ANTITUMOR EFFECT OF ORALLY ADMINISTERED EXTRACTS FROM FRUIT BODY OF *GRIFOLA FRONDOSA* (MAITAKE)

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We conducted a footpad swelling test to clarify whether a delayed type hypersensitivity (DTH) reaction against tumor antigen is potentiated by oral administration of D fraction extracted and purified from fruit body of *Grifola frondosa* (maitake), with a ratio of β-glucan to protein of 7 : 3. When the antigen was injected in D fraction administered tumor-bearing mice (D-mice), footpad swelling was observed, suggesting that a DTH reaction against the tumor antigen was potentiated by oral administration of D-fraction. 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. Even after CTL were eliminated, tumor neutralizing activity was at no time impaired, indicating that spleen cells other than CTL play a leading role in tumor cytotoxicity.

We hypothesize from the above results, that a DTH reaction, manifested by the combined action of delayed hypersensitivity T cells (Tdh) and macrophages (Mφ), was potentiated by oral administration of D-fraction. Even when CTL are absent, there is a high degree of tumor involution. But D-fraction did not activate gut-associated lymphoid tissue (GALT) and Peyer's patch (PP), by oral administration.

Key words : Delayed-type hypersentsitivity, Antitumor activity, Oral administration, Footpad swelling test

It is known that lentinan, schizophyllan, PSK and other substances extracted from mycelia or the fruit bodies of fungi belonging to basidiomycetes exhibit antitumor effect when administered intravenously or intraperitoneally. However, these materials, with the exception of PSK, have been reported to be ineffective when given orally. In the previous paper, the authors reported that involution of a transplanted allogenic tumor in mice occured when the fruit-bodies of *Lentinus edodes* (shiitake, raw mushroom of lentinan[1~3]). Furthermore, it has been observed that D-fraction (a polysaccharide-protein complex, with a 7 : 3 ratio of β-glucan to protein) obtained from hot-water extracts of the fruit bodies of maitake, inhibits tumor growth when orally administered[4]. This inhibition was confirmed to be derived from the tumor neutralizing reaction induced in normal mice by the implantation of spleen cells from tumor-bearing mice which were treated with D-fraction.

Moreover, we have reported that either cytotoxic activity of macrophages and cytotoxic T cells against tumor cells or the production of interleukin-1 by macrophages and cytotoxicity inductive ability of macrophages with Lyt-2⁻ cells were enhanced by D-fraction in an *in vitro* system[4]. It is well known that there are two types of cytotoxic reaction associated with cytotoxic T cells (CTL, Lyt-2⁺ expressed) which bond with the tumor cells associated with specific antigen and destroys these cells directly; the second is a non-specific cytotoxic reaction (delayed-type hypersensitivity, DTH) conducted by macrophages which activated by lymphokine-produced DTH, inducible T cells (Tdh, Lyt-2⁻

expressed). Recently, Hamaoka et al. reported that a DTH reaction, nonspecific immune response induced by Tdh, is manifested in the body and that the tumor is rejected by this reaction[5~7]. Therefore, we used an *in vivo* system to study whether this DTH reaction is activated by orally administered D-fraction.

MATERIALS AND METHODS

(1) Regents : Mitomycin C was obtained from Kyowa Hakko Kogyo, Anti-Lyt-2.1 monoclonal antibody and Low-Tox-M rabbit complement from Cedariane Lab. (Canada), FBS and phytohemagglutinin (PHA-P) from Gibco Lab. (U.S.) and various antibiotics form Sigma Chemicals (U.S.).

(2) Preparation of extractable materials : The process used for extracting materials from powdered fruit bodies of *Grifola frondosa* by Mushroom Research Institute of Japan (Kiryu, Gumma) was described in a previous paper[2].

(3) Mice and tumor : MM-46 carcinoma was transplanted into C3H/HeN mice.

(4) Measurement of living cells : A 0.2 % w/v trypan blue acqueous solution was mixed with a 4.25 % w/v NaCl solution at a ratio of 4 : 1 just before use, and adjusted for stain. This solution was mixed with a cell suspension at a ratio of 1 : 1, and the number of non-staining cells were counted by cytometer.

(5) Tumor implantation and administration of D-fraction : The MM-46 tumor cells (1×10^6) were inoculated subcutaneously to the right axillary region of male C3H/HeN mice (5 w), and from the following day on, 0.5 ml each of D-fraction, adjusted to a sugar concentration of 1.5 mg/ml, was administered orally 10 times on alternate days. After completion of treatment, breeding was further continued for 8 days and mice in which tumors had involuted to more than 70 % when compared with normal mice were termed D-mice, then used for the footpad swelling and Winn assay.

(6) Footpad swelling test[8,9]: Intraperitoneal MM-46 tumor cells were collected from abdominal fluid and washed three times by centrifugation (1,500 rpm, 5 min.) in ice-cold RPMI-1640 medium. After adjusting to 10^7 cells/ml with RPMI-1640 medium containing 5 % FBS, 100 μg of MMC was added and incubated at 37℃ for 20 min. The cells were then washed 5 times in ice-cold RPMI-1640 medium and adjusted to 5×10^7 cells/ml. Then 10^6 cells were injected subcutaneously to the right planta of mice. Forty-eight hours after implantation, the thickness of the planta was measured and the difference from the measurements before implantation was expressed as the degree of swelling.

(7) Winn assay[8] : We prepared a whole spleen cell suspension and a spleen cell suspension (Lyt-2$^-$ spleen cells) without Lyt-2 cells. After killing the mice by dislocating cervical vertebrae, the spleen was extirpated, suspended with RPMI-1640 medium and filtered through a 40-mesh stainless sieve : 5 ml of 10-fold diluted RPMI-1640 medium was added to this mixture erythrocytes (hypotonically). Next, 0.5 ml of anti-Lyt-2.1 monoclonal antibody was added to 10 ml of whole spleen cell suspension which was then adjusted to 3×10^7 cells/ml, then incubated at 4℃ for 60 min. After centrifugation at 1,200 rpm for 5 min, cells were resuspended in 10 ml of RPMI-1640 medium containing rabbit complement, and Lyt-2$^+$ cells were impaired by incubation at 37℃ for 60 min. Then cells were immediately cooled in ice and washed by centrifugation at 1,200 rpm for 5 min. The cells suspended in the RPMI-1640 medium were used as Lyt-2$^-$ spleen cells.

RESULTS

In order to examine whether a DTH reaction against tumor antigen is potentiated *in vivo* by orally administered D-fraction, a footpad swelling test was conducted. The three experiment groups consisted of 5 mice each : (i) normal mice, (ii) tumor-bearing saline administered mice (control) and (iii) tumor-bearing D-fraction administered mice (D-mice). The tumor cells treated with MMC were injected subcutaneously as antigen to the right footpad of the mice and the degree of swelling was measured after 48 h. As shown in Fig. 1, when the antigen was injected in D-mice, swelling of the footpad by more than 0.15 mm was observed [9]. But no such swelling was observed in the normal and control mice. These results suggest that D-fraction treatment potentiates a DTH reaction against tumor antigen in tumor-bearing mice.

If this DTH reaction plays an important role in

tumor rejection, we hypothesize that when a spleen cell group, from which cytotoxic T cells have been eliminated, is implanted in normal mouse, tumor growth inhibition is observed.

A Winn assay was conducted using whole spleen cells from tumor involuted D mice and Lyt-2 spleen cells in which Lyt-2$^+$ cells corresponding to CTL were eliminated.

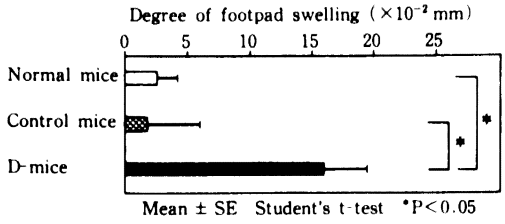

Fig. 1. Effect of D-fraction administration on footpad swelling test

The results are summarized in Table 1. When whole spleen cells were used, the tumor-growth was completely inhibited in mice in inoculated with a mixture of spleen cells obtained from D mice and tumor cells (E/T = 1 : 100). There are two types of T cells : one is CTL expressing L3T4$^-$ and the other, helper T cell (Th) and Tdh expressing L3T 4$^+$, Lyt-1$^+$2$^-$. In a Winn assay was conducted in the same manner using these cells, which are termed effector cells. The results are shown in Table 2.

Even after CTL were eliminated from whole spleen cells, tumor neutralizing activity was not

Table 1. Tumor neutralization test by spleen cells (Winn assay)

Effector cells	Whole spleen cells (E/T=100)	
Recipient mice	C3H (normal)	
	tumor weight (mg)	TIR (%)
Controls	79 ± 35 —*	70.7
D-mice	22 ± 1 —*	99.3
Tumor cells only[a)]	270 ± 121	–

[a)] : Tumor cells were inoculated without splenocytes
Significance of difference (t-test) : *P<0.05
Mean ± SE TIR (tumor-growth inhibition ratio)

Table 2. Tumor neutralization test by Lyt-2$^-$ splenocytes from whole spleen cells

Effector cells	Lyt-2$^-$ splenocytes (E/T=15)	
Recipient mice	C3H (normal)	
	tumor weight (mg)	TIR (%)
Control	85 ± 27 —*	67.3
D-mice	0 ± 0 —**	100.0
Tumor cells only[a)]	260 ± 63	–

[a)] : Tumor cells were inoculated without splenocytes
Significance of differences (t-test) : *P<0.05, **P<0.01
Mean ± SE TIR (tumor-growth inhibition ratio)

impaired. The results indicate that spleen cells other than CTL play a leading role in tumor cytotoxicity. In this experiment, the fractionation of T cells by nylon wool was not performed, because antigen presenting cells (APC) are required for the recognition of antigen by $L3T4^+$, $Lyt\text{-}2^-$ cells. As described above, there are Th and Tdh in T cells expressing $L3T4^-$, $Lyt\text{-}2^-$ Tdh induced nonspecific cytotoxic activity of macrophages through interleukin-2 (IL-2) production. The result of the Winn assay was obtained when effector and target cells were mixed and administered to the recipient. But in this experimental system, the passibility that tumor involution occured due to the acting host immune system after implantation cannot be rejected. Also the results seen in Table 2 which were obtained when CTL-eliminated spleen cells were implanted as effector cells together with tumor cells in the recipient, we consider that in this experimental system CTL was induced from CTL precursor existing the recipient 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, we prepared a system in which CTL induction had not occured in the recipient body (Table 3).

This is, ICR-nu/nu mouse without matured T cells was used as the recipient, 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 accompanied by spleen cells defective CTL form D mice. These results indicate that a DTH reaction, manifested by the collaborative action of Tdh and Mϕ, was potentiated by an oral administration of D-fraction, and that even when CTL is absent, the involution of a tumor is highly likely to occur. Furthermore, based on the fact that orally administered D-fraction involuted tumors in site far from the intestinal lumen with immunoactivation, we assumed the existence of a transmission system. In other words, we supposed that the local immune system in gut-associated lymphoid tissue (GALT) existing in the whole area from stomach to rectum was activated, and that this signal induces the activation of the central immune system which participates in tumor rejection. We therefore examined whether lymphocytes in GALT are activated by D-fraction by growing response against phyto-hemagglutinin (PHA), the mitogen of T cells, as an index. After mesenteric lymph nodes and Peyer's patch (PP) were collected from mice in there groups (normal, tumor-bearing saline-administered and tumor-bearing D-fraction-administered mice), lymphocytes were isolated and the rate of living cells was counted by staining with trypan blue (Table 4).

After confirmation that there were no differences

Table 3. Tumor neutralization test by spleen cells from ICR nu/nu mouse

Effector cells	$Lyt\text{-}2^-$ splenocytes (E/T=15)	
Recipient mice	ICR-nu/nu	
	tumor weight (mg)	TIR (%)
Control	111 ± 40 **	87.4
D-mice	66 ± 23 **	92.5
Tumor cells only[a)]	878 ± 212	—

[a)] : Tumor cells were inoculated without splenocytes
Significance of differences (t-test) : $^*P<0.05$, $^{**}P<0.01$
Mean ± SE TIR (tumor growth inhibition ratio)

Table 4. Viability of lymphocytes obtained from mesenteric lymphnode (MLN) and Peyer's patch (PP)

Mice	Viability (%)	
	MLN	PP
Normal	79	68
Normal D fraction administered	90	74
Tumor bearing	86	68
Tumor bearing D fraction administered	84	74

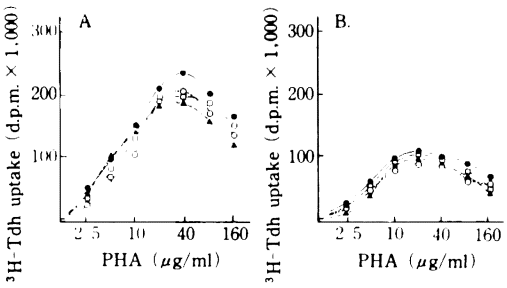

Fig. 2. Proliferative response to PHA by lymphocytes obtained from mesenteric lymphnode and Peyer's patch
A; Mesenteric lymphnode, B; Peyer's patch. normal (○·····○), normal-D administration (●——●) tumor bearing (□–·–·–□), tumor bearing-D administration (▲-----▲)

in the survival rates for MLN and PP, the cells were incubated for 48 h in the presence of PHA at 2.5—160 μg/ml and intra-cellular uptake of ^{3}H-thymidine was examined (Fig. 2). It was observed that not all lymphocytes of MLN and PP were activated by PHA. These result indicate that, at least in this experimental system using PHA, immunoactivation of GALT did not occur by oral administration of D-fraction.

DISCUSSION

We have already reported that when D-fraction extracted and purified from maitake was given orally[2] host immunoactivation also occured and that involution of allogenic tumors was also induced by intraperitoneal administration. Recently, a delayed-type hyper-sensitive reaction (DTH reaction), in which Lyt-2$^-$ Tdh and Mϕ participate has been shown to be important[5~7,10]. We undertook this experiment to study whether this DTH reaction is potentiated by orally administered D-fraction.

As shown in Fig. 2, DTH reaction is that Tdh expressing L3T4$^+$, Lyt-2$^-$ recognized and bonds antigen-specifically with complex of antigen and class II MHC which was expressed on the surface of antigen-presenting cells (APC), producing MAF and other interleukins.

It is well-known that Mϕ acquires an antigen-nonspecific cytotoxic activity by this produced MAF. One method of detecting this reaction is the footpad swelling test. This phenomenon is induced when antigen-specific Tdh existing in peripheral blood is activated by antigen stimulation, and Mϕ is accumulated and infiltrated by lymphokines. However, it cannot be said that this swelling always shows a quantitative DTH reaction, so that the results obtained should be considered qualitative[9]. The results (Fig. 1) showed a DTH reaction in the tumor-bearing D-fraction administered group with swelling of 0.15 mm. In contrast, there was no reaction in the tumor-bearing D-fraction non-administered group or in the normal mouse group. These results suggest that the induction of Tdh, which exists in peripheral blood and acts specifically against tumor antigen, was enhanced by the administration of D-fraction. It is well known that there is a Jones-Mote reaction in footpad swelling reaction, however, this reaction is not manifested in mice[5]. In our study, the swelling in tumor-bearing D-fraction administered mice was thought to derive from the DTH reaction.

Secondly, to examine whether Tdh participates in

tumor involution of the administration of D-fraction, a Winn assay was conducted (Table 1). When whole spleen cells were used, increased neutralizing activity was exhibited in tumor-bearing D-fraction administered mice. Lyt-2 spleen cells were obtained after elimination of CTL from whole spleen cells by treatment with anti-Lyt-2 monoclonal antibody and complement. As shown in Table 2, nodecrease of the activity was observe when these cells were used. This suggests that Lyt-2$^-$ cells have tumor neutralization. However, since effector and target cells were implanted simultaneously in normal mouse, host immune response in the recipient should be considered. That is, when Th, existing in spleen as Lyt-2$^-$ cells, is implanted together with tumor cells as antigen, the possibility that CTL is newly induced from CTL precursors in the recipient by Th produced IL-2 and antigen stimulation may arise. We then conducted an experiment with nude mice having no matured T cells. Again, tumor neutralizing activity in tumor-bearing D-fraction administered mice was detected (Table 3). This result indicated that DTH reaction induced by the collaborative action of Tdh and macrophages possibly plays a leading role in tumor rejection. We consider that tumor rejection systems other than the DTH reaction are natural killer cells (NK) and lymphokine activated killer cells (LAK). However, the possibility of the participation of NK is small because MM-46 tumor cells are resistant to NK[5]. Therefore, the possibility that Lyt-2$^-$ Th produced IL-2, and LAK induced by this production, impair tumor cells cannot be ruled out. However, when the result of footpad test is considered, it is reasonable to conclude that DTH reaction with Lyt-2$^-$ Tdh plays a leading role in neutralizing activity. The influence of D-fraction against GALT was examined with reference to the general immunoactivation pathway after oral administration of D-fraction. The main function of GALT is to respond to extraneous antigen when ingested orally[11], and it is known to exist independently of the general immune system through blood circulation. In this GALT, PP is presumed that M cells capture the antigen which has invaded the intestinal lumen, lymphocytes which are sensitized by this uptake in PP enter into MLN while differentiating and maturing, circulate from the intestinal lumen over the whole body, returning to the intestinal mucosa selectively[12].

We studied the growth response reaction to PHA, T cell mitogens, as an index whether lymphocytes of MLN and PP are activated by the administration of D-fraction. However, under these experimental conditions, growth increase was not observed (Fig. 2).

From these results, we used to set up further experiment.

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マイタケ子実体抽出物の経口投与による抗腫瘍機作

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担子菌から抽出した多糖タンパク複合体であるシゾフィランやレンチナンは腹腔内投与法によって抗腫瘍性を示すが，これらは経口投与ではこのような作用を発現しないことが知られている。筆者らはマイタケ子実体より熱水抽出したD-画分（多糖：タンパク＝7：3）を経口投与したとき，細胞傷害性T細胞の活性や，マクロファージのインターロイキン-1産生能が増強されることにより腫瘍の増殖抑制を示すことを報告した。近年，遅延型過敏症反応(DTH 反応)が腫瘍の排除作用を示すことがいわれている。そこで，本実験では，D-画分を経口投与し，このDTH 反応が増強されるか否かについて検討した。その結果，footpad 試験より腫瘍抗原に特異的に働く Tdh の誘導が増大されたり，spleen 細胞中から anti-Lyt-2 モノクローナール抗体の処理により CTL を除去した Lyt-2-spleen 細胞による腫瘍中和反応 (Winn assay) が増強されることが認められた。以上の結果は Lyt-2-の Tdh による DTH 反応により腫瘍増殖抑制がおこっていることを推定させるものである。

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