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Antitumor Activity Exhibited by Orally Administered Extract from Fruit Body of *Grifola frondosa* (Maitake)

IKUKO HISHIDA, HIROAKI NANBA* and HISATORA KURODA

Laboratory of Microbiology, Kobe Women's College of Pharmacy, Motoyama, Higashinada, Kobe 658, Japan

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The acid-insoluble, alkali-soluble, hot-water-extractable polymer (a polysaccharide containing approximately 30% of protein; D-fraction) obtained from the fruit bodies of *Grifola frondosa* (maitake) exhibited antitumor activities against allogenic and syngeneic tumors on oral administration to mice. Winn assay conducted to examine the tumor growth-suppressing effect revealed a complete inhibition of the tumor by the oral administration of D-fraction. This fact indicates that stimulation of the immune response system triggered by the tumor-bearing state is activated by D-fraction. Consequently, the activity of D-fraction on cells associated with immune response was examined. The cytolytic activity and interleukin-1 productivity of macrophages or T cells which exhibit antigen-specific cytotoxicity were enhanced. D-fraction was found to potentiate the delayed-type hypersensitivity response which is associated with tumor growth suppression.

Keywords——*Grifola frondosa*; antitumor activity; β-glucan(D-fraction); delayed-type hypersensitivity (DTH); oral administration

Many antitumor-active polysaccharides derived from basidiomycetes have been reported, including lentitan, shizophyllan, PSK and others, most of which principally consist of similar β-glucans. Most of these polysaccharides, excluding PSK, exhibit antitumor activity *via* the activation of host immune systems when administered intravenously or intraperitoneally. However, they have been reported to be ineffective when given orally. The authors have isolated from the fruit body of *Grifola frondosa* (maitake), a β-glucan (MT-2) which carries many 1,3-linked oligosaccharied side chains branching from the 1,6-linked main chain. The authors reported previously[1,2] that MT-2 exhibited antitumor activity by stimulating the immune system of mice, when administered intraperitoneally. Miyazaki[3] and Mizuno *et al.*[4] obtained a lentinan-like polysaccharide from this fruit bodies and reported that it was inactive when administered orally, whereas an antitumor effect was exhibited when it was administered intraperitoneally or intravenously. In contrast, the author[5,6] have observed that the immune systems in mice were stimulated, leading to the regression of tumors, when powdered fruit body of *Lentinus edodes* (shiitake) was orally administered to mice. Moreover, Ohkuma *et al.*,[7] reported that stimulation of the immune system occured when a protein–polysaccharide complex extracted with hot water from *Flammulina velutipes* was administered orally to tumor-bearing mice. In this context, we have examined the antitumor activity of extracts from the bodies of maitake.

Materials and Methods

(1) Preparation of Extractable Materials——The process used for extracting materials from the powdered fruit body of *Grifola frondosa*, supplied by the Mushroom Research Institute of Japan (Kiryu, Gumma), was based on the described by Chihara *et al.* for the extraction of lentinan from shiitake.[8] As shown in Fig. 1, the powder (600 g) was mixed with 3000 ml of deionized water, and heated at 100 °C for 10 h. After centrifugation at 6000 rpm for 10 min, the supernatant was used as pre-A fraction. An equal amount of 99% EtOH was added to this pre-A fraction and left at

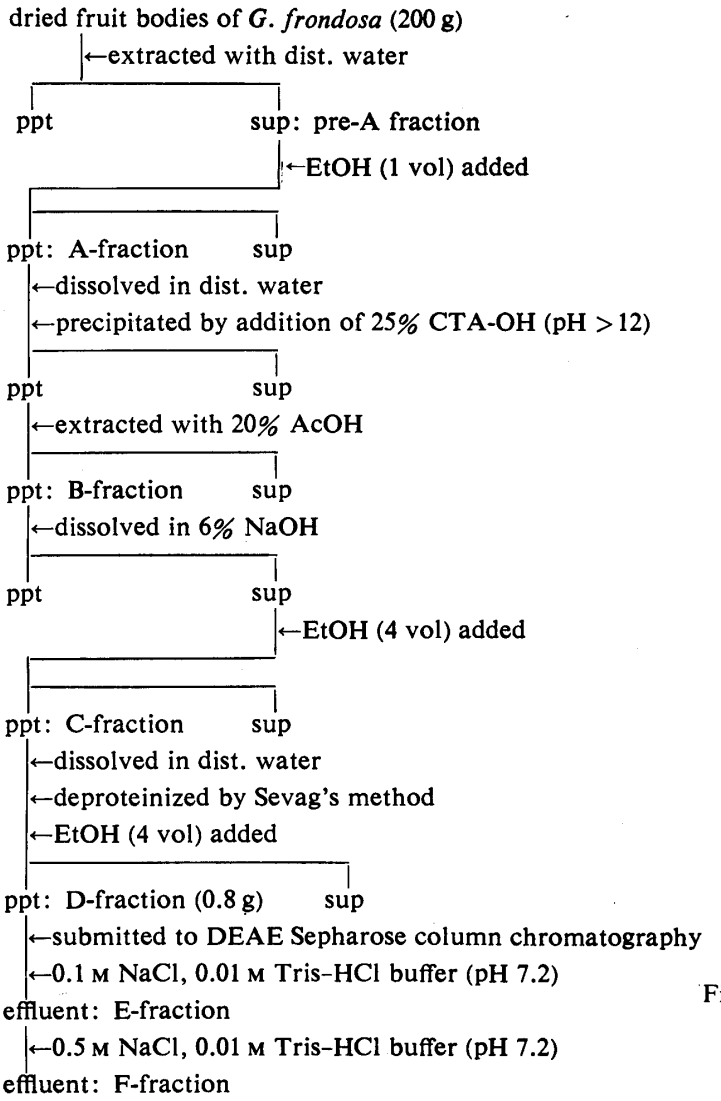

Fig. 1. Extraction of Polysaccharide from *G. frondosa*

4 °C for 24 h. The precipitate A-fraction was separated and dissolved in deionized water. Then, 25% (CTA-OH) (cetyltrimethylammonium hydroxide) was added dropwise with stirring until the pH exceeded 12 and the mixture was allowed to stand at 4 °C for 12 h to give a precipitated. This pellet was treated with 20—50% acetic acid to remove the acid-soluble fraction, and the residual fraction was obtained as B-fraction. This fraction was treated with 6% NaOH to separate the alkali-soluble fraction. This solution was combined with 4 volumes of EtOH to obtain the precipitate polymer. This pellet (C-fraction) was dissolved in an equal amount of distilled water and deproteinized with $CHCl_3$–MtOH (9 : 1). The solution was again treated with 4 volumes of EtOH. The obtained precipitate pellet (D-fraction) was a light brown and amorphous powder, which was positive in the anthrone test, and contained approximately 30% protein as determined by Lowry's method. The D-fraction was charged on a diethylaminoethyl (DEAE)-Sepharose column (5 × 40 cm), and eluted with 0.01 M Tris–HCl buffer containing 0.1 or 0.5 M NaCl (pH 7.2) to give E-fraction and F-fraction successively.

(2) Mice and Tumors——Sarcoma-180 was transplanted into ICR mice, MM-46 carcinoma and MH-134 hepatoma into C3H/HeN (C3H) mice, IMC carcinoma into CDF_1 mice and B-16 melanoma into C57BL/6 mice.

(3) Tumor Growth-Inhibitory Effect——Tumor cells (1×10^6) were implanted subcutaneously in the right axillary region of mice, and from 24 h after administration, 0.5 ml of each fraction, adjusted to a sugar concentration of 3.0 mg/ml, was orally administered on alternate days. After the completion of the administration, the solid tumors were extirpated and weighed to obtain the tumor growth inhibition rate (TIR), which was calculated according to the previous paper.[1]

(4) Winn Assay[9]——MM-46 tumor cells (1×10^6) were inoculated in the axillary region, and from 24 h after inoculation, 0.5 ml of D-fraction, adjusted to a sugar concentration of 1.5 mg/ml, was orally administered 10 times on alternate days. After the completion of the administration, the spleen was extirpated from these D-fraction-administered mice (hereafter termed simply D-mice). The obtained 5×10^6 spleen cells were mixed with 1×10^6 MM-46 tumor cells. The mixed cell suspension was implanted into the right axillary region of normal C3H mice

(recipients), and after 14 d, solid tumors were extirpated and weighed. On the other hand, two experimental systems were employed to compare tumor growth inhibition in D-mice. In the first, the tumor was extirpated from C3H mice inoculated with a mixture of MM-46 tumor cells and the spleen cells of MM-46 tumor-bearing C3H mice which had been given saline orally. In the second, the growing tumor was isolated from C3H mice implanted with only MM-46 tumor cells as control mice.

(5) Preparation of Macrophages (Mϕ)——Five-week-old C3H (or CDF_1) male mice were killed by dislocating cervical vertebrae and peritoneal cells were obtained by washing with Hanks' solution. After centrifugation at 1200 rpm for 5 min, the precipitated cells were collected and adjusted with RPMI-1640 medium to 1×10^5 cells/ml. These cells were incubated for 45 min at 37 °C in an atmosphere containing 5% CO_2 gas, and non-adherent cells were eliminated by washing with Hanks' solution. Mϕ which were adsorbed selectively on the plate wall were collected.

(6) Preparation of Whole Spleen Cells—— The extirpated spleen from C3H male mice (5 weeks old) was washed and disintegrated sufficiently in RPMI 1640 medium. Then, the cells were passed through a 40-mesh stainless steel screen. The cells were collected by centrifugation at 1200 rpm for 10 min and 0.2 ml of a 10-fold dilution of Eagle's MEM was added to lyse contaminating erythrocytes hypotonically for 10 s, then 2 ml of Eagle's MEM (2-fold concentration) was added promptly and mixed. The whole was centrifuged at 1200 rpm for 10 min, and the obtained cells were adjusted to 1×10^7 cells/ml and used as the whole spleen cell suspension.

(7) Preparation of T Cells——Whole spleen cell suspension was charged on a nylon wool column (1×15 cm) moistened with RPMI-1640 medium. After incubation for 60 min at 37 °C in a humidified atmosphere of 5% CO_2 in air, the T cells were eluted with RPMI-1640 medium. The eluted T cells were collected by centrifugation at 1200 rpm for 5 min.

(8) Preparation of Lyt-1$^+$ T Cells[10]——Anti-Lyt-2.1 monoclonal antibody (50 μl) was mixed with 1×10^6 T cell/ml. After incubation at 37 °C for 60 min in an atmosphere of 5% CO_2, in air, the suspension was washed with RPMI-1640 medium by centrifugation. The cells obtained were incubated with 50 μl of Low·Tox-M rabbit complement for 60 min at 37 °C. Then cells obtained by subsequent centrifugation were stained with trypan blue, and a preparation containing 1×10^5 viable cells was made.

(9) Labeling of the Target Cells——As described in the previous paper,[2] 2×10^6 tumor cells were mixed 50 μl of 40μCi/ml ^{3}H-uridine and after incubation for 60 min at 37 °C in an atmosphere containing 5% CO_2 gas, free ^{3}H-uridine was sufficiently rinsed out with RPMI-1640 medium. The same medium was further added to prepare a suspension containing 1×10^4 cells/ml.

(10) Cytotoxicity Test——The effector cells obtained from D-mice were suspended in RPMI-1640 medium (1×10^5 cells/ml), to which were added labeled tumor cells to make the E/T ratio = 10 : 1 or 50 : 1. The cell suspension was cultured at 37 °C for a predetermined period of time and after centrifugation, 1 ml of the supernatant was mixed with 10 ml of Univagel II reagent (Nakarai Chemicals Co.). The radioactivity of the obtained suspension was measured with an Aloka LSC-700 scintillation counter.

(11) Preparation of Solution Containing Interleukin-I (IL-1)[11]——Mϕ obtained from D-mice were adjusted to 1×10^5/ml with RPMI-1640 medium which did not contain serum, and 2 ml of this suspension was placed on a 12-well flat plate. After incubation for 72 h at 37 °C in an atmosphere of 5% CO_2 in air, the supernatant was filtered through a 0.45 nm Millipore filter for sterilization and stored at −80 °C. This was thawed as required.

(12) Measurement of Lymphocyte Growth Reaction[11]——The thymus was extirpated from normal male C3H mice which had been killed by bleeding from the femoral vein and carotid artery. Cells were teased out in RPMI-1640 medium and after being passed through an 80 mesh sieve, were used as the cell suspension. The suspension was adjusted to 1×10^7 /ml with RPMI-1640 medium containing 0.7 mg/ml peanut lectin coagulant. After incubation for 45 min, 5 ml of 20% fetal bovine serum (FBS)-added RPMI-1640 medium was overlaid, and after further incubation for 90 min, PNA-noncoagulated cells were collected. These cells were washed with RPMI-1640 medium not containing serum, and cells were resuspended in 2 ml of galactose solution (2.7 g of D-galactose, 2 ml of FBS, 15 ml of MEM medium, 3 ml of 20 mM HEPES and 75 ml of distilled H_2O). After 10 min, sufficient rinsing was conducted with RPMI-1640 medium, and the suspension were adjusted to 5×10^6 cells/ml with RPMI-1640 medium and divided into 0.1 ml aliquots on a 96-well flat plate (Corning Glass Works). IL-1 solution (0.05 ml) obtained as described (11) was added at two dilutions with 0.05 ml of RPMI-1640 medium or 0.05 ml of PHA-P at 10 μg/ml. This mixture was incubated for 72 h. At 8 h before the end of incubation, 0.1 μCi of ^{3}H-thymidine was added and mixed by shaking the plate. Then 100 μl of this reaction fluid was filtered through a Millipore filter, which was dried after being washed with 1.5 ml of 5% trichloroacetic acid (TCA) solution. The radioactivity was measured (see preceding section (10)).

Results

As a preliminary test, after implantation of an allogeneic Sarcoma-180 tumor into ICR mice, each extracted fraction was administered, in an amount of 1.5 mg sugar content, orally 10 times on alternate days. On the 12th day after the completion of the administration, solid tumors were extirpated and weighed to obtain the TIR. When D-fraction was administered

TABLE I. Tumor Growth-Inhibitory Activity of Extracts from *G. frondosa*

Fraction	Tumor weight (g)	TIR (%)
Control	2.28±1.16	—
Pre-A	2.14±1.09	2
A	2.31±1.66	—
B	1.86±0.13	18
C	2.31±1.36	—
D	0.96±0.67[a)]	58

(10 mice × 3)

Significance of differences (*t*-test) *a*) $p<0.05$.

TABLE II. Determination of Optimal Dose of Fraction for Tumor Growth Inhibition (ICR-Sarcoma-180)

Sugar content (mg/ml)	Tumor weight (g)	TIR (%)	F-Distribution
0 (Control)	12.77±3.81	—	—
0.2	5.12±2.75[a)]	54	7.55
0.4	5.60±1.81[a)]	56	3.26
0.75	5.00±1.17[a)]	61	1.37
1.5	4.00±1.47[a)]	69	2.16

Significance of differences (*t*-test) *a*) $p<0.05$.

TABLE III. Tumor-Inhibitory Effects of D-Fraction on Syngeneic Tumors

Mouse	Tumor	Tumor weight (g)		TIR (%)
		Control	D-Fraction	
C3H	MM-46	6.09±3.83	2.24±2.30[a)]	64
CDF_1	IMC	6.47±2.92	1.63±1.90[b)]	75
C57BL/6	B-16	5.70±1.80	4.10±2.04	27

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

58% TIR was obtained but practically no antitumor effect was observed with E- or F-fraction (obtained by the passage of D-fraction through an ion-exchange column). Therefore, the authors deal exclusively with D-fraction in this paper. The result is shown in Table I. The D-fraction at doses ranging from 0.2 to 1.5 mg of the sugar was administered to determine the optimal dose and the result is given in Table II. While each of these doses was significantly effective at a probability level of 0.01, no significant difference was observed between these doses. However, intragroup variance was the least at the 0.75 mg dose (25 μg/g of mouse body weight). Therefore this dose of D-fraction was used in this experiment.

Table III indicates the growth-inhibitory effect of the D-fraction against some syngeneic tumors. MM-46 carcinoma in C3H mouse and IMC carcinoma in CDF_1 regressed after oral administration of D-fraction under these conditions (TIR, 64% and 75%), while the tumor growth inhibition was as low as 27% in the case of B-16 melanoma in C57BL/6 mouse. Winn assay was conducted to examine whether immune systems were associated with the manifestation of these antitumor effects. MM-46 carcinoma tumor cells were subcutaneously implanted in C3H mice, and D-fraction was orally administered according to the previous

TABLE IV. Tumor Inhibition by Splenocytes from D-Fraction Administered Mice (Winn Assay)

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

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

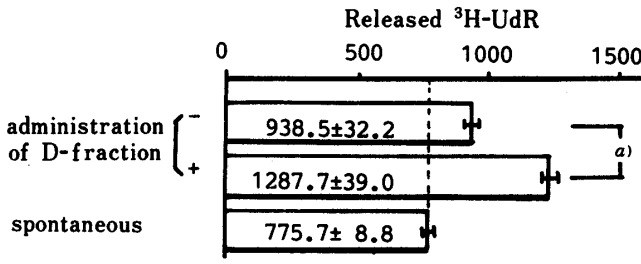

Fig. 2. Effects of D-Fraction on MM-46 Tumor Cell-Lytic Activity of Macrophages Obtained from MM-46 Tumor-Bearing Mice

Significance of differences (*t*-test) *a*) $p<0.05$.

procedures. Thus mice in which the tumor growth was suppressed 60 to 70% were termed 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 d, the extirpated tumors were weighed. Table IV 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 activity of the immune response system which had been induced by the implantation of the tumor was further stimulated by the administration of D-fraction.

Therefore, we examined how the cells of the immune system were activated by D-fraction in an *in vitro* system. First, intraperitoneal Mϕ obtained from D-mice or from tumor-bearing mice not given D-fraction were mixed with ^{3}H-uridine-labeled tumor cells (at an E/T ratio = 1/50) and after incubation for 48 h, the released radioactivity in the medium was measured. As shown in Fig. 2, Mϕ obtained from normal mice did not exhibit any cytotoxicity to tumor cells, but Mϕ obtained from MM-46 tumor-bearing mice did show cytotoxicity. Administration of D-fraction further potentiated this activity.

Next, the action of D-fraction against cytotoxic T cells (Tc cells), which show antigen-specific activity, was examined. The T cell suspension which was obtained from spleen cells of D-mice or control mice by passage through a nylon-wool column was mixed with ^{3}H-uridine-labeled MM-64 or MH 134 tumor cells as syngeneic tumor cells and IMC tumor cells as allogeneic tumor cells, (E/T = 1/10). After incubation for 4 h, the radioactivity released into the supernatant was measured; the results are summarized in Fig. 3. The activity of Tc cells obtained from D-mice, the MM-46 tumor of which had regressed, exhibited approximately 3.7-fold potetiation when compared with Tc cells obtained from MM-46 tumor-bearing mice which had not received D-fraction (dpm of tumor-bearing D-mice Tc cells − dpm of normal mice Tc cells/dpm of tumor-bearing mice Tc cells − dpm of normal mice Tc cells). However, administration of D-fraction did not potentiate cytotoxic activity against MH-134 cells, having a same major histocompatibility complex (MHC), or against IMC cells, having a different MHC between the tumor cells and host cells. This indicates the activation of Tc cells with antigen-specific cytotoxicity due to the administration of D-fraction. In general, this Tc

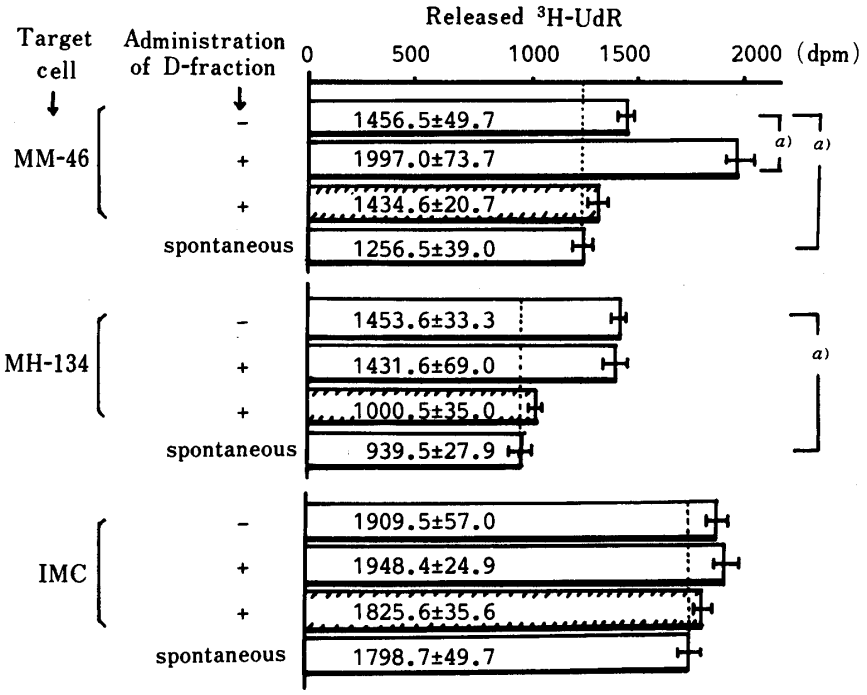

Fig. 3. Effects of D-Fraction on Cytolytic Activity of T Cells Obtained from MM-46 Tumor-Bearing Mice against Various Tumor Cells

□, MM-46 tumor-bearing mice; ⬚, normal mice. Significance of differences (t-test) *a*) $p<0.05$.

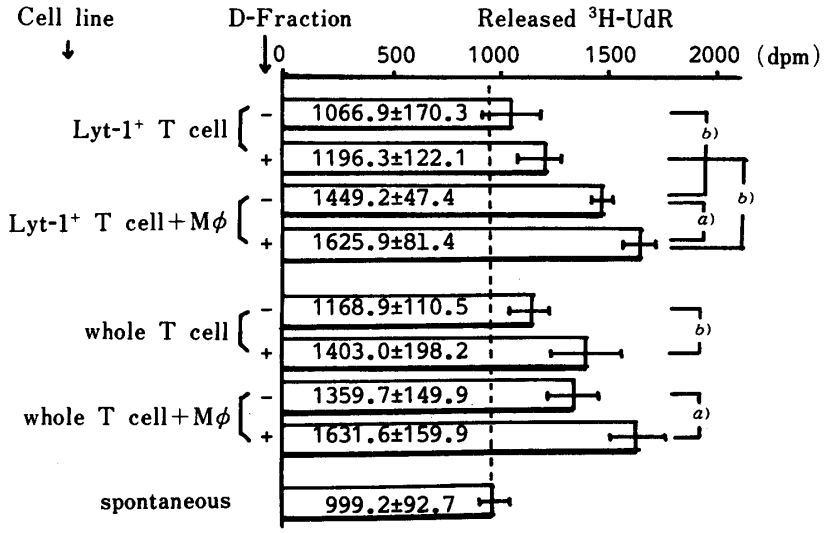

Fig. 4. Effects of D-Fraction on Cytolytic Activity of (Lyt-1$^+$T cell + Macrophage) System

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

cell is due to Lyt-2$^+$ T cells, while delayed-type hypersensitivity (DTH) response, on the other hand, is associated with Lyt-1$^+$ T cells.

Consequently, we examined whether the DTH response is actually associated with tumor growth inhibition, and how D-fraction influences DTH response. Lyt-1$^+$ T cells were obtained by removing Lyt-2$^+$ T cells by treatment with anti-Lyt-2.1 monoclonal antibody and complement. A system exclusively containing this T cell fraction or also containing Mϕ obtained from normal mouse were prepared, and to each was added ^{3}H-uridine-labeled MM-

TABLE V. Effects of D-Fraction on Interleukin-1 Production by Mϕ Obtained from MM-46 Tumor-Bearing Mice

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 a)

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

46 tumor cells. After incubation for 48 h, released radioactivity was measured. The results are illustrated in Fig. 4. Although Lyt-1$^+$ T cells alone exhibited no cytotoxicity in either the D-fraction-administered group of the D-fraction-nonadministered group, cytotoxicity was observed in the presence of normal mouse Mϕ which exhibit no cytotoxicity alone. Futhermore, this cytotoxicity was potentiated by the administration of D-fraction. These results suggest that DTH response is associated with tumor growth suppression and that the response is enhanced by D-fraction. When the same experiment was conducted using whole T cells, without separating Lyt-1$^+$ and Lyt-2$^+$ T cells, the potentiation of cytotoxicity was enhanced even in the absence of Mϕ. This can be interpreted as resulting from a concented action between Tc cells of Lyt-2$^+$ and DTH-inducing T cells of Lyt-1$^+$ (Tdh cells). These results indicate that the immune systems were stimulated by D-fraction.

Thus, we examined the influence of D-fraction on the production of interleukin-1 (IL-1). The activity of IL-1 in the supernatant of cultured Mϕ obtained from D-mice or from tumor-bearing saline-dosed mice was measured in terms of the uptake of ^{3}H-thymidine by T cell obtained from normal mouse. Table V indicates the results. It is seen that 3.2-fold potentiation of IL-1 activity was attained by the administration of D-fraction. This result suggests that the increase in Mϕ IL-1 production promoted the activation of the T cell group.

Discussion

Generally, it is well known that some polysaccharides obtained from basidiomycetes spp. show immune-stimulating activity.[12] Therefore, the concentrations of the extractable material were expressed in terms of polysaccharide contents. The D-fraction at a dose of 0.75 mg of polysaccharide exhibited antitumor activity. Since the fractionation from pre-A through D-fraction executed in this study followed the procedures adopted in the extraction of alkali-soluble, but acid-insoluble polysaccharides (β-glucan), the fact that D-fraction obtained by this method exhibited a tumor growth-suppressing effect means that a component having an antitumor activity was purified stepwise until an effective concentration was reached at the D-fraction. If a more concentrated solution were administered, other fractions might exhibited a similar effect. However, judging from the concentrations used in this study (all the solutions used for oral administration were viscous) a higher concentration would be difficult to give orally.

On the other hands, we confirmed the presence of a substance which inhibited immune stimulation in the pre-A fraction. It appears that this substance may have been gradually removed in the subsequent fractionations. The D-fraction was further fractionated into E, F and residual fractions. The obtained E-fraction corresponds to MT-2,[1] which exhibits a strong antitumor activity when injected intraperitoneally, but proved ineffective when given orally. This dependence on the effect on the route of administration remains to be investigated. Next, the Winn assay was employed to examine whether the tumor-suppressing effect of the

D-fraction is due to stimulation of the immune system. As shown in Table VI, when a mixture of tumor cells and spleen cells obtained from a tumor-bearing mouse was injected, significantly higher TIR was obtained as compared with the case of mice inoculated with tumor cells alone. This result suggested that the tumor-bearing mice showed an immune response to the tumor, inducing spleen cells which have cytotoxicity against the tumor cells. Since this action was further potentiated by the administration of D-fraction and a complete inhibition of tumor growth resulted, we assumed that the oral administration of D-fraction strongly activated the cellular immune system which was stimulated by tumor-implantation. Thus, the cytotoxic activity was examined in an *in vitro* system. When D-fraction was administered, Mϕ, T cells and Tdh cells were all activated significantly as compared with the control mouse. As shown in Fig. 3, the activity of Tc cells, which exhibit antigen-specific cytotoxicity, is potentiated, whereas natural killer cells or lymphokine-activated killer cells which exhibit antigen-nonspecific activity, are not influenced. Figure 4 illustrates the results on the cytotoxicity induced by DTH response. Namely, Lyt-1^+2^- T cells obtained from tumor-bearing mouse showed practically no cytotoxicity alone, but when Mϕ, exhibiting no cytotoxicity, were added to this system, cytotoxicity was observed. Futher, in a system consisting of Lyt-1^+2^- obtained from D-mouse and Mϕ, this action was significantly potentiated. These results indicate that Tdh cells, induced in the tumor-bearing state, produced Mϕ activating factor through contact with antigen tumor cells, and that DTH response was promoted by D-fraction dosing. If such a DTH response could occur also the *in vivo* system, macrophage activating factor production would be initiated through antigen-specific Tdh response, and the activated Mϕ would nonspecifically show cytotoxicity against heterologous cells surrounding the Mϕ.

Solid tumors in a host have cell surface antigens.[13] Fujiwara *et al.*, suggested the possibility that DTH response-activated Mϕ play a greater role in antitumor activity than Tc cells, having a higher antigen-specificity[14]. If so, it would be important to observe the DTH response against tumor cells after the administration of D-fraction. The next phase of the present experiment was to examine interleukin-1 production caused by the administration of D-fraction. As shown in Table V, production of IL-1 was increased by D-fraction, and this IL-1 was considered to act on T cells, promoting the production of IL-2, and elevating the induction of Tc cells or Tdh cells, thus stimulating the whole immune system.

Although the mechanism involved is yet to be elucidated, the fact that D-fraction actually enhanced the production of IL-1 by Mϕ collected from tumor-bearing mouse may give a clue to the settlement of this problem. It is believed that gut-associated lymphoid tissue (GALT) is present in the digestive tract. In GALT, T cells, along with B cells, are present.[15] Moreover, it was reported that phagocytic epithelial cells in the epithelium of the digestive lumen, together with M cells and Mϕ existing in Peyer's patch, exhibit phagaocytic and antigen-presenting activities.[16] Therefore, D-fraction is considered to be subjected to phagocytosis by these cells, resulting in their activation and leading to the promotion of IL-1 production by Mϕ existing in GALT, followed by an increase in the production of IL-2. The IL-2 thus produced is considered to enter the blood circulation and then to be associated with the stimulation of the immune system. We intend to investigate the influence of D-fraction on Mϕ obtained from the intestinal lumen to obtain evidence for the above suggestion.

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