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Minoru Moriyama*

*Okayama University,

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Minoru Moriyama

Abstract

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KEYWORDS: lentinan, lipopolysaccharide, migration inhibition activity, immunochemotherapy

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ANTITUMOR EFFECT OF POLYSACCHARIDE LENTINAN ON MH-134 ASCITES HEPATOMA IN C₃H/HE MICE

Minoru MORIYAMA

Department of Surgery, Okayama University Medical School, Okayama 700, Japan

(Director: Prof. K. Orita)

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Abstract. Lentinan inhibited the proliferation of MH-134 ascites hepatoma transplanted subcutaneously. The best result occurred when 1 mg-2 mg/kg of lentinan was administered for 10 consecutive days from the eighth day after tumor transplantation. Tumor proliferation was 33 % inhibited as measured by the average tumor diameter. The average survival (days) when chemotherapy with mitomycin-C (MMC), 5-FU and Ara-C in combination with lentinan, was administered concurrently in the second week of the tumor transplantation was 29.2 days as compared to 20.5 days in the untreated group, 25.1 days in the group given lentinan alone, and 22.0 days in the group receiving chemotherapy alone. When lentinan was administered in combination with bacterial lipopolysaccharide (LPS), the group given lentinan for 5 consecutive days from the third day after tumor transplantation and 30 μ g LPS i.p. on the thirteenth day, had 70 % inhibition of tumor as measured by the average tumor weight. The antitumor activity of lentinan was studied by following changes in macrophage migration inhibition activity (MI). In the untreated group, MI activity disappeared on the 14th day after tumor transplantation. In the group treated with lentinan, spleen cells had positive activity suggesting a restorative action of lentinan on the immune suppression accompanying tumor growth.

Key words : lentinan, lipopolysaccharide, migration inhibition activity, immuno-chemotherapy.

Lentinan, an extract from the edible mushroom *Lentinus edodes*, is a glucan which has a molecular weight of approximately 400,000 and which possesses a β (1 \rightarrow 3) bond. It has a marked antitumor effect against sarcoma 180 transplanted to SWM/MS mice (1). This is thought to result from restoring the lowered immune capacity of the tumor-bearing host to normal (2). In normal mice, lentinan does not promote the series of immune responses represented by the delayed type of skin reaction and the skin transplantation reaction (3). In contrast to anti-cancer drugs the toxicity of which is determined by the amount administered, the optimal dose of lentinan is related to cell-mediated immunity *in vivo* (4) resulting in a decrease in activity if the drug is given in amount more or less than the optimal dose. Moreover, it is known that the effectiveness of lentinan varies with the strain of mouse and kind of tumor used (5).

Therefore, in the present study, the optimal dose and the timing of administration of lentinan, and the effects of lentinan combined with chemotherapy,

and with LPS (an immuno-modulator), were studied. The effect of lentinan on changes in the MI activity of axillary lymph nodes and spleen cells of cancer-bearing mice was investigated.

MATERIALS AND METHODS

Animals. C₃H/He male mice were purchased from Experimental Animal Dealer in Shi-zuoka. The mice were 6 weeks old and weighed approximately 20 g.

Tumor. MH-134 ascites hepatoma derived from C₃H mice was maintained in the peritoneal cavity of mice of the same strain from generation to generation. At the time of use, live cells unstained with trypan blue were counted and 5×10^5 or 1×10^6 cells were transplanted subcutaneously in the backs of mice of the same strain.

Drugs. Polysaccharide lentinan was supplied by Ajinomoto Co., Inc., Tokyo. Mitomycin C and 5-Fluorouracil were purchased from Kyowa Hakko Co., (Tokyo) and Cytosine arabinoside from Nippon Shinyaku Co., (Kyoto). LPS was a product of Difco, Detroit, Michigan, of *E. coli* 0111: B₄ was diluted to the concentration of 500 μ g/ml with PBS and kept at -20°C until used. All drugs were administered i. p.

Administration of lentinan alone. After transplanting 1×10^6 cells, average tumor diameter were measured against lapse of time for the lentinan treated and untreated groups. The tumor growth inhibition rate was calculated by taking the size of the untreated group as 100%. Taking the day of transplantation as Day 0, i. p. lentinan was administered daily from Day 1 to Day 10 in doses of 0.5 mg/kg, 1 mg/kg, 2 mg/kg and 5 mg/kg. Next, the dose was fixed at 1 mg/kg and the results for a pre-administration group of Day -10 to Day -1 , the group of Day 1 to Day 10, and the group of Day 8 to Day 17 were compared.

Combination of lentinan with chemotherapy. The chemotherapy consisted of MFC multiple drug therapy using the three drugs MMC, 5-FU and Ara-C. A preliminary experiment to determine the sublethal dose in mice was performed taking the dose given to a human adult as the standard dose. The results indicated that 2.5 times the dose of each drug was needed, therefore, MMC 0.2 mg/kg, 5-FU 25 mg/kg and Ara-C 2 mg/kg were each given i. p. on Day 3 and Day 6 or on Day 9 and Day 12. The dose of lentinan was fixed at 2 mg/kg/day and given i. p. from Day 7 to Day 14, a total of 8 times. In the experiment where lentinan was combined with MFC, administration of both drugs on the same day was avoided. Lentinan was given 8 times during the period Day 6 to Day 15. The antitumor effect was evaluated from the length of survival (in days).

Combination of lentinan and LPS. The sublethal dose for LPS was 30 μ g/mouse (Table 3). This dose was administered once i. p. either on Day 8 or on Day 13. The dose of lentinan was fixed at 2 mg/kg/day. Mice receiving lentinan alone were given it from Day 8 to Day 15. In the group receiving LPS and lentinan, lentinan was given daily i. p. injections from Day 3 to Day 7 or from Day 9 to Day 15, to avoid giving the two drugs on the same day. The central portion of solid type tumors in mouse develops hemorrhagic necrosis if LPS is administered, therefore, evaluation of the antitumor effect was made by comparing tumor weights on the 21st day after transplantation.

Macrophage migration inhibition test (MI). Tumor antigens were prepared by disrupting MH-134 cells with ultrasonication, and centrifugation at 30,000g for 30 min. The protein content of the supernatant thus obtained was determined according to the method of Folin-Lowry, then adjusted to 100 μ g/ml concentration before use. Peritoneal exudate cells (PEC) were prepared by injecting 20 ml liquid paraffin into the guinea-pig peritoneal cavity. Lapa-

rotomy was performed one week later, the peritoneal cavity washed, and PEC were collected. A suspension 1×10^7 cells/ml was made in TC-199 solution. Lymphocytes were collected from axillary lymph nodes and the spleen. The spleen cells were used after destroying the red cells. The MI test were based on the direct capillary tube method used routinely in our clinic for human cancer. 2×10^6 test lymphocytes and 8×10^6 PEC were mixed, placed in 8 separate capillary tubes, and centrifuged at 80g for 3 min. The capillary tubes were then cut at right angles to the boundary line between the supernatant and the cell layer and the cell layer portions fixed with silicon-grease in a micro petri-dish. For the test group, 1 ml of medium containing soluble antigen and 20 % FCS was added. For the control group, 1 ml of medium without antigen was added. Each was covered with a cover-glass and incubated at 37°C for 24 h in a CO₂ chamber. The macrophage migration from the tip of each test tube was projected on a viewer, its area measured, and the MI calculated by the following formula:

$$\text{MI} (\%) = \frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}} \times 100$$

From a preliminary experiment using non-sensitized lymphocytes, under 85 % was taken as indicating positive activity.

RESULTS

The optimal dose of lentinan was calculated from the growth curves after doses of 0.5, 1.0, 2.0 and 5.0 mg/kg (Fig. 1). In the 1 mg- and 2 mg-group, tumor growth was clearly inhibited.

The average tumor diameter on Day 21 (Table 1) showed an inhibition rate of 25-26 % for the 1 mg- and 2 mg-group. This was significantly different ($P < 0.05$) from those of the other 3 groups (including the control). Therefore, 1 mg- 2 mg/kg/day of lentinan was taken as the optimal dose. The antitumor effects were compared to the timing of administration. The best result was obtained in the group given lentinan from Day 8 to Day 17 (Fig. 2). The tumor inhibition rate was 33 %, a significant difference ($P < 0.05$) from the control group and the preadministration group (Table 2).

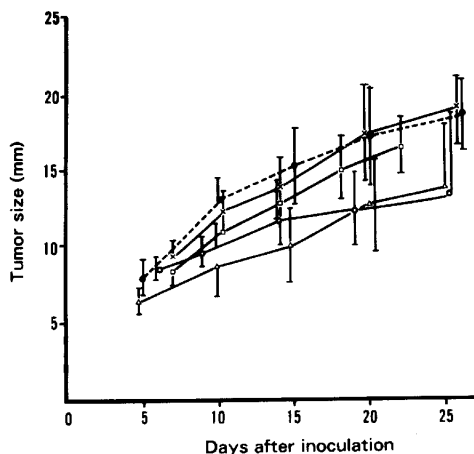


Fig. 1. Antitumor effect of lentinan on MH-134 hepatoma 1×10^6 cells of MH-134 hepatoma were transplanted subcutaneously on Day 0 in C₃H/He mice. Lentinan in doses of 0.5, 1.0, 2.0 and 5.0 mg/kg/day was administered in each experimental group during the period of Day 1 to Day 10. Each group consisted of 10 to 12 mice.

- : control
- ×-----×: 0.5 mg/kg
- △-----△: 1.0 mg/kg
- : 2.0 mg/kg
- : 5.0 mg/kg

TABLE 1. ANTITUMOR ACTIVITY OF LENTINAN ON MH-134 TRANSPLANTED IN C₃H/HE MICE

Exp. group	Dose (mg/kg×days)	Days of lentinan injection	Average ^a tumor size (mm)	Tumor ^b inhibition ratio (%)
①	0.5 × 10	+1 ~ +10	15.8 ± 3.64 ^c	3
②	1 × 10	+1 ~ +10	12.2 ± 3.38	25 ^d
③	2 × 10	+1 ~ +10	12.0 ± 3.98	26 ^e
④	5 × 10	+1 ~ +10	15.7 ± 2.97	4
Control			16.3 ± 4.15	—

a. Average tumor diameter in each group on Day 21 after tumor transplantation; b. Tumor inhibition rate for each group, taking the tumor diameter of the untreated group as 100%; c. Average tumor size ± SD.; d,e. P < 0.05 v.s. other three groups including control

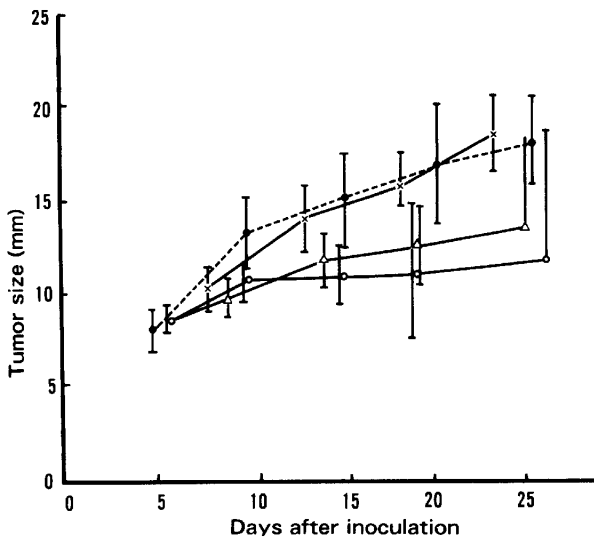


Fig. 2. Antitumor effect of lentinan on MH-134 hepatoma. On Day 0, 1×10^6 cells of MH-134 were transplanted subcutaneously to mice. Lentinan in a dose of 1 mg/kg was administered from Day -10 to Day -1 before the tumor cell inoculation. It was also injected from Day 1 to Day 10, and from Day 8 to Day 17, and the antitumor effects were compared.

●-----● : control
 ×——× : lentinan 1.0 mg/kg Day -10 to Day -1
 △——△ : lentinan 1.0 mg/kg Day 1 to Day 10
 ○——○ : lentinan 1.0 mg/kg Day 8 to Day 17

TABLE 2. ANTITUMOR ACTIVITY OF LENTINAN ON MH-134 TRANSPLANTED IN C₃H/HE MICE

Exp. group	Dose (mg/kg×days)	Days of lentinan injection	Average ^a tumor size (mm)	Tumor inhibition ratio (%)
①	1 × 10	-10 ~ -1	16.4 ± 1.37 ^b	—
②	1 × 10	+1 ~ +10	12.2 ± 3.37	25 ^c
③	1 × 10	+8 ~ +17	10.9 ± 4.27	33 ^d
Control			16.2 ± 2.50	

a. Comparison of the average tumor diameter in each group on Day 21 after tumor transplantation; b. Average tumor size ± S.D.; c,d. P < 0.05 v.s. other two groups including control

The effect of lentinan administered together with MFC therapy (6) was studied by comparing the average survival times of groups given lentinan alone (the sixth group), MFC alone (the second and the fourth groups), and lentinan combined with MFC (the third and fifth groups) (Fig. 3). The fifth group using lentinan and MFC showed the best life-prolongation effect (29.2 days). This was significantly different ($P < 0.05$) from the second group (22.0 days), the fourth group (23.0 days) and the control group (20.5 days). It was also better than the third group which was given MFC prior to the addition of lentinan (25.0 days) and the sixth group which receiving lentinan alone (25.1 days) but the difference was not statistically significant.

Prior to using the combination of lentinan with LPS, the dose of LPS was determined by single intraperitoneal injections of various doses and cell counts and assessing the toxic effect from the survival rates (Table 3). With a dose greater than $100 \mu\text{g}$, the toxic effect was extremely high even in normal mice. Even with a dose of $50 \mu\text{g}$ the toxic effect increased as the tumor burden proceeded from the 8th to the 13th day after transplantation. Therefore, the sub-lethal dose used in the present experiment was a single i.p. dose of $30 \mu\text{g}$. The timing of administration was studied in six experimental groups. Both lentinan and LPS were administered to three groups over various time schedules. Other

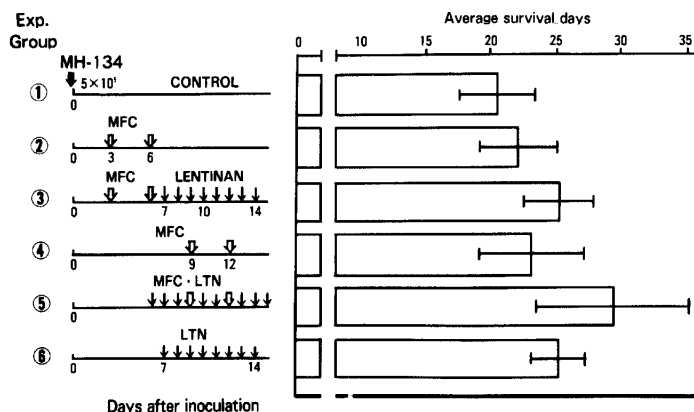


Fig. 3. Additive effect of lentinan and chemotherapeutic agents. After transplanting 5×10^5 cells of MH-134 tumor on Day 0, comparison was made of the average survival times in the group given the combination of lentinan and MFC, the group given lentinan or MFC alone, and the untreated group. MFC was given to the second and the third group on Day 3 and Day 6, and to the fourth and the fifth group on Day 9 and Day 12. As for lentinan, 2 mg/kg was given to the third and the sixth group on Day 7 up to Day 14. In the fifth group, in order to avoid the administration of lentinan on the same day with MFC, lentinan was given 8 times during Days 6 to 15, all i.p.

↓ : MH-134 5×10^5 cells

⇓ : MFC; MMC 0.2 mg/kg + 5-FU 25 mg/kg + Ara-C 2 mg/kg

↓ : lentinan 2 mg/kg/day

TABLE 3. TOXIC EFFECT OF LPS ON NORMAL AND MH-134 BEARING MICE

Days of LPS treatment ^a	No. of animals alive 48 h. after LPS treatment				
	Total in experimental group Dose/mouse of LPS				
	200 μ g	100 μ g	50 μ g	30 μ g	10 μ g
8 days after tumor transplant ^b	0/5	0/7	2/6	19/20	10/10
13 days after tumor transplant	0/5	0/6	3/7	12/12	7/7
Normal mice	0/5	3/5	5/5	6/6	

a. LPS : single administration, i.p. b. MH-134 : 5×10^5 cells transplanted s.c.

groups were given either lentinan or LPS alone. LPS (30 μ g/mouse) given i.p. to a mouse in which a solid tumor had been established caused hemorrhagic necrotic changes in the center of the tumor and temporary shrinkage of tumor diameter within 24-48 h, regardless of whether lentinan was given or not. None of the tumors disappeared completely in any of the experimental groups, and the remnant tumor re-proliferated. Comparison of tumor weight and tumor inhibition rate on Day 21 in each group showed that even when lentinan was administered consecutively for one week beginning the day after LPS administration, the results were no better than those for lentinan given alone (the third and fourth groups in Table 4).

There was no evidence of synergism in the first group in which LPS was administered after the lentinan given in the early period of from Day 3 to Day 7, but in the second group in which LPS was given on Day 13, 5 days after the last lentinan administration, the tumor inhibition rate was 70.9 % and a marked delay in regeneration of the residual tumor occurred. This result for the second group was significantly different ($P < 0.05$) from the fourth group (lentinan alone) and the fifth and sixth groups (LPS alone).

TABLE 4. EFFECT OF COMBINATION USE OF LENTINAN AND LPS

Exp. group	No. of mice	Treatment		Average tumor size (mm)		Average tumor weight (g) ^c	Tumor inhibition ratio (%)
		Lentinan ^a	LPS ^b	Day 7	Day 12		
①	11	Day 3 to Day 7 ^a	Day 8	7.2 \pm 1.7	11.5 \pm 1.8	1.52 \pm 0.42	50.3
②	19	Day 3 to Day 7	Day 13	7.1 \pm 1.3	13.3 \pm 2.3	0.89 \pm 0.52	70.9 ^e
③	10	Day 9 to Day 15	Day 8	7.4 \pm 1.1	10.4 \pm 2.4	1.43 \pm 0.19	53.3
④	10	Day 8 to Day 15	—	7.0 \pm 1.8	13.3 \pm 3.3	1.45 \pm 0.26	52.6
⑤	14	—	Day 8	7.1 \pm 1.5	12.1 \pm 2.3	2.90 \pm 0.69	5.2
⑥	12	—	Day 13	7.0 \pm 1.5	16.0 \pm 2.7	1.62 \pm 0.50	47.1
Control	14	—	—	7.0 \pm 1.2	16.2 \pm 3.2	3.06 \pm 1.05	—

a. lentinan 2 mg/kg/day, i.p.; b. LPS: 30 μ g/mouse, i.p.; c. average tumor weight on Day 21; d. MH-134 5×10^5 cells were transplanted on Day 0; e. $P < 0.05$ v.s. other five groups

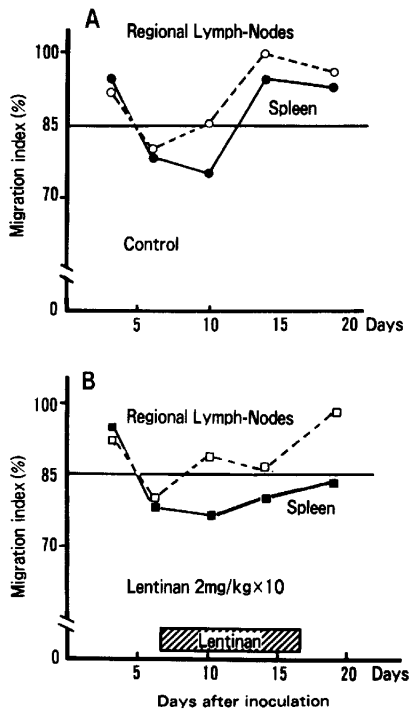


Fig. 4. Effect of lentinan on changes of MI activity in MH-134 tumor bearing mice.

To C_3H/He mice, 5×10^5 tumor cells were inoculated subcutaneously on the back on Day 0. Control group (A), lentinan treated group (B); each group reveals the change of MI activity of regional lymphnodes and spleen cells on Days 3, 6, 10, 14 and 19.

The MI index is taken on the abscissa, and those of less than 85 % are taken as MI positive. Lentinan was given in the dose of 2 mg/kg/day from Day 7 to Day 16, 10 times i.p.

○, ● : control group
 □, ■ : experimental group
 — : spleen
 - - - : regional lymphnodes

Next, the antitumor activity of lentinan was studied *in vitro* by the direct MI test (Fig. 4). When 5×10^5 cells of MH-134 were transplanted on Day 0 in C_3H/He mice, the tumors became palpable by Day 5, and grew rapidly thereafter. The host had died from the tumor by Day 25. In the untreated group, the MI activity of regional lymph nodes reached a maximum of 79 % on Day 6. The activity of spleen cells became positive on Day 6 and reached a peak of 76 % on Day 10. The MI activity of both regional lymph nodes and spleen cells disappeared on Day 14. In the lentinan-treated group, the activity of regional lymph nodes showed changes similar to the untreated group, but the activity of the spleen cells remained positive even after Day 14. This is interpreted as indicating prevention to a certain extent of the suppression of cell-mediated immunity accompanying tumor growth by the lentinan treatment.

DISCUSSION

Favorable results have been obtained recently for cancer treatment as a result of advances in surgical technique, radiation therapy and chemotherapy. However, the healing rate in advanced stages of cancer, recurrences after operation, and metastasis formation still leaves much to be desired. Since the weak-

ened resistance of the patient poses a severe problem at present, cancer immunotherapy in recent decades has attempted to manipulate the immunopreventive mechanism of the cancer-bearing host and to enhance and maintain the cancer extinction function.

Immunotherapy can roughly be divided into two modalities: active immunotherapy where the body is actively sensitized by antigen to elicit *in vivo* resistance, and passive immunotherapy where resistance is received from another body in which immunity is already established.

In addition, there are specific and nonspecific methods. However, at present, there are few methods that give rapid results for human cancer that is extremely complicated or that occurs in the presence of various clinical limitations. Special attention has been given to nonspecific immunotherapy which can be expected to prolong life by suppressing tumor growth and restoring decreased immunity.

Bacterial products such as BCG, BCG-CWS, *Corynebacterium parvum* and OK-432; PS-K, lentinan and LPS composed mainly of polysaccharide; or levamisole that is a low molecular chemical substance, are drugs that are now used in nonspecific active immunotherapy. However, differences in experimental procedure, dose, route of administration and timing of administration have resulted in contradictory results.

The immuno-response may be enhanced or inhibited, as reported by many workers and showing that the action mechanisms of immunopotenciators are little understood.

Lentinan is a polysaccharide having a molecular weight of about 400,000 and is considered to be an almost pure chemical substance (4). Its antitumor effect includes 100 % inhibition of S-180 solid tumor transplanted to ICR-JCL or SWM/MS mice, but only 35 % when the same tumor is transplanted to C₃H/He mice, and no complete tumor disappearance. In regard to other tumors, it is almost ineffective against Ehrlich tumor, 40 % effective against MM 102 tumor, about 65 % effective against CCM adenocarcinoma, and not all effective against 558 adenocarcinoma (5). In the case of MH-134 ascites hepatoma iso-transplanted subcutaneously to C₃H/He mice, the tumor sizes indicated an inhibitory effect of about 30 % (Table 2). These results suggest that the anticancer spectrum of lentinan alone is not wide.

In S-180 cancer-bearing ICR mice, lentinan administered prior to tumor transplantation caused inhibition of about 80 %. This is lower than the 100 % inhibition caused by giving lentinan immediately after transplantation (5). There is also a report that in a similar experiment in which the dose of lentinan was increased in the second week after the tumor had become established, the tumor inhibition rate improved (7). Our results are in almost complete agreement with this (Fig. 2). Levamisole given to mice with MH-134 hepatoma after the tumor has proliferated somewhat and the host immunity has started to decrease has

been reported to have an antitumor effect by preventing the decrease (8-9). Our results support this very interesting finding.

The antitumor mechanism of lentinan has been variously explained as activation of an alternative complement pathway (10), activation of macrophages (11), tumor destruction by cytotoxic T cells (12), or potentiation of antibody dependent cell-mediated cytotoxicity (ADCC) via antibody production from the activation of helper T cells (13). The changes in MI activity suggest that the suppression of cell-mediated immunity is prevented by lentinan administration (Fig. 4).

This agrees with reports that lentinan restores the delayed type of hypersensitivity (DTH) (14) and promotes the recovery of T cell inhibition in Ehrlich cancer-bearing mice (15). Drugs that restore the decreased immunological function of cancer-bearing mice to normal levels have the protein-polysaccharide PS-K (16), but it is unclear whether the mechanisms involved in the action of PS-K and lentinan are the same or different. Levamisole is also known to have similar properties from a study of cell-mediated immunity in patients with cancer of the gastrointestinal tract (17).

However, since the clinical management of cancer at present does not include the use of nonspecific immunopotentiating agents as first choice except in a very few cases, treatment has to rely on a combination of surgery, radiation, and chemotherapy. Hence, in addition to studying the mechanisms involved in immunotherapy, combination therapy and the most advantageous use of the various chemotherapeutic agents urgently needs to be rationalized.

Therapy using a combination of various immunoadjuvants and chemotherapy has already been reported for BCNU with BCG in leukemia (18), and C. Parvum with cyclophosphamide (CY) in mouse fibrosarcoma (19). Recently, however, the importance of the timing of administration of these various drugs in immuno- and chemotherapy has been pointed out.

For example, in the combination of PS-K and CY to KMT-17 tumorbearing rats, marked life-prolongation occurred when PS-K was given on Day 1 and CY on Day 3 (20). The combination of lentinan with CY to S-180 solid tumor was most effective when both drugs were administered simultaneously in the second week of transplantation. With 3-methylcholanthrene-induced mouse autochthonous tumor, however, CY followed by lentinan 2-3 weeks later gave better results (21). The combination of lentinan and MFC given together during the second week after tumor transplantation gave significantly better life-prolongation than the group treated with MFC alone (Fig. 3). However, the results for this combination were not significantly different from those for the group treated with lentinan alone. This seemed to be due to the fact that lentinan prevented, to a certain extent, the decrease in the host immunity caused by MFC therapy, but it is also possible that the dose of MFC was excessive.

The better results obtained when lentinan and MFC were given together than when MFC was given separately remains unclarified, and it is necessary to

study more precisely changes in the immunity of the host at each stage of administration.

As the combination of chemotherapeutic agents with immunoadjuvant is in generally use, better antitumor effects ought to derive from the combined use of immunopotentiating agents which modulate *in vivo* immunity by different mechanisms.

LPS has been considered an interesting modulator of immune response for a long time. It's combination with lentinan and improved timing of administration has led to marked antitumor effects (Table 4); namely, lentinan given consecutively from Day 3 to Day 7 after tumor transplantation, a 5-day respite, when LPS on Day 13 resulted in a marked inhibitory effect as high as 70 %. Various mechanisms have been suggested for the antitumor action of LPS, such as tumor necrosis factor (TNF) liberated into blood leading the tumor to hemorrhagic necrosis (22), LPS enhancing the function of tumor specific T cells (23), or LPS, as a B cell mitogen, potentiating ADCC to activate macrophages that can serve as an effector cell (24).

The marked effects attained by the optimal timing of the use of lentinan and LPS may have been brought about by lentinan restoration of helper T cell activity that had been suppressed by the cancer burden, and potentiation of the ADCC route by LPS. Looking at this in another way, increase of several kinds of serum protein 4 to 7 days after lentinan administration was closely related to the appearance of antitumor activity (25), and hence to the timing of combination use of LPS.

Before immunochemotherapy can be used clinically for the treatment of cancer, more basic data in the relationship between timing and the effect of drugs on the immunity of the cancer-bearing host are needed.

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