Piedade people regularly consume a kind of mushroom that is unknown elsewhere.

The story is probably a latter date fabrication to render the healing powers of this mushroom more plausible. In reality, the inhabitants of Piedade have never eaten this mushroom, which is, today, not even common in their area. Furumoto was rather captivated by its excellent organoleptic properties which reminded him of the famous Matsutake, a delicious edible but rare mushroom in Japan. He therefore sent samples of the Brazilean mushroom to several Japanese universities, and he also consulted the well-known Belgian agaricologist, Dr Paul Heinemann, who identified the species as *A. blazei* Murrill. Subsequently, after 10 years of sustained efforts, Japanese mycologists managed to cultivate the mushroom. Initially, they called it 'Kawariharatake', which corresponds more or less with its habitus, until the day that a child, delighted by the elegant stature of these agarics, exclaimed: 'they look like real princesses!'. From that day, the Japanese call them 'Princess mushrooms' or 'Himematsutake'.

A literature search reveals that the medicinal properties of this mushroom have mainly been studied by Japanese pharmacologists. Not surprisingly, it is also Japanese companies who have marketed *A. blazei*-based medicinal drugs.

Description and taxonomic position

One will look in vain for *A. blazei* in European and American field guides. Heinemann gives the following description: **Cap** 5–11 cm broad, first convex, then plane in age, surface pale brown to brown with fine scales. Margin inrolled when young. **Stalk** 6–13 cm long, 1–2cm thick, cylindrical, hollow, white, but yellowing when crushed. **Veil** membranous whitish to brownish with brownish particles on underside. **Gills** very close, free, whitish becoming brownish, then chocolate-brown. **Flesh** firm, white, turning yellow-orange when cut. Odour sweet, of almonds. Spore print chocolate brown.

The photos (Figs 1–3) show the robust cultivated form. The carpophores are reminiscent of those of The Prince (*A. augustus*), and they share indeed its excellent taste and flavour, and its yellowish bruising flesh. There are, however, notable differences, especially at the mycelium stage. Certain mycologists rather compare *A. blazei* to *A. subrufescens*, a species that only stains very slightly yellowish, but which also has a pronounced almondy flavour. This 'Almond Mushroom' also prefers a high temperature, and is therefore common in the East coast States of America. It is interesting to note that about 100 years ago this mushroom was cultivated in California, where it soon lost against the competition of the good old white Button mushroom. Other specialists maintain that the agaric cultivated in Brazil is not identical with *A. blazei* as it was originally described by Murrill. They rather see a close relationship with *A. silvaticus*, in spite of the fact that this is a red bruising species! To render to Caesar what belongs to Caesar, one is inclined to re-baptize the mushroom and call it *Agaricus brasiliensis.*

Cultivation

Since *A. blazei* likes warmth and light, it is an excellent mushroom to cultivate outdoors. Indeed, from about 20 years ago the Brazileans have cultivated the mushroom in the hot season using bagasse, a waste product from sugar cane manufacture, as a convenient substratum. After composting, this bagasse, enriched with 1,5–2% nitrogen (as urea, manure or ammonium nitrate), provides a good yield, but it is also possible to grow the mushroom on pasteurised horse dung. The American specialist Paul Stamets also obtains good results when using enriched sawdust: 5 pounds of this substratum yield 1 lb of mushrooms! When the mycelium has wholly invaded the composted substratum, fruitbody formation is induced by covering it with a thin layer of casing soil. The growth of the mushrooms requires a temperature of 25–27°C and a relative humidity of 75–85%. The flushes occur three times with 2–3 week intervals. *Agaricus blazei* may grow as single fruitbodies, but it more often forms clusters. Clearly, the cultivation method has a marked influence on the aspect and composition of the mushrooms. In general, composted bagasse and horse manure will yield fruitbodies with darker coloured caps than those obtained from cultures on sawdust. The best time to harvest the mushroom is when the gills are still covered by the partial veil. In this condition they can be sold as first quality, but to ensure their long shelf life they should be rapidly stored at a temperature of 3–4°C.

The agarics can be sold fresh, but most of the harvest is dried (Fig. 4). The best quality consists of closed veil and thick fleshed fruitbodies, cut length-wise and dried in a warm air current. The Brazileans call it Cogumelo do Sol (Sun mushroom). For the U.S.A., Stamets has proposed the common names 'The King Agaricus' or the 'Almond Portobello'. The latter name is well chosen since it capitalizes on the popularity of the giant-sized form of the ordinary Button mushroom which is called Portobello.

Nutritional qualities

The dried mushrooms retain about 7 per cent of moisture. The dry matter has the following average composition: 38 per cent protein, 40 per cent carbohydrates, 3 per cent fat, and about 7 per cent of mineral compounds including 2,5 per cent potassium, 1 per cent phosphorus and 0,1 per cent magnesium. Moreover, *A. blazei* contains nutritionally important amounts of B vitamins, niacin, and even vitamin D plus the essential elements iron, manganese, zinc and copper. Just like the other flavescent agarics, the mushroom has the regrettable tendency to concentrate certain heavy metals of which cadmium is the most dangerous. In the course of their research, the present authors observed that the amount of this toxic metal in Brazilean cultivars generally remained well below the legal limits. The same can be said about their mercury and lead content. However, some consignments of dried *A. blazei* from China were found to contain excessive amounts of cadmium, although the mercury, lead and arsenic concentrations were quite acceptable.

Figure 1 (page 31). The freshly picked fruitbodies of cultivated *A. blazei*.
Figure 2 (page 31). An abundant flush of the Cogumelo do Sol (Sun mushroom) as it is called in Brazil.
Figure 3 (page 31). Three Brazilean scientists observing *A. blazei* Murrill. From left to right: Dalva Santana (entomologist), Angela Amazonas (mycologist), who are both working at Embrapa Florestas, a Research Institute dealing with Forestry, belonging to the Ministry of Agriculture, in Colombo, Paraná, and Renato Rau, pharmacologist at the Institute for Technology of Paraná, situated in Curitiba, the Capital of Paraná State. (All taken at the mushroom farm of grower Aldinei Mussy, Guarapava, Paraná, Brazil.)

Figure 1

Figure 2

Figure 3

Exploiting the gastronomic potential

Eating this agaric is a first order gastronomic experience! Immediately after harvest, its almondy flavour may be a bit too strong, but it decreases to a most pleasant level during the following days. The colour of the fresh mushrooms turns golden yellow upon cooking, but this phenomenon disappears 1–2 days after picking. Stamets recommends cooking the sliced mushrooms simply in olive oil at high temperature, and to season with salt, soy sauce and tamari. The texture of the cooked mushrooms is far better than those of the ordinary Button mushrooms or Oysters. The gastronomic potential of *A. blazei* has not yet been sufficiently explored. This poses a challenge to the French 'Chefs de cuisine' who will undoubtedly develop a series of succulent recipes for this extraordinary mushroom.

Medicinal properties

As already mentioned, *A. blazei* contains high levels of beta glucans, immunostimulating polysaccharides which are selectively cytotoxic on tumor cells. Consequently, many companies on the Internet advertise and sell not only the dried mushroom, but also preparations containing enriched fractions of the active principles. The accompanying publicity often exaggerates their healing powers, but its beneficial action in the treatment of various forms of cancer, arteriosclerosis, diabetes and chronic hepatitis seems to have been well established by clinical research. In Japanese pharmacies one already finds a whole array of medicinal drugs based on mushrooms. The photograph shows three products which were purchased in Tokyo. The drug AGARICUS consists of a water-soluble granulate constituting undoubtedly the more or less purified beta glucan fraction (Fig. 5). Studies on the medicinal virtues of *A. blazei* are still in progress, especially in Japan, but now also in the U.S.A.

Is there a future for *A. blazei* on the European markets?

In Japan, *A. blazei* is already the centre of an industry worth 600 million US dollars annually. The mushroom is cultivated commercially in Brazil, Japan, China and Korea. Lately, Paul Stamets is growing it in Olympia, Washington, and there are also cultivators in California and Hawaii. Europe has not awakened yet to the potential of this new haut-de-gamme gourmet mushroom. In Switzerland, The Netherlands and Denmark one has just started some experimental cultures. Some information can be found on the Internet about *A. blazei*, but mainly extensive publicity about its healing properties. In fact, the only sales argument are its (often exaggerated) medicinal virtues. Its culinary appeal is almost never mentioned.

To inspire confidence, a drug should be expensive. No wonder that the price of the dried mushroom fluctuates between 50 and 100 US dollars! However, the case of the Hen-of-the-Woods (*Grifola frondosa*) demonstrates that consumption of a once rare and expensive mushroom can spread widely in a short time. About 10 years ago, this mushroom, also known as 'Maitake', was sold as an expensive medicine, but only in Japan and China. Since European and American growers have mastered its culture, it is sold_among others_at the Swiss market for about the same price as Chanterelle mushrooms. Indeed, the price of *A. blazei* could decrease rapidly, once the Chinese start to export the mushroom to Europe. In that case one would expect the mushroom growers of the Old World to extend their activities to include *A. blazei*!

Reference

Stamets, P. (2000). Call it Himematsutake or call it the Almond Portobello_It is special. *Mushroom, the Journal of Wild Mushrooming (USA)* **18(3),** 10–13.

Figure 4. The mushrooms as sold: cut length-wise and dried.

Figure 5. Japanese medicines derived from mushrooms and recommended for treatment against cancer: next to AGARICUS (containing the beta glucan fraction), one observes;'Super Maitake', a product on the basis of *Grifola frondosa*, enriched with vitamin C. The 'Mesima Pure', a most expensive drug, is manufactured from *Phellinus linteus*, a Polypore parasitizing mulberry trees.