

Original research

Antitumor activity of orally administered "D-fraction" from Maitake mushroom (*Grifola frondosa*)

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It is well-known in Japan that some medicinal mushrooms contain polysaccharide compounds which demonstrate antitumor activity. The author obtained the acid-insoluble, alkali-soluble, hot-water-extractable polysaccharide (containing about 30% of protein) "D-fraction" from the fruiting body of the Maitake mushroom which exhibited antitumor activity against allogenic and syngeneic tumors on oral administration to mice. A Winn assay revealed a complete tumor inhibition indicating that stimulation of an immune response triggered by the tumor-bearing state is activated by D-fraction. The cytolytic activity and interleukin-1 productivity of macrophages or T cells which exhibit antigen-specific cytotoxicity were enhanced. Footpad swelling test against D-mice (D-fraction administered tumor-bearing mice) suggested that delayed type hypersensitivity (DTH) reaction against tumor antigen was potentiated. Winn assay was carried out with whole spleen cells from D-mice and Lyt-2⁻ spleen cells in which Lyt-2⁺ cells corresponding to cytotoxic T cells (CTL) were eliminated by treating with anti-Lyt-2⁺ monoclonal antibody and complement. It confirmed that tumor neutralizing activity was at no time impaired even after CTL were eliminated, concluding that a DTH reaction, manifested by the combined action of delayed hypersensitivity T cells (Tdh) and macrophages was potentiated by oral administration of D-fraction.

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Introduction

IN JAPAN OVER THE LAST 20 YEARS, a number of oriental mushrooms have been studied due to anecdotal reports of their medicinal effects, and several have been shown to possess antiviral and anticancer properties . Some of the mushroom extracts, such as lentinan, shizophyllan and PSK (a drug formula), have been approved as anticancer drugs by the Japanese government and are widely employed.

Mushroom substances extracted from mycelia or the fruiting bodies of fungi belonging to basidiomycetes exhibit antitumor effects when administered intravenously or intraperitoneally. However, most of these materials are reported ineffective when given orally (1,2). In contrast, the author has observed that an immune response in mice was induced, leading to the regression of transplanted tumors, when the powdered fruit body of *Lentinus edodes* (Shiitake) was orally administered (3,4).

DRUG/DOSE	Daily injection x 10 days from first day of implantation of cancer	Daily injection x 10 days from first day of implantation of cancer
Maitake/ 1.0 mg I 0.5 mg	79.9% I 69.1%	86.6% I 86.0%
PSK/ 300mg (Kawaratake)	-4.7%	-7.1%
Lentinan/ 1.0 mg (Shiitake)	33.6%	54.4%

TABLE 1. Anti-tumor effect of mushroom extracts against allogenic tumor (Sarcoma 180)

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FRACTION	TUMOR WEIGHT (g)	TIR (%)
Control	2.28	--
Pre-A	2.14	2
A	2.31	--
B	1.86	18
C	2.31	--
D	0.27	88

(10 mice x 3)

TABLE 2. Tumor growth-inhibitory activity of extracts from *G. frondosa* (ICR-Sarcoma 180) TIR= tumor inhibition rate.

This tumor regression was even more profound when powdered *Grifola frondosa* (Maitake) was orally administered (5). In this context, the antitumor activity of orally administered extract (D-fraction) from Maitake mushroom on cells associated with immune response is examined and the involvement of DTH reaction is also investigated.

Against Allogenic and Syngeneic Tumors

Polysaccharides consisting of a ß-1,3 glucopyranoside main chain with ß-1,6-linked glucose branches isolated from various fungi are reported to possess antitumor activities (6-9). The author has isolated from the fruiting body of Maitake a ß-glucan which carries many 1,3-linked oligosaccharide side chains from the 1,6-linked main chain. The superior antitumor activity in Maitake extract as shown in *Table 1* could be partly due to the difference of the polysaccharide structure; the degree of branching is greater than others (10). In this test, mice were injected with various extracts of medicinal mushrooms for 10 days starting from the 1st or 9th day after implantation of Sarcoma 180. The tumor inhibition rate (TIR) of Maitake was 86.6%. On the other hand, PSK has shown no effect even with 300 mg/Kg/day and lentinan, approved as an anti-stomach cancer drug in Japan, has shown relatively weak inhibition effect. The author conducted the same test in 1980 on *Ganoderma lucidum* (Reishi mushroom), a legendary mushroom in China known as the elixir of immortality over two millennia, but it demonstrated very little activity in the test (TIR 5.2% with 1.0mg dose and 36.8% with 50mg dose).

The D-fraction of Maitake extract was acquired in the process as shown in *Figure 1* (11). As a preliminary test, after implantation of an allogenic Sarcoma-180 tumor into ICR mice, each extracted fraction was orally administered, in an amount of 1.5 mg/ml, 10 times on alternate days. On the 12th treatment day, solid tumors were extirpated and weighed to obtain TIR. The result is shown in *Table 2*. When D-fraction was administered, 88% TIR was observed. However, in the

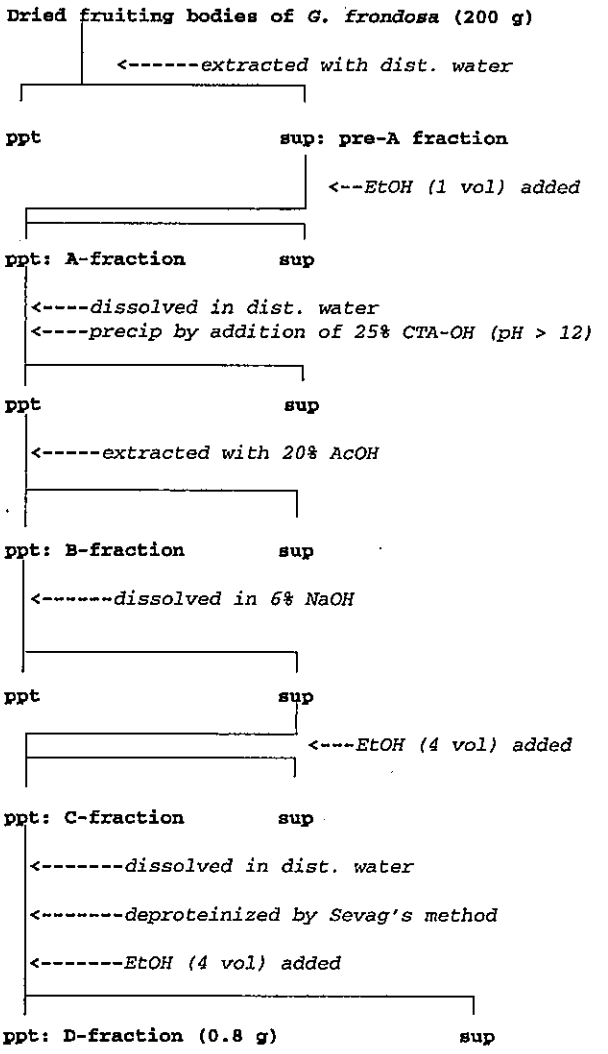

FIGURE 1. Extraction of polysaccharide from *G. frondosa*

experiment using an allogenic tumor, possible involvement of allograft rejection due to a difference of major histocompatibility antigen (MHA) could not be ruled out. Table III indicates the growth inhibitory effect of the D-fraction against some syngeneic tumors. MM-46 carcinoma in C3H mice and IMC carcinoma in CDF mice regressed after oral administration of D-fraction under these conditions (TIR 64% and 75%), while the tumor inhibition was as low as 27% in the case of B-16 melanoma in C57BL/6 mice. A Winn assay was conducted to examine whether immune responses were associated with the manifestation of these antitumor effects. MM-46 carci-

MICE	TUMOR SYSTEM	GROWTH INHIBITION	
		Oral administration	Intraperitoneal
C3H	MM-46 carcinoma (breast)	64.0% (1.5 mg)	83.2% (1.0 mg)
CDF1	IMC carcinoma (skin)	75.0% (1.5 mg)	47.7% (1.0 mg)
C57BL/6	B-16 melanoma (skin)	27.3% (1.5 mg)	25.6% (1.0 mg)

TABLE 3. Effects of D-fraction on interleukin-1 production by macrophages obtained from MM-46 tumor bearing mice

Treatment	Tumor weight (g)	
Spleen from MM-46-bearing C3H mice	0.44 ± 0.24	a)
Spleen from D-fraction administered MM-46-bearing C3H mice	0.00 ± 0.00	b)
MM-46-bearing C3H mice (control)	1.44 ± 1.11	a)

Significance of differences (t-test) a) $p < 0.05$, b) $p < 0.01$

TABLE 4. Tumor inhibition by splenocytes from D-fraction administered mice (Winn Assay)

noma tumor cells were subcutaneously implanted in C3H mice, and D-fraction was orally administered (D-mice). A spleen cell suspension obtained from these D-mice or MM-46 tumor-bearing mice was mixed with MM-46 carcinoma tumor cells, and the cell suspension was inoculated into normal C3H mice. After breeding the mice for 14 days, the extirpated tumors were weighed. *Table 4* shows the results. While significant regression was observed in tumors obtained from the mice implanted with the mixture of tumor cells and spleen cells obtained from tumor-bearing mice, complete tumor destruction was observed in mice inoculated with the mixture of tumor cells and spleen cells obtained from D-mice. These results suggest that the immune response which had been induced by the implantation of the tumor was further stimulated by the administration of D-fraction.

Cytolytic Activity and DTH Response

The author confirmed that the cytotoxic activity of macrophages and cytotoxic T cells against tumor cells and the production of interleukin-1 by macrophages with Lyt-2$^-$ cells were enhanced by D-fraction *in vitro* (11). In the *in vitro* test, the activity of Tc cells obtained from D-mice, in which the MM-46 tumor had regressed, exhibited approximately 3.7-fold potentiation when compared with Tc cells obtained from MM-46 tumor-bearing mice which had not received D-fraction. *Table 5* is the result of D-fraction effect on the production of interleukin-1 (IL-1). It is seen that a 3.2-fold potentiation of IL-1 activity was attained by the administration of D-fraction. This result suggests that the increase of IL-1 production in macrophages promoted the activation of the T cell group. It is known that there are two types of cytotoxic reactions. One is associated with cytotoxic T cells (CTL, Lyt-2$^+$ expressed)

Mouse	Uptake of ^{3}H-thymidine (dpm)	
Tumor-bearing, non administration of D-fraction	3122.3 ± 423.7	a)
Tumor-bearing, administration of D-fraction (D-mouse)	9877.4 ± 224.5	a)
Non tumor-bearing, administration of D-fraction	4414.4 ± 404.4	

Significance of differences (t-test) a) p<0.01

TABLE 5. Effects of D-fraction on interleukin-1 production by macrophages obtained from MM-46 tumor bearing mice

Treatment		Footpad Swelling (x 10^{-2}mm)	
Tumor-bearing	Saline	3.5 ± 1.8	*
	D-Fraction	15.7 ± 0.9	*
Normal		3.3 ± 1.1	

*Mean ± SE , Student's test * p < 0.05*

TABLE 6. Effects of D-fraction on on footpad swelling test

which bond with the tumor cells associated with specific a antigen and destroy these cells directly; the other is a non-specific cytotoxic reaction (delayed-type hypersensitivity: DTH) conducted by macrophages activated by lymphokine-produced DTH-inducible T cells (Tdh, Lyt-2$^-$ expressed). It is reported that this DTH (nonspecific immune response induced by Tdh), is manifested in the body and that the tumor is rejected by this reaction (13). The author next used an *in vivo* system to examine the DTH reaction in orally administered D-fraction by conducting a footpad swelling test and Winn assay.

The three experimental groups consisted of 5 mice each: (i) normal mice, (ii) tumor-bearing saline administered mice (control) and (iii) tumor-bearing D-fraction mice (D-mice). The tumor cells treated with MMC were injected subcutaneously an antigen to the right footpad of the mice and the degree of swelling was measured after 48 hours. As shown in *Table 6,* when the antigen was injected in D-mice, swelling of the footpad by more than 0.15 mm was observed (14). No such swelling was observed in the normal and control mice. These results suggest that D-fraction treatment potentiates a DTH reaction against tumor antigens in tumor-bearing mice. We hypothesized that if this DTH reaction played an important role in tumor rejection, tumor growth inhibition would be observed when a spleen cell group, from which CTL was eliminated, is implanted in normal mice.

A Winn assay was conducted using Lyt-2$^-$ spleen cells in which Lyt-2$^+$ cells corresponding to CTL were eliminated. As shown in Table VII, even after CTL were eliminated from spleen cells, complete tumor inhibition was observed, indicating that spleen cells other than CTL play a leading role in tumor cytotoxicity. This result was obtained when effector and target cells were mixed and administered to the recipient. In this experimental system, however, the possibility that tumor involution occurred due to the acting host immune system after

Effector cells	Lyt-2⁻ Splenocytes (E/T=100)	
Recipient mice	C3H (normal)	
	tumor weight (mg)	Inhibition (%)
Tumor-bearing — Saline	851.2 ± 37.1 *	67.3
Tumor-bearing — D-Fraction	0.1 ± 0.1 **	100.0
Tumor cells only a)	2601.3 ± 63.8	0.0

a) Tumor cells were inoculated without splenocytes

Student's t-test $^{*}p < 0.05$ $^{**}p < 0.01$

TABLE 7. Tumor neutralization test by Lyt-2 splenocytes from whole spleen cells

implantation cannot be rejected. Also, from the results seen in *Table 7,* which were obtained when CTL-eliminated spleen cells were implanted as effector cells together with tumor-cells in the recipient, we can consider that CTL could be induced from CTL precursors, inducing CTL tumor cytotoxicity when Th having the same phenotype as Tdh reacted with tumor antigen and produced IL-2.

Thus, to clarify this theory, the author prepared a system in which CTL induction had not occurred in the recipient body *(Table 8).* ICR-nu/nu mice without matured T cells were used as recipients. As shown in *Table 8,* spleen cells obtained from D-mice also showed strong tumor neutralizing activity with TIR 92.5%. Consequently, it was clarified that, even under conditions in which CTL induction in the recipient cannot possibly occur, the neutralization of the tumor is accomplished by CTL-defective Lyt-2 splenocytes.

These results indicate that a DTH reaction, manifested by the collaborative action of Tdh and macrophages, was potentiated by oral administration of D-fraction, and that even when CTL is absent, the involution of a tumor is highly likely to occur.

Discussion

The author previously reported that extracts from the fruiting body of the Maitake mushroom showed antitumor action against allogenic and syngeneic tumors by not only directly activating the various effector cells (macrophages, Natural Killer cells, cytotoxic T cells, etc.) to attack tumor cells, but also by potentiating the activities of various mediators including lymphokines and IL-1 to enhance cellular immune functions and to prevent a decrease of immune functions in the tumor-bearing host (12). These mechanisms seem to be similar to that of lentinan isolated from the Shiitake mushroom and possibly other mushroom extracts that possess similar antitumor activity. Unlike other mushroom extracts, however, Maitake inhibits tumor growth even when orally administered. In this paper, the author has reported that the stimulation of immune response is enhanced by oral administration of Maitake D-fraction. The enhancement of the cytotoxic activity and IL-1 productivity by macrophages and of T cells which exhibit antigen-specific cytotoxicity was explained. The potentiation of the DTH reaction by the combined action of Tdh and macrophages was also elucidated.

Effector cells		Lyt-2⁻ Splenocytes (E/T=15)		
Recipient mice		ICR nu/nu		
		Tumor weight (mg)		Inhibition (%)
Tumor-bearing	Saline	111 ± 40	**	87.4
	D-Fraction	66 ± 13	**	92.5
Tumor cells only a)		878 ± 99		0.0

a) Tumor cells were inoculated without splenocytes

Student's t-test ** $p < 0.01$

TABLE 8. Tumor neutralization test by spleen cells from ICR-nu/nu mouse

As a microbiology researcher as well as a mycologist, the author's current interest focuses on the Maitake mushroom. Apparently, this legendary and very tasty mushroom has many more medicinal effects than just its antitumor activity. Current research by the author appears to indicate that the mushroom may also possess anti-retroviral, hypoglycemic and anti-hypertensive effects.

As for the anti-HIV activity, the National Institute of Health of Japan announced the results of its screening tests on sulfated Maitake extract under its standard Microplate Method on January 23, 1991. Also, in January 1992, the U.S. National Cancer Institute in Frederick, MD, finalized its in-vitro screening of anti-HIV Drug Testing System under its Developmental Therapeutics Program and confirmed the anti-HIV activity of sulfated Maitake extract which was, in fact, as strong as that of AZT. The abstract of the author's research conducted at his laboratory in Kobe, Japan was presented at VIII International AIDS Conference in Amsterdam in July, 1992.

1./9. Ohno N, Ito K, Takeyama T, Suzuki I, Sato K, Oikawa S and Miyazaki T. Chem. Pharm. Bull., 35,3395 (1985)
2. Mizuno T, Ohsawa K, Hagiwara N and Kuboyama R. Agri. Biol. Chem, 50, 1979 (1986)
3. Nanba H, Mori K, Toyomatsu T and Kuroda H. Chem. Pharm. Bull., 35, 2453 (1987)
4. Nanba H and KurodaH. Chem. Pharm. Bull., 35,2459 (1987)
5. Mori K, Toyomatsu T, Nanba H and Kuroda H, Int'l Symposium Scientific and Tech. Aspects of Cultivating Edible Fungi, The Penn Univ., July 1986
6. Maeda YY, Hamuro J, Yamada YO, Ishimura K and Chihara G. IMMUNOPOTENTIATION, ed. by G. E. W. Wolstenholme et al., Excerpta Medica, North Holland, 1973, P.259
7. Chihara G, Maeda YY, Hamuro J, Sasaki T and Fukuoka F. Nature (London), 222, 687 (1969).
8. Komatsu N, Okubo S, Kikumoto S, Kimura K, Saito G and Sasaki S. Gann, 60, 137 (1969)
10. Nanba H, Hamaguchi A, and Kuroda H. Chem. Pharm. Bull, 35, 1162-1168 (1987)
11. Hishida I, Nanba H, and Kuroda H. Chem. Pharm. Bull, 36, 1819-1827, (1988)
12. Adachi K, Nanba H, Kuroda H. Chem. Pharm. Bull, 35, 262-270, (1987)
13. Fujiwara H, Furuzawa M, Yoshioka T, Nakajima H, Hamaoka T. **The role of tumor-specific Lyt-1⁺2⁻T cells in eradicating tumor cell *in vivo*, (i) Lyt-1⁺2⁻T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation *in vivo*.** J. Immunol., 133:1671-1679, 1984
14. Yamada Y, Nanba H and Kuroda H. Chem. Pharm. Bull, 38, 790-796 (1990)