MINIREVIEW

Mushrooms, Tumors, and Immunity: An Update

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There is significant interest in the use of mushrooms and/or mushroom extracts as dietary supplements based on theories that they enhance immune function and promote health. To some extent, select mushrooms have been shown to have stimulatory action on immune responsiveness, particularly when studied *in vitro*. However, despite their widespread use for potential health benefits, there is a surprising paucity of epidemiologic and experimental studies that address the biologic activities of mushrooms after oral administration to animals or humans. There have been a number of studies that have addressed the ability of mushrooms to modulate mononuclear cell activation and the phenotypic expression of cytokines and their cognate receptors. There have also been a number of attempts to determine antitumor activities of mushrooms. Such studies are important because many of the components of mushrooms do potentially have significant biologic activity. All data, however, should be tempered by the possibility that there are toxic levels of metals, including arsenic, lead, cadmium, and mercury as well as the presence of radioactive contamination with ^{137}Cs. In this review, we will present the comparative biology with respect to both immunological and antitumor activities of mushroom extracts and also highlight the need for further evidence-based research. Exp Biol Med 229:393–406, 2004

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Mushroom extracts have been increasingly sold as dietary supplements based on the claim, generally with a paucity of data, that they enhance immune functions and promote health (1). Two general approaches are possible in the investigation of potential health benefits associated with the consumption of mushrooms or their extracts: (i) epidemiological studies and (ii) experimental studies addressing the biological activities of mushroom extracts or whole mushrooms after oral administration to experimental animals and, eventually, humans. At the time of our earlier review (1), there were essentially no epidemiological data, and few studies had been conducted with unfractionated mushroom extracts or whole mushrooms. Instead, most research had taken the approach of isolating pharmaceutically active mushroom compounds. Among them, large polysaccharides, most of them β-(1→6)-branched β-(1→3)-linked glucans, had been found to exhibit significant antitumor activity. The β-glucans that had been studied most extensively were lentinan from *Lentinus* (*Lentinula*) *edodes*, schizophyllan (sonifilan) from *Schizophyllum commune*, grifolan (GRN) from *Grifola frondosa*, and SSG from *Sclerotinia sclerotiorum*. The basic chemical structure of the beta glycan lentinan is illustrated in Figure 1. The available evidence indicated that the anticarcinogenic properties of these polysaccharides were attributable to enhancement of the numbers and/or functions of macrophages, NK cells, and subsets of T cells, that is, to the modulation of both innate and adaptive immunity.

There has been surprisingly little additional research on some of the mushroom polysaccharides that had previously garnered so much attention. Instead, a variety of different mushroom species have been investigated and there are now more studies addressing the oral administration of mushroom extracts as well as of semipurified and purified polysaccharides. After a brief summary of studies addressing only the antitumor activities of mushrooms, a major portion of this review will be devoted to research on the immunomodulatory activities of orally administered mushroom extracts, fractions, and compounds, followed by a section on parenterally administered mushroom polysac-

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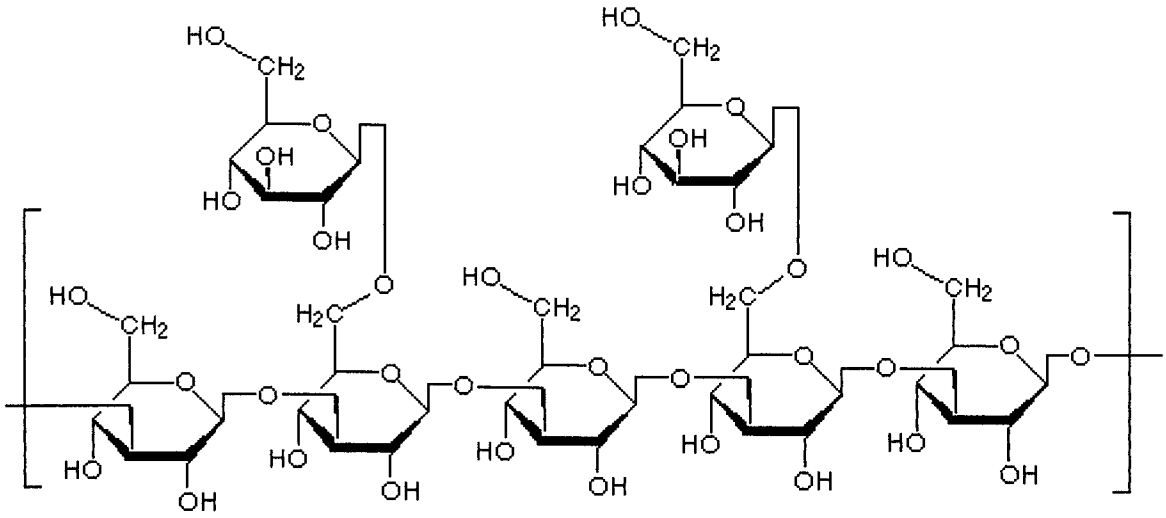

Figure 1. Chemical structure of beta glycan lentinan.

charides and the cell surface receptors mediating their effects. Of note, much of the recent research has addressed the ability of mushrooms or some of their constituents to modulate the cytokine profile, and this capacity has been investigated not only in various models of tumorigenesis but also in a variety of other states and diseases in which modulation of the immune response may be beneficial.

Epidemiological Data

There has only been one epidemiological study assessing the association between mushroom intake and cancer, more specifically gastric cancer (2). This case-control study from Korea, an area with a high prevalence of gastric cancer, was based on 136 patients and an equal number of controls. It found an inverse correlation between mushroom intake and the risk of gastric cancer. Using the risk of gastric cancer in relation to low mushroom intake as the reference value, the odds ratio was 0.38 (95% confidence interval [CI] 0.21–0.66) for medium intake and 0.30 (CI 0.15–0.62) for high intake after adjustment for sex, age, socioeconomic status, family history of gastric cancer, and duration of refrigerator use. Unfortunately, this study does not provide the unadjusted odds ratios and lacks details on statistical methods, making it difficult to fully assess the strength of the reported association.

It is possible that mushrooms constitute such a small portion of the diet in many regions that their effect on the risk of various cancers will be difficult to detect. It seems that higher mushroom consumption occurs primarily in regions where people can gather wild mushrooms. Wild mushrooms may be contaminated with substantial levels of toxic metals such as arsenic, lead, cadmium, and mercury as well as radioactive substances such as ^{137}Cs (3–5) because many mushroom species have the ability to accumulate relatively high concentrations of metals. Such high levels of toxic compounds may offset whatever health benefits a diet high in mushrooms could potentially confer.

Why Whole Mushrooms or Mushroom Extracts May Be More Beneficial Than Isolated Constituents

Carcinogenesis can be broken down into three stages: initiation, promotion, and progression. The initiation phase involves exposure to a mutagen and often requires its subsequent metabolic transformation into a biologically active form. This exposure, even if resulting in permanent damage to DNA, is often insufficient by itself to cause cancer. At least in chemically induced tumors in experimental animals, a tumor promoter is often required to stimulate cell division and result in the formation of small, benign tumors. A similar promotion phase is thought to exist in naturally occurring cancers, but the actual events are still only poorly understood. Progression to malignancy occurs when the tight controls that normally govern cell cycle progression break down, resulting in the uncontrolled proliferation of cancerous cells. It also involves the ability of these cells to invade surrounding tissue and to eventually metastasize. The modulation of the host immune system attributed to mushrooms, particularly to various mushroom polysaccharides, is likely to affect primarily the promotion and progression stages. Other substances contained in mushrooms may be able to interfere with tumor initiation through a variety of mechanisms, such as enhancing the host's antioxidant capacity or upregulating phase I and phase II enzymes involved in the metabolic transformation and detoxification of mutagenic compounds (6). Yet other mushroom constituents may inhibit promotion or progression by exerting direct cytotoxicity against tumor cells (7), interfering with tumor angiogenesis (8), or upregulating other nonimmune tumor-suppressive mechanisms.

An aqueous extract of *Agaricus blazei* Murill given in the drinking water to rats and mice before chemical cancer induction exhibited antimutagenic effects but was ineffective when administered in the postinduction period (9–11). These studies are of particular interest because the extraction consisted of leaving 25 g of powdered dry fruiting body in 1 l of water at room temperature for 2 hrs, which is the way that *A. blazei* is popularly prepared by people taking it for its health benefits. Interestingly, the same strain of *A. blazei* fed in dry powdered form to rats at 10% of the diet exhibited significant antimutagenic activity even when given in the postinitiation period, suggesting that some active constituent was lost during the aqueous extraction (12). Similar antimutagenic effects were reported for diets containing powdered *Lentinula edodes* (shiitake) (13, 14).

The lipid fraction of *A. blazei* was found to contain a compound with antitumor activity, subsequently identified as ergosterol (8). The lipid fraction of *Grifola frondosa* exhibited antioxidant activity and inhibited the cyclooxygenase enzymes, COX-1 and COX-2 (15). Ergosterol was again identified as one of the most active constituents. Oxidative damage is strongly implicated in the development of many chronic diseases, including cancer. The inducible form of COX, COX-2, also appears to play an important role in certain cancers. Its inhibition can result in the inhibition of tumor development, and it appears to be beneficial even in some established tumors (16).

There are also several reports of mushrooms containing more than one polysaccharide with antitumor activity (1). An interesting example is *A. blazei*. It contains an antitumor glucan with a β-(1→6) backbone (17), which differs from the β-(1→3) backbone with 1→6 branches shared by many other antitumor glucans. In addition, an α-1,6- and α-1,4-glucan complex (18) 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 (19). The 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.

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 but through different mechanisms. Thus, they could potentially provide additive, or even synergistic, effects in the prevention and treatment of cancer. A similar scenario can be envisioned in a variety of other diseases. It should not be overlooked, however, that individual mushroom constituents could also interfere with each other's effects. This is illustrated by a study in which increasing fractionation of an *A. blazei* extract enhanced some biological activities but abolished others (20). Ethanol fractions obtained from the hot-water extract of mycelium or dried fruiting body of this mushroom induced TNF-α and IL-8 secretion in rat bone marrow macrophages. Interestingly, further fractionation with increasing ethanol concentrations resulted in the reduction of this cytokine-inducing ability in mycelial extracts but enhanced it in the fruiting-body extract. Whereas the first fractions did not induce nitric oxide (NO) production, fractions obtained by precipitation of fruiting-body extract with high ethanol concentrations stimulated macrophages to produce significantly higher levels of NO than controls.

Thus, careful studies comparing the activity of isolated compounds with those of whole-mushroom extracts are necessary to determine whether whole mushrooms provide benefits that go beyond those achievable with isolated constituents. To date, such direct comparisons have occasionally been performed during the original fractionation of mushrooms, but they generally use the same amount of material regardless of its state of purification and do not attempt to administer equivalent amounts of a specific constituent. A recent study investigated the inhibitory effects of oral lentinan and crude shiitake extract on colon carcinoma growth in mice and found lentinan markedly more effective than the crude extract (21). The authors used what they called an "equivalent volume" of crude extract and lentinan. It is not clear in the paper whether the volume of vehicle used for the administration was equivalent or whether the amount of lentinan in the crude extract was the same as that used in the studies with pure lentinan.

Antitumor Activity of Mushrooms and Their Constituents

Our earlier review concentrated on the four mushrooms, *Lentinus (Lentinula) edodes*, *Schizophyllum commune*, *Grifola frondosa*, and *Sclerotinia sclerotiorum*, because a majority of research available at the time had focused on those four species, particularly their respective β-glucans, lentinan, schizophyllan (also called SPG, sonifilan, or sizofiran), grifolan, and SSG. In recent years, little additional research has been conducted with these four mushrooms, but a host of other species has been investigated. Many of these investigations have been focused on the antitumor activities of mushrooms and their constituents, and Table 1 illustrates the large variety of species being explored. It also highlights that, even in the same tumor model, different mushroom species and their polysaccharides are effective at vastly differing doses (ranging from 10 mg/kg to 1000 mg/kg).

A more systematic approach to the evaluation of mushroom antitumor activity is clearly needed. In our opinion, this approach should include comparisons of different mushrooms in the same tumor model and the same mushroom in different tumor models. Given the effectiveness of some mushroom compounds at doses of a

Table 1. Antitumor Activities of a Variety of Mushrooms and/or Their Constituents

Mushroom/constituent	Dose and route of administration	Mouse strain	Tumor model	% Inhibition (or increase in life span)	Reference
Agaricus blazei/ATF = acid treated fraction	1 mg/mouse intratumorally into the right flank on Days 3, 4, and 5 after MethA injection into the right and left flank	BALB/c	MethA, double-grafted	70% in both flanks	(7)
Fraction containing mostly (1→4)-α-D-glucan and (1→6)-β-D-glucan				100% in both flanks	
Agaricus blazei		ICR	Sarcoma 180	No effect and 77-90	(86)
Polysaccharide fractions	0.5 or 2 mg/mouse i.p., 5 doses on alternate days starting 7 days after tumor implantation				
Hot water extract	2 mg p.o., 35 doses (no further details provided)		Sarcoma 180	47	
Sparassis crispa/several polysaccharide fractions	0.020, 0.1, or 0.5 mg/mouse i.p., three doses on Days 7, 9, and 11 after tumor implantation	ICR	Sarcoma 180	0.02 mg: 54 to 84; 0.1 mg: 95 to 100; 0.5 mg: 91 to 99	(87)
Lyophyllum decastes Sing.					
Ethanol precipitate of hot water extract	10 mg/kg i.p. for 10 d starting 24 hrs after tumor implantation	ICR	Sarcoma 180	88	(22)
Polysaccharide fractions IV-2 and IV-3	10 mg/kg i.p. for 10 days starting 24 hrs after tumor implantation		Sarcoma 180	97 with complete regression in 9/10 mice	
Lentinus edodes (Shiitake)					
Lentinan	3 mg/mouse/day p.o. starting 7 days before K36 inoculation	AKR	K36 murine lymphoma	94	(21)
Crude extract	"Equivalent volume" to lentinan p.o. starting 7 days before K36 inoculation		K36 murine lymphoma	55	
Lentinan	As above		Various colon carcinoma cell lines	90-93	
Phellinus rimosus (Berk) Pilat.					(88)
Ethyl acetate	50 mg/kg p.o., for 10 consecutive days starting 24 hrs after tumor implantation	Swiss albino	Ehrlich ascites carcinoma	65% increase in life span	
Methanol				33% increase in life span	
Aqueous				No effect	
Ethyl acetate	50 mg/kg p.o., for 10 consecutive days starting 24 hrs after tumor implantation	Swiss albino	Dalton's lymphoma ascites	96	
Methanol				84	
Aqueous				88	
Ethyl acetate	50 mg/kg p.o., for 10 days starting 13 days after tumor implantation	Swiss albino	Dalton's lymphoma ascites	64	
Methanol				49	
Aqueous				57	

Table 1. *Continued*

Mushroom/constituent	Dose and route of administration	Mouse strain	Tumor model	% Inhibition (or increase in life span)	Reference
Pleurotus pulmonarius (Fr.) Quél. methanol extract	250, 500, or 1000 mg/kg i.p., five doses on alternate days starting 24 hrs after i.p. tumor cell injection	BALB/c	Ehrlich ascites carcinoma	No effect	(89)
	250, 500 or 1000 mg/kg i.p. 10 doses on consecutive days staring 24 hrs after s.c. tumor cell injection		Ehrlich ascites carcinoma[a]	52, 67, and 82 (volume); 50, 64, 81 (weight)	
Lepista inversa (Scop.: Fr.) Pat./CE = methanol crude extract	75 mg/kg i.p., starting 3 days after tumor transplantation	DBA/2	L1210 (lymphocytic leukemia)	50% increase in lifespan	(90)
	75 mg/kg i.p., starting 5 days after tumor transplantation		3LL (Lewis lung carcinoma)	No significant effect	

[a] The Materials and Methods mention Dalton's lymphoma ascites, which is the solid tumor model the same group of researchers used in another study (88). In this study, however, the results refer exclusively to Ehrlich ascites carcinoma.

few micrograms per mouse, there seems to be little reason for further investigation of similar compounds that need to be administered at 100-fold higher doses—unless they can be demonstrated to have unique effectiveness in a specific model.

Only a few of the studies listed in Table 1 investigated any immunomodulatory mechanisms, and the results indicated that these mushrooms and their constituents work by similar mechanisms as those discussed in our previous review, namely enhancement of both nonadaptive and adaptive immunity, that is, macrophage (22), NK cell (7), as well as lymphocyte numbers and/or activity (21). Of particular note, several of the listed studies reported that oral administration of mushroom extracts or fractions was effective in inhibiting tumorigenesis.

Oral Administration of Mushroom Extracts to Human Cancer Patients

A randomized, placebo-controlled, double-blind study was conducted with orally administered polysaccharide peptides (PSP) isolated from *Coriolus versicolor* in a total of 68 patients with advanced (stages III or IV) non–small-cell lung cancer (23). The patients received three capsules of 340 mg each (or placebo) three times daily for 4 weeks. Leukocyte and neutrophil counts rose significantly after PSP treatment, whereas they decreased in the control group. Total IgG and IgM levels were significantly increased in the PSP group but not in the placebo group, with the difference between the groups being statistically significant. There were, however, no complete or partial responses to PSP treatment. The number of patients that withdrew from the study, mostly due to significant deterioration, was significantly higher in the placebo group ($n = 8$) compared with the PSP group ($n = 2$), suggesting that ingestion of PSP was associated with a reduced rate of deterioration.

A phase I/II study was conducted with Ganopoly (a crude polysaccharide fraction of *Ganoderma lucidum* (Curt.: Fr.) P. Karst, 600 mg given orally three times daily for a total dose of 1800 mg/day in patients with advanced cancer (24). No partial or complete responses occurred, but some patients reportedly experienced palliative effects. Immune parameters were assessed in 75 of the 143 patients originally enrolled and were found not to be affected by the mushroom fraction. In a subgroup of 32 patients with stable disease for $\geq$12 weeks, however, the lymphocyte mitogenic response to PHA and ConA was significantly increased, as was the NK activity.

In a case series of eight patients with various cancers (mostly stage III, 1 stage II, 1 stage IV), who were given 100 mg of D-fraction, a polysaccharide isolated from *Grifola frondosa*, daily for up to 34 months, there was an, at times marked, increase in NK activity (25). Based on a list of reduced or enhanced parameters, the same group of authors reported that a positive response occurred in 23 of 36 cancer patients who took a combination of MD-fraction

(which appears to be identical to the D-fraction) and maitake (26). The dosage and length of dosing was not specified, but it seems to have differed considerably among patients.

In another study, patients with prostate cancer were supplemented with a shiitake mushroom (*Lentinus edodes*) extract consisting of oligosaccharide (α-1,4-glucan), polysaccharide (β-1,3-glucan) and proteins (27). In this open-label, nonrandomized study, none of the patients had a complete or partial response (reduction of prostate-specific antigen levels of >50%), indicating that this mushroom extract was not an effective treatment for prostate cancer. However, the authors did note that several of the patients in the treatment group had stable PSA levels.

Animal Studies with Whole-Mushroom Extracts or Mushroom Polysaccharides Given Orally

Among the numerous studies investigating the effects of oral administration of mushroom compounds or extract, the report by Ng and Yap (21) is of particular interest. These authors found that lentinan, a polysaccharide purified from *Lentinus edodes*, significantly inhibited tumorigenesis when given by gavage prior to inoculation with lymphoma cells. Slightly less efficacy was observed when oral lentinan administration was begun on the day of inoculation (28). This contrasts with research available at the time of our earlier review indicating that lentinan, a polysaccharide isolated from *L. edodes*, was ineffective in inhibiting tumor growth when given orally. Ng and Yap (21) developed a new and simpler procedure for the purification of lentinan that gives a higher yield, but a less purified product (88% purity compared with ≥99 %), than that used in previous studies. Although the contaminating substances, mostly proteins and some lipids, did not exhibit antitumor activity by themselves, the possibility cannot be excluded that they somehow modified the activity of lentinan in such a way as to make it active after oral administration, whereas the previously used highly purified fraction exhibited no inhibitory properties.

Current research on the immune modulation exerted by mushrooms has gone beyond the mechanisms involved in their antitumor activities and has begun to address their potential use in other diseases and clinical states in which modulation of certain immune parameters may be beneficial. One of the risks of radiation and chemotherapy in the treatment of cancer patients is the development of leukopenia, which substantially increases the risk of infections. Hence, several recent studies have addressed the question of whether mushroom extracts or constituents can enhance hematopoiesis, and potential mechanism(s) by which they might do so.

In irradiated BALB/c mice, a water-soluble extract from *Lentinus lepideus* given orally for 24 days enhanced hematopoiesis to the extent that the number of colony-forming units-granulocytes/macrophages (CFU-GM) had already increased to the levels seen in nonirradiated controls by Day 8, while the number of erythroid burst-forming units (BFU-E), though considerably higher than in PBS-treated irradiated animals, was only half that seen in nonirradiated controls, but had fully recovered by Day 24 (29). The serum concentrations of IL-6, IL-1β, and granulocyte/macrophage colony-stimulating factor (GM-CSF) were reportedly significantly increased compared with irradiated and nonirradiated controls. The highest levels and greatest increases, however, were seen on Day 24, whereas significant repopulation was observed within the first 8 days. Thus, the relevance of these cytokines in the enhancement of hematopoiesis in this model is not clear. It is worth noting that peripheral blood may not be the ideal compartment for assessing cytokines involved in hematopoiesis occurring in the bone marrow.

In cyclophosphamide-induced leukopenia, oral administration of a polysaccharide fraction from *Sparassis crispa*, called CA1-fraction and consisting of a 1,6-branched 1,3-β-D-glucan fraction, at doses of 50, 100, or 200 μg/mouse, accelerated the rate of recovery of a variety of leukocyte subsets (30). *In vitro*, addition of CA1 to splenocytes from cyclophosphamide-treated animals resulted in a significantly increased IL-6 and interferon (IFN)-γ production compared with saline-treated cells. Interestingly, a similar increase was not observed in cells from normal animals.

The authors reported similar results with i.p. administration of SCG from *Sparassis crispa* Fr. (31). SCG was reportedly purified from SCCA, but it is unclear whether SCCA is identical with the fraction previously called CA1. The optimal dose of SCG, which appears to have been given as a single injection, ranged between 250 μg/mouse and 1000 μg/mouse. In contrast, a daily dose of between 50 μg/mouse and 100 μg/mouse was determined to be optimal for oral CA1. Although the authors stated that CA1 was administered daily, it is not clear if this included the entire 11 days that the study lasted. Regrettably, this lack of detail in the reporting of the methods, along with the differences in study design, make a direct comparison of the results after oral and i.p. administration difficult. The cumulative dose of oral CA1, which is presumably less purified than SCG, appears to have been similar to the one-time dose given i.p. This may indicate that oral administration is at least as effective as i.p. injection, a hypothesis that would be interesting to test.

The effect of oral administration of ethanol extracts of four different mushrooms was also examined in a model of type IV hypersensitivity in which mice were sensitized by application of oxazolone to the shaved skin, then challenged with application to the right ear (32). When given daily for 3 days at a dose of 250 mg/kg before the challenge, oral administration of ethanol, but not water, extracts of *Hypsizygus marmoreus*, *Flammulina velutipes*, *Pholiota nameko*, and *Pleurotus eryngii* significantly inhibited oxazolone-induced hypersensitivity. Interestingly, two of these extracts (*H. marmoreus* and *P. nameko*) markedly

enhanced the hypersensitivity reaction when given 3 days before sensitization.

A proteoglycan isolated from *Proteus linteus* given orally to male DBA/1 mice reduced the peak severity of collagen-induced arthritis (CIA), although it did not accelerate its resolution (33). Unfortunately, some of the details of this study are not clear, and there are discrepancies between the results presented in some of the figures and the accompanying text. However, what seems to emerge from the study is that oral administration of the proteoglycan, particularly when given after the booster injection of collagen type II, was associated with decreased IFN-γ production in inguinal lymph nodes (LN) and, to a far lesser extent, in Peyer's patch (PP), but not in mesenteric LN. TNF-α synthesis was decreased in the postadministration, but not in the preadministration, group in all three tissues. IL-4 and IL-10 production, as well as IL-12 synthesis, were not affected in any of these tissues. TGF-β synthesis was slightly, but significantly, increased in mesenteric LN in the preadministration and in PP in the postadministration group. This, together with the finding that collagen type II-specific IgG2a was reduced in both dosing schedules, led the authors to conclude that the proteoglycan from *P. linteus* induced a shift toward a Th2 cytokine pattern. However, given the absence of changes in IL-12, IL-4 and IL-10 production, this interpretation needs to be viewed with caution.

Studies with Fractions or Isolated Polysaccharides Given Parenterally

In contrast with the reduction in severity of CIA by a proteoglycan isolated from *P. linteus*, the D-fraction of *Grifola frondosa* administered i.p. to male DBA/1J mice starting after the second immunization with collagen type II markedly exacerbated the severity of CIA (34). Type-II collagen-specific IgG production and expression of the B cell activation marker, CD69, did not differ from controls. Various parameters of cellular immunity (percentage of $CD4^+$ T cells in lymph nodes, CD69 expression on $CD4^+$ T cells, T cell proliferation in response to ConA) also did not reveal significant differences in the D–fraction-treated group. There was, however, significantly increased production of IL-1β, GM-CSF, and TNF-α by splenic, though not peritoneal, macrophages of D–fraction-treated animals compared with saline- or dextran-treated controls. Both TNF-α and IL-1β produced in the synovial joints are strongly implicated in the pathogenesis of experimental collagen-induced as well as human rheumatoid arthritis. Whether their elevation in the spleen, but not peritoneum, reflects macrophage activation in the synovium remains to be established. In contrast with the lack of effect of D-fraction on peritoneal macrophage IL-1β production, others observed enhanced synthesis of IL-1β by peritoneal macrophages from D–fraction-treated ICR mice infected with *L. monocytogenes* compared with untreated or vancomycin-treated animals (35). The statistical significance of this difference was not reported. If reproducible, these results raise the question of whether some of the discrepancies between this and the preceding study may be attributable to the different mouse strains used and/or to differences in the action of D-fraction in the two distinct disease models.

When C3H/HeN mice received daily i.p. injections of D-fraction for 19 days starting 24 hrs after implantation of MM-46 carcinoma cells, tumor growth was significantly reduced (by 82%) compared with PBS-treated controls (25). This was accompanied by a significant increase in the release of TNF-α and IFN-γ by ConA-stimulated whole spleen cells and a 1.3-fold increase in the intracellular TNF-α expression in NK cells. The statistical significance of this difference was not stated. *In vitro* incubation of Raw 264.7 macrophages with high concentrations of D-fraction (500–1000 μg/ml) resulted in marked release of IL-12. Whether IL-12 plays a role in enhancing the cytotoxicity of NK cells against tumors *in vivo* remains to be established.

In an experimental model of septic shock induced by intraperitoneal injection of *Eschericia coli* LPS, i.p. administration of 100 mg/kg of an acidic polysaccharide from *Phellinus linteus* before LPS treatment resulted in prolonged survival (36). This was associated with significantly reduced serum concentrations of TNF-α and IL-12; IL-1β synthesis tended to be decreased, while IL-6 levels tended to be increased, but these latter two effects were not statistically significant.

In healthy male ICR mice, the profile of cytokine mRNAs and kinetics of their induction in peritoneal exudate cells (PEC) and splenocytes were compared after i.p. injection of lentinan or a polysaccharide-protein complex (PSPC) from *Tricholoma lobayense* that contains ~40% polysaccharides, consisting of a variety of hexoses and pentoses, and 30% protein (37). Administration of both compounds was associated with a strong upregulation of IL-1α, IL-1β, and TNF-α in PEC. The expression of IFN-α rose slightly in PEC from PSPC-treated animals, but strongly after lentinan treatment. In contrast, in splenocytes, IFN-γ expression was upregulated to a greater extent after PSPC administration than after lentinan treatment. In addition, the kinetics of IL-1β and TNF-α expression in spleen differed considerably between PSPC- and lentinan-treated animals.

Type 1 Versus Type 2 Immune Responses. The production of two distinct cytokine patterns was originally recognized in subsets of helper T cells (Th). The set designated as Th1 is characterized by IL-2, IFN-γ, and IL-12 production and activates macrophages. The other set, designated as Th2, is characterized by IL-4, -5, -6, -10, and -13 synthesis and promotes humoral immunity. Since then, it has been recognized that cell subsets other than Th cells can produce similar cytokine patterns, which has prompted the classification of the respective immune responses into type 1 and type 2, rather than Th1 and Th2.

Several groups of researchers specifically addressed the question of whether mushroom polysaccharides can induce type 1 or type 2 cytokine responses. One of these studies

compared the effects of three different mushroom β-glucans on the production not only of IL-4 and IFN-γ but also of the IgG subclasses (38). Isotype switching to IgG1 is induced by IL-4 and inhibited by IFN-γ. Conversely, switching to IgG2a is induced by IFN-γ and inhibited by IL-4.

The β-glucans investigated were grifolan from *Grifola frondosa*, a glucan from *Sclerotinia sclerotiorum* (SSG), and sonifilan from *Schizophyllum commune*. When administered together with trinitrophenyl ovalbumin (TNP-OVA), these β-glucans were found to differ substantially in their ability to induce the two subclasses of TNF-OVA–specific IgG, with the most marked differences observed 20 and 24 days after the first immunization. SSG strongly induced IgG2a, but not IgG1. Grifolan and the single helical conformer of sonifilan induced both types, though to differing degrees. In contrast, the triple helical conformer of sonifilan did not significantly affect the production of either subclass. For grifolan and both conformers of sonifilan, TNP-OVA–specific antibody subclass production by splenocytes harvested on Day 12 correlated rather poorly with the synthesis of IFN-γ and IL-4. There was, however, a good correspondence between the increase in IFN-γ production by splenocytes from mice injected with SSG and *in vitro* and *in vivo* IgG2a production. These results suggest that different β-glucans vary in their ability to induce type 1 or type 2 immune responses.

Several groups of researchers have shown that the intracellular glutathione (GSH) redox status not only of T cells themselves (39), but also of macrophages and dendritic cells that present antigens to T cells, plays an important role *in vitro* and *in vivo* in determining the polarization toward a type 1 or type 2 immune response (40–42). GSH supplementation and high intracellular GSH levels result in a type 1 response characterized by increased IFN-γ and IL-12 and decreased IL-4, IL-6, and IL-10 production. In contrast, GSH depletion is associated with the induction of a type 2 response with decreased IL-12 and IFN-γ release and increased IL-4, IL-6, and IL-10 synthesis. Conversely, treatment of macrophages with Th1 cytokines (IFN-γ) induces reductive macrophages with elevated intracellular GSH content, while IL-4 treatment results in oxidative macrophages characterized by reduced GSH content (41).

Adherent splenocytes from mice injected with SSG were found to contain significantly higher levels of GSH than control splenocytes (43). Consistent with the study by Suzuki *et al.* (44), SSG induced IFN-γ and IL-12 synthesis and upregulated the production of the IFN-γ–dependent IgG2a isotype. These findings, therefore, support an association between high intracellular GSH concentrations and a Th1 cytokine response.

Further confirmation comes from a study with lentinan (45). Peritoneal macrophages from mice that had received lentinan i.p. not only had similar intracellular GSH levels as those of animals that were injected with GSH-monoethyl-ester but also exhibited essentially the same functional characteristics (45). Macrophages from both groups produced high levels of IL-12 and NO in response to stimulation with IFN-γ and/or LPS, whereas no IL-12 and little NO production was detectable in macrophages from saline-treated controls. The synthesis of IL-6 did not differ significantly between lentinan- and saline-treated animals. In contrast, LPS administration decreased the intracellular GSH level and, correspondingly, macrophages from LPS-treated mice stimulated with IFN-γ did not release detectable amounts of IL-12 and produced less NO, but significantly higher levels of IL-6, than macrophages from saline-treated controls. When splenic $CD4^+$ T cells isolated from lentinan-treated animals were stimulated with anti-CD3, they released significantly more IFN-γ than saline-treated controls, while IL-4 levels were the same in both groups. Of note, two different mouse strains, C57BL/6 and DBA/2, were used for these experiments, but the authors did not always specify which strain was examined in what experiment. This is unfortunate because, under neutral priming conditions, C57BL/6 mice are characterized by a genetic predisposition toward a type 1 default cytokine pattern (low IL-4, high IFN-γ production), whereas DBA/2 mice produce both high IL-4 and IFN-γ (46). Thus, it would have been of interest to determine whether lentinan is able to polarize the response toward a type 1 pattern in both types of genetic backgrounds.

The question of whether a mushroom polysaccharide is able to override the underlying propensities of different mouse strains toward a type 1 or type 2 cytokine pattern was addressed by another group of researchers. They examined the effects of the D-fraction polysaccharide from *Grifola frondosa* on cytokine profiles in MM-46 carcinoma-bearing C3H/HeN (47) and BALB/c mice (48). In C3H/HeN mice, a type 1 immune response predominates, whereas BALB/c mice are characterized by a genetically determined predominance of a type 2 default cytokine secretion pattern.

In C3H/HeN mice, i.p. administration of this β-glucan resulted in an enhanced ratio of IFN-γ–expressing to IL-4–expressing spleen and lymph node $CD4^+$ T cells (47). Splenocytes and lymph node cells from D-fraction–treated animals secreted significantly greater amounts of IL-12 and IL-18 (which synergize in the induction of IFN-γ), and TNF-α in response to stimulation with ConA than did cells from tumor-bearing controls. In contrast, IL-1β synthesis was decreased.

When administered i.p. to BALB/c mice, the D-fraction induced the production of IFN-γ by splenocytes and lymph node cells, while decreasing the synthesis of IL-4 (48). This was associated with a significant increase in the intracellular IFN-γ expression and concomitant decrease in the IL-4 expression in splenic and lymph node $CD4^+$ T cells. Antigen-presenting cells were found to secrete significantly more IFN-γ and IL-12p70. These two studies do not represent a direct comparison of the effect of D-fraction in the two different mouse strains and differ somewhat in their experimental design. Nonetheless, the striking similarity of the results suggest that the D-fraction of *G. frondosa* can

induce a type 1 immune response in tumor-bearing mice regardless of the type of default cytokine pattern to which the individual strain is predisposed.

The role of dendritic cells in the induction of a type 1 immune response was further investigated in the same model (49). After stimulation with ConA, splenic DC from tumor-bearing animals treated with D-fraction produced significantly higher amounts of IL-12p70 and IFN-γ than those from saline-treated controls. Lymph node DC also produced significantly more IFN-γ, but the increase in IL12p70 release was not statistically significant. Administration of D-fraction was associated with a significant increase in the proportion of $CD8\alpha^+$ DC, but it did not affect the $CD8\alpha^-$ fraction. It has been reported that DC expressing the CD8α molecule drive predominant Th1 responses, whereas DC lacking CD8α expression preferentially induce a Th2 response (50, 51). Injection of DC isolated from tumor-bearing mice treated with D-fraction into normal mice that were subsequently inoculated with colon 26 carcinoma cells completely inhibited tumor development in the recipients. Cells from tumor-bearing animals not treated with D-fraction also exerted some inhibitory effects (39%) compared with cells from normal mice. These results further confirm that DC has an important function in the protection from tumors and that the effects of D-fraction are at least partially mediated by enhancing the $CD8\alpha^+$ subset of DC involved in directing the immune response toward a type 1 pattern.

The preceding studies along with what had been known previously show that several mushroom polysaccharides are potent inducers of proinflammatory cytokines, whereas others are able to downregulate them. These studies also suggest that some might induce a type 1 immune response, whereas others favor a type 2 polarization. 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.

The ability to enhance the production of proinflammatory cytokines may be beneficial in certain infectious diseases but can have negative consequences in diseases with pathologies in which such cytokines are known to play a central role. The exacerbation of CIA in D-fraction–treated animals clearly illustrates that. Similarly, the polarization of the immune response toward a type 1 or type 2 pattern may be valuable in some situations and detrimental in others. This highlights the need for more systematic studies of the cytokine profiles associated with the administration of various mushrooms and their constituents before they are arbitrarily tried in various disease states. Such a systematic approach will have to take into account the genetic background of the animals used, will have to establish a careful dose-response curve, and should ideally include a comparison of different routes of administration.

β-Glucan Receptor(s)

The induction of cellular responses by mushroom and other β-glucans is likely to involve their specific interaction with one or more cell surface receptors. This has been the focus of intense research in recent years. Unfortunately, one quite commonly finds the results obtained with one or two β-glucans extrapolated to β-glucans in general, which are then referred to as "β-glucan." It should by now be evident that such a substance does not exist. Even β-glucans of similar structure, molecular weight, and solution conformation exhibit vastly differing biological activities *in vitro* and *in vivo* and these differences are yet more pronounced when structurally less similar β-glucans are included in the discussion. To complicate matters even more, much of what is currently known about the molecular interaction of β-glucans and various cell types comes from studies conducted with zymosan. Zymosan is a particle obtained from yeast (*Saccharomyces cerevisiae*) consisting of a variety of different substances, including mannans, glucans, glucosamine, and glycoproteins and is, therefore, not ideally suited for the investigation of β-glucan–specific activities. If, however, a specific effect resulting from the binding of zymosan to a cell can be inhibited by a variety of β-glucans, it can be concluded that these β-glucans bind to the same receptor(s) as the β-glucan part of the zymosan particle.

Previously reviewed studies suggested that the complement receptor type 3 (CR3 = CD11b/CD18) was a prime candidate for the β-glucan receptor on human monocytes, neutrophils, and NK cells. Since then, independent confirmation of a role for CR3 as a β-glucan receptor has been provided by the findings that preincubation of human monocytes or murine peritoneal macrophages with antibodies against CD11b and/or CD18 partially inhibited some of the functional consequences of β-glucan binding (52, 53). The β-glucan receptor site was localized to a lectin site C-terminal of the I-domain of CD11b (54). The same site was identified as "the" β-glucan receptor on mouse leukocytes, which exhibited functional characteristics comparable with those of the human equivalent (55). The same group of authors has shown in a series of experiments that incubation of NK cells or neutrophils with small soluble β-glucans primed the CR3 receptor in such as way as to enhance the cytotoxicity against iC3b-opsonized target cells that were otherwise resistant to CR3-mediated cytotoxicity (55, 56). Thus, CR3 plays a dual role, and its role as a receptor for certain β-glucans primes it for its function as an iC3b-receptor for iC3b-coated target cells. This mechanism was subsequently shown to be involved in the antitumor activity of two soluble polysaccharides isolated from zymosan and consisting primarily of either glucose or mannose (57). Treatment with these polysaccharides inhibited tumor growth in normal BALB/c mice implanted with various mammary tumor cell lines but was ineffective in CR3-deficient mice, confirming the central role of CR3 in this process. Whether this mechanism plays a role in the

mushroom β-glucan–mediated antitumor effects remains to be established. The effect of mushroom polysaccharides on tumor-specific antibody production has also only rarely been addressed, but a recent study indicated that sonifilan did not have a significant effect on this parameter in sarcoma 180-bearing mice (58).

There are also data arguing against an exclusive, or even an important role, for CR3 as a β-glucan receptor. Binding of several different β-glucans was observed in undifferentiated U937 cells, which do not express CR3 (59). Similarly, the production of O_2^- and TNF-α by the rat NR8383 alveolar macrophage cell line was enhanced after stimulation with PGG-glucan, even though this cell line does not express detectable levels of CR3 (60). In contrast, rat peritoneal macrophages exhibited considerable surface expression of CR3, but required ~50-fold higher concentrations of PGG-glucan for stimulation of respiratory burst activity. Bone marrow dendritic cells from CD11b- (Mac-1)-deficient mice exhibited the same responses to zymosan as did wild-type bone marrow dendritic cells, and soluble β-glucans inhibited these responses to a similar degree in cells from both types of animals (61). While these data do not rule out the possibility that CR3 constitutes a binding site for β-glucans, they strongly suggest that a molecule distinct from CR3 mediates some of the functional responses to β-glucans.

Several lines of evidence suggest that there is more than one β-glucan receptor and that, whereas some cells express only one type, others have at least two (59, 62). We agree with others (63) that, in addition to the conformation dependence of their receptor interactions, the enormous functional diversity of β-glucans also argues for the presence of several receptors on at least some cell types. This is, however, not a universally accepted concept (54, 64).

In recent years, several candidates in addition to CR3 have been identified. These include dectin-1 and its human homologue, sometimes referred to as the β-glucan receptor (βGR) (61, 64–66), a scavenger receptor (67), and glycosphingolipid lactosylceramide (63, 68). In our previous review, we also summarized data presented by another group of researchers, which indicated the presence of two non-CR3 β-glucan receptors on human monocytes (69). These were subsequently partially characterized as a 160-kDa and a 180-kDa complex, the 180-kDa receptor consisting of polypeptides of 95, 60, and 20 kDa, whereas the 160-kDa receptor was a multimer of two polypeptides of 27 kDA and 20 kDa (70). It appears that none of the recently proposed candidates have similar molecular masses.

Dectin-1 is a type II membrane receptor composed of an extracellular C-type lectin domain and a cytoplasmic domain containing an immunoreceptor tyrosine-based activation motif (ITAM). It was identified as a β-glucan receptor by its ability to bind to unopsonized zymosan in conjunction with the fact that this binding could be inhibited to varying degrees by a variety of β-(1-3), β-(1-6), β-(1-3),(1-6), β-(1-3),(1-4), and α-(1-6) glucans (65). Binding of a β-glucan to dectin-1 is a necessary, but not sufficient, step in the induction of NF-$_\kappa$B activation and TNF-α release (61, 66). The β-glucans of fungal cell walls are thought to be pattern-recognition molecules for the innate immune system, and in the last few years, toll-like receptors (TLRs) have emerged as a major group of pattern-recognition receptors, that is, molecules that recognize conserved molecular patterns that are shared by a large group of microorganisms (71, 72). Recent experimental results indicate that, for the activation of NF-$_\kappa$B and induction of TNF-α synthesis, dectin-1 needs to cooperate with TLR-2 (61, 66), possibly as part of a heterodimer with TLR6 (73).

In contrast, the activation of NADPH and production of reactive oxygen species is a direct consequence of β-glucan recognition by dectin-1 and does not require TLR-2 (61). Nonetheless, prestimulation of RAW cells with known activators of TLR-2 or TLR-4 could further enhance the dectin-induced production of ROS, and these data also indicate that dectin-1 can cooperate with TLRs other than TLR-2.

Although it was originally thought that the expression of dectin-1 was largely restricted to dendritic cells, it was subsequently shown that monocytes, macrophages, neutrophils, and a subset of T cells also express this β-glucan receptor (74). There seems to be some disagreement, however, concerning the levels of expression on these cell types, with some authors detecting the highest level on DC (61, 75), whereas others find higher expression on monocytes, macrophages, and neutrophils than on DC (74). Interestingly, no expression was detected on NK cells (74), although β-glucan binding to NK cells has been reported (76) and enhanced NK activity after administration of β-glucans is a common finding. As mentioned above, NK cells express CR3, which is primed by binding of β-glucans for its function in the killing of iC3b-target cells. Taken together, these findings suggest that CR3, but not dectin, is the primary β-glucan receptor on NK cells.

With the exception of brain, muscle, and skin, most murine tissues contain detectable dectin-1 transcript (74), and similar findings were reported from human tissues (77). Although murine small intestine was found to contain dectin-1 mRNA (74), it is not clear whether intestinal epithelial cells or dendritic cells are responsible for this dectin-1 expression. Expression of TLR2, 3, and 4 was demonstrated in several intestinal epithelial cell lines (78, 79). The presence of other TLRs in these cells has yet to be reported.

There have been numerous reports of immune modulation associated with oral ingestion of mushroom β-glucans. We previously discussed data suggesting that large polysaccharide fragments can be detected in serum after oral ingestion of polysaccharides. Many of the immune modulating activities of mushroom polysaccharides, however, require polymers larger than those reported to date.

Hence, it must be assumed that ingested β-glucans interact with either intestinal epithelial cells and/or intestinal DC, ultimately resulting in the priming or activation of other immune cells. In this context, it is of interest that intestinal DC, which are known to express dectin-1 and TLRs, were recently shown to be able to directly sample the content of the intestinal lumen without interfering with the integrity of the epithelial layer (80). This provides them with a route of bacterial uptake (and possibly of bacterial or fungal products) that is independent of M cells. Whether this ability of intestinal DC plays a role in the immunomodulating effects of orally administered β-glucans and what exactly that role may be remain to be established.

Structure-Activity Studies

It was recently reported that various β-glucans, including some mushroom polysaccharides (schizophyllan and scleroglucan), bound to receptors on the human promonocytic cell line U937 with greatly different affinities (59). The U937 cells were undifferentiated and were confirmed not to express CR3, but other findings strongly suggested the presence of at least two other β-glucan receptors. Scleroglucan, a highly branched (1-3)- β-D-glucan from *Sclerotium glucanum*, exhibited the highest affinity (IC_{50} = 23 nM for the inhibition of glucan phosphate binding), followed by schizophyllan (11 μM) and laminarin (21 μM). Though based on a rather limited number of different β-glucans, these findings suggest that the solution conformation (triple versus single helix) and, to a lesser extent, degree of branching, molecular weight, and possibly polymer charge, play a role in determining the affinity for one or more glucan receptors. Affinity alone, however, does not seem to determine the cellular response to a β-glucan. Although scleroglucan exhibited a higher affinity for the U937 β-glucan receptor(s) than did glucan phosphate, the two polysaccharides activated NF-$_\kappa$B to a similar degree (81). It has been suggested that the ability of β-glucans to cross-link receptors is an important determinant of efficient activation of leukocytes (60, 62).

The importance of the triple versus single helical structure of β-glucans for some of their biological activities was further underscored by the finding that denaturing of the triple helical structure of scleroglucan resulted in the loss of the ability to induce TNF-α production in human monocytes (53). Scleroglucan samples with a molecular weight of $<50 \times 10^4$ g/mol or $> 110 \times 10^4$ g/mol were most effective. In this study, stimulation of TNF-α synthesis was partly inhibited by antibodies against CD14 or CD11b, the latter indicating that CR3 was one of the receptors involved in the β-glucan–induced activation. Others, however, observed that the synthesis of a variety of cytokines, including TNF-α, from differentiated U937 cells, THP-1 cells, or human PBMC was stimulated by a single helical conformer of schizophyllan, but not by the native triple-helical conformer (82), whereas the induction of hematopoiesis appeared to be independent of the nature of the helical conformation (83). Yet another group of researchers reported that linear, but not branched, β-glucans induced NF-κB activation in RAW macrophage cell lines (84). It seems likely that at least some of the discrepancies in these results are due to the use of cells expressing distinct β-glucan receptors or combinations of receptors, each with different specificities of β-glucan structural features.

Concluding Remarks

Collectively, the literature published over the past decade supports the concept that certain mushrooms and mushroom extracts may have potent anticarcinogenic actions. Regrettably, the interpretation of many of these studies is complicated (due to a lack of experimental details and a lack of positive and negative controls). Publishers and reviewers in this field could greatly contribute to the improvement of both aspects by carefully evaluating the methodology of submitted research results and by providing editorial assistance to authors whose native language is not English.

The investigators themselves could significantly advance the field by being consistent in the use of designations and abbreviations for compounds, or fractions, they isolate from mushrooms. Most important, there is a need in the field for detailed information on the extraction procedure and, if at all possible, a thorough analysis of the chemical composition of the extract under investigation. This would not only enhance reproducibility, but would eventually make it possible to correlate specific chemical constituents or combinations of constituents with particular biological activities. The importance of a biochemical characterization of extracts is underscored by the findings that extracts prepared from different lineages of *L. edodes* differed in their ability to protect mice from the mutagenic effects of *N*-ethyl-*N*-nitrosourea (14). Similar results have been reported with extracts from different lineages of *A. blazei* (9). A subsequent study by the same group of researchers indicated that the time of harvest was another important factor in determining the biological activity of mushrooms because open basidiocarps showed different chemopreventive activity from closed basidiocarps of the same lineage of *A. blazei* (12). In addition, storage conditions have been shown to influence the polysaccharide content of mushrooms (85). The dependence of the chemical composition of essentially all botanical products on species or strain as well as growing, harvesting, processing, and extraction procedures is well established and should receive more attention in mushroom research.

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