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Naoto Moriya* Hiroaki Niwa†

Kunzo Orita‡

*Okayama University,

†Okayama University,

‡Okayama University,

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Abstract

Using C3H/He mice, the antitumor effect of lipopolysaccharide (LPS) alone and in combination with Lentinan extracted from Lentinus edodes was studied. The influence of LPS on cellular immunity and the antitumor effect of the tumor necrosis factor (TNF) were also examined. LPS, which was administered into mice with tumor, induced hemorrhagic necrosis of the tumor within 48 h, demonstrating a high antitumor effect. When LPS was used in combination with Lentinan, the tumor growth was significantly inhibited as compared to that in the control mice. The combination of LPS and Lentinan prevented a decrease in the delayed type hypersensitivity in tumor bearing mice. Application of rabbit serum containing TNF resulted in hemorrhagic necrosis of the tumor within 48 h, as with LPS.

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ANTITUMOR EFFECT OF BACTERIAL LIPOPOLYSACCHARIDE (LPS) ALONE AND IN COMBINATION WITH LENTINAN ON MH-134 TUMORS IN C3H/He MICE

Naoto MORIYA, Hiroaki MIWA and Kunzo ORITA

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

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Abstract. Using C3H/He mice, the antitumor effect of lipopolysaccharide (LPS) alone and in combination with Lentinan extracted from *Lentinus edodes* was studied. The influence of LPS on cellular immunity and the antitumor effect of the tumor necrosis factor (TNF) were also examined. LPS, which was administered into mice with tumor, induced hemorrhagic necrosis of the tumor within 48 h, demonstrating a high antitumor effect. When LPS was used in combination with Lentinan, the tumor growth was significantly inhibited as compared to that in the control mice. The combination of LPS and Lentinan prevented a decrease in the delayed type hypersensitivity in tumor bearing mice. Application of rabbit serum containing TNF resulted in hemorrhagic necrosis of the tumor within 48 h, as with LPS.

Key words : antitumor effect, lipopolysaccharide, Lentinan, tumor necrosis factor.

There have been numerous reports on the antitumor effect of bacterial lipopolysaccharide (LPS), but because of its severe side effects, this compound has not been used clinically. The antitumor mechanism of LPS is not fully understood, though there have been reports that macrophages activated by LPS are cytotoxic to the tumor cells and that T lymphocytes are necessary for LPS to exhibit an antitumor effect. Recently the tumor necrosis factor (TNF) was found to be a host mediator of the antitumor action of LPS (1). The purpose of the present study was to investigate the influence of LPS on cellular immunity and the antitumor effect of TNF. In an experimental tumor system we tested the antitumor effect of LPS alone and in combination with Lentinan, which is an antitumor polysaccharide extracted from Lentinus edodes, and also investigated the influence of these drugs on the delayed type hypersensitivity (DTH). Furthermore, we compared the antitumor effect of TNF with that of LPS.

MATERIALS AND METHODS

Experimental tumors. Tumor cells used in this experiment were MH-134 tumor cells and Meth A sarcoma cells, which were maintained in the peritoneal cavity of C3H/He and BALB/c mice, respectively. Viable tumor cells (5×10^5), counted by the trypan blue dye exclusion method, were transplanted subcutaneously into the backs of 6 to 8 week-old C3H/He and BALB/c mice.

Drugs. E. coli 0111, Be (Difco), LPS was diluted to a concentration of 500 μg/ml with phosphate buffered saline (PBS) and stored at −20 ℃ until use. Lentinan was supplied by Ajinomoto Co., Inc., and BCG was purchased from Nippon BCG K.K..

Preparation. of TNF. Viable BCG, 3×10^8 viable organisms, were injected intravenously into New Zealand White rabbits weighing 2.0 − 2.5 kg. Two weeks after the injection, 100 μg of LPS was injected intravenously, and two hours later blood was collected. The serum was inactivated at 56 ℃ for 30 min and then stored in ampuls at −20 ℃ until use.

Administration of LPS alone. LPS, 30 μg/mouse, which is the optimal dose (2), was administered intraperitoneally into mice on the day of transplantation (Group I), and on the 8th day, the 13th day and on both the 8th and 13th days (Groups II, III and IV, respectively). The diameters of the tumors were measured on the 7th, 12th and 21st days, and the weights of the tumors were measured on the 21st day.

Administration of LPS in combination with Lentinan. Doses were fixed at 30 μg/mouse for LPS and 2 mg/kg for Lentinan, which are the optimal doses (2). Lentinan alone was administered from the 1st to the 8th day after the transplantation of MH 134 hepatoma cells in Group A, LPS only on the 12th day in group B, and in the combination treatment (Group C), Lentinan was administered from the 1st to the 8th day and LPS on the 12th day. The diameters of the tumors were measured until the 21st day, and the weights of the tumors were measured on the 21st day.

Administration of TNF. On the 7th day after tumor transplantation, 0.7 ml/mouse of rabbit serum containing TNF was administered to the tumor-bearing mice through the caudal vein. The diameters and weights of the tumors were measured at the same times as in the LPS experiments.

Assay of delayed type hypersensitivity (DTH). DTH was assayed using the picryl chloride method decribed by Asherson *et al.* (3) (Table 1).

TABLE 1. SCHEDULE OF THE DELAYED TYPE HYPERSENSITIVITY ASSAY

	Days after tumor transplantation				
	Healthy control	Tumor[a] control	LPS	Lentinan	Lentinan[b,c] + LPS
Sensitization with 6.0 % picryl chloride in alcohol	0, 9, 18	0, 9, 18	0, 9, 18	0, 9, 18	0, 9, 18
Challenge with 1.0 % picryl chloride in olive oil	7, 15, 25	7, 15, 25	7, 15, 25	7, 15, 25	7, 15, 25
Measurement of the increase in ear thickness	8, 16, 26	8, 16, 26	8, 16, 26	8, 16, 26	8, 16, 26

a : MH134 hepatoma cells 5×10^5 were transplanted s.c.

b : Lentinan, 2.0 mg/kg/day, was injected i.p. on day 12

c : LPS, 30 μg/mouse, was injected i.p. from day 1 to day 8

RESULTS

Effects of LPS alone on MH-134 tumors. Hemorrhagic necrosis of tumors were found within 48 h in mice in Groups II, III and IV. A temporary reduction in

the diameter of the tumors was noted, but a complete regression was observed in only about 10 % of the mice. Tumors in mice treated with LPS were significantly smaller than those in control mice ($p<0.05$). The difference in the weight of tumors between mice in Group IV (1.33 ± 0.19 g) and control mice (2.74 ± 0.42 g) was significant ($p<0.01$). No significant difference was noted between the mice in Groups II, III and IV and control mice (Table 2).

TABLE 2. ANTITUMOR EFFECT OF LPS ON MH134 TUMOR BEARING C3H/He MICE[a]

Group	No. of mice	LPS[b]	Tumor size (mm)			Tumor weight (g)
			Day 7	Day 12	Day 21	
I	8	Day 0	6.8± 0.31	11.2± 0.56	19.9± 0.73	2.17± 0.24
II	28	Day 8	8.0± 0.28	10.2± 0.45***	17.5± 0.84*	1.75± 0.19*
III	14	Day 13	8.0± 0.56	11.2± 1.20	15.8± 1.76*	1.50± 0.26*
IV	15	Day 8,13	8.2± 0.36	11.5± 0.53	16.3± 0.90*	1.33± 0.19**
Control	20	——	8.4± 0.34	12.9± 0.60	20.3± 1.12	2.74± 0.42

a : MH134 hepatoma 5×10^5 cells were inoculated on day 0 (Mean ± S.E.)
b : LPS, 30 μg/mouse, was injected i.p.
*** : $p<0.005$ v.s. control ** : $p<0.01$ v.s. control, * : $p<0.05$ v.s. control

Effect of LPS in combination with Lentinan. In the mice treated with the combination of LPS and Lentinan, the average tumor weight was 1.07 ± 0.29 g, which was significantly lower than that of the control mice (2.96 ± 0.54 g) ($p<0.01$) (Table 3).

TABLE 3. ANTITUMOR EFFECT OF THE LPS AND LENTINAN COMBINATION ON MH134 TUMOR BEARING C3H/He MICE

Group	No. of mice	Treatment		Tumor size (mm)		Tumor weight (g)
		Lentinan	LPS	Day 7	Day 21	
A	7	Day 1-8		8.1± 0.34	19.0± 1.13	2.00± 0.23
B	5		Day 12	9.5± 0.62	18.3± 2.01	1.36± 0.24
C	9	Day 1-8	Day 12	8.1± 0.29	16.6± 1.46	1.07± 0.29**
Control	8			8.8± 0.30	21.4± 1.82	2.96± 0.54

LPS, 30 μg/mouse. was injected i.p. (Mean ± S.E.)
Lentinan, 2.0 mg/kg/day, was injected i.p.
** : $p<0.01$ v.s. control,

Antitumor effect of TNF. Four of six C3H/He mice showed hemorrhagic necrosis of tumors. The average diameter of the tumors on the 21st day was 21.5 ± 0.61 mm which was significantly less than that of the control mice (25.0 ± 0.85 mm) ($p<0.01$) (Table 4). In the experiment using BALB/c mice, hemor-

TABLE 4. ANTITUMOR EFFECT OF RABBIT TUMOR NECROSIS FACTOR (TNF) AND LPS ON MH134 TUMOR BEARING C3H/He MICE

Treatment group	No. of hemorrhagic necrosis	Tumor size (mm)		Tumor weight (g)
		Day 7	Day 21	
TNF[a]	4/6	9.6 ± 0.27	21.5 ± 0.61**	2.93 ± 0.21
LPS[b]	4/6	10.6 ± 0.89	22.5 ± 0.72*	3.25 ± 0.25
Saline[c]	0/6	9.9 ± 0.06	25.0 ± 0.85	3.38 ± 0.43

(Mean ± S.E.)

a : TNF, 0.7 ml/mouse, was injected i.v. on day 7
b : LPS, 30 μg/mouse, was injected i.v. on day 7
c : Saline, 0.7 ml/mouse, was injected i.v. on day 7
** : $p < 0.01$ v.s. control * : $p < 0.05$ v.s. control

TABLE 5. ANTITUMOR EFFECT OF RABBIT TUMOR NECROSIS FACTOR (TNF) AND LPS ON METH A TUMOR BEARING BALB/c MICE

Treatment group	No. of hemorrhagic necrosis	Tumor size (mm)		Tumor weight (g)
		Day 7	Day 21	
TNF[a]	4/10	8.7 ± 0.31	18.8 ± 0.83	2.26 ± 0.35
LPS[b]	3/10	9.2 ± 0.38	19.7 ± 0.84	2.73 ± 0.40
Saline[c]	0/10	8.9 ± 0.52	20.3 ± 1.16	2.99 ± 0.43

(Mean ± S.E.)

a : TNF, 0.7 ml/mouse, was injected i.v. on day 7
b : LPS, 30 μg/mouse, was injected i.v. on day 7
c : Saline, 0.7 ml/mouse, was injected i.v. on day 7

TABLE 6. EFFECT OF THE COMBINED USE OF LPS AND LENTINAN ON THE INCREASE IN EAR THICKNESS

Group	Day after tumor inoculation (mean ± S.E.)		
	7	15	25
Healthy control	2.22 ± 0.17	3.50 ± 0.20	3.99 ± 0.40a
Tumor control	1.60 ± 0.18	1.75 ± 0.25	1.21 ± 0.30
Lentinan	2.00 ± 0.23	2.69 ± 0.29*	1.32 ± 0.34
Lentinan + LPS	2.04 ± 0.21	3.52 ± 0.24**	1.65 ± 0.32

a : Ear thickness ($\times 10^{-2}$ mm)
* : $p < 0.05$ v.s. Tumor control. ** : $p < 0.01$ v.s. Tumor control

rhagic necrosis was observed in three of ten mice treated with LPS and in four of ten mice treated with TNF. There was a temporary inhibition of tumor growth, but no significant antitumor effect was observed (Table 5).

Effects on delayed type hypersensitivity (DTH). DTH was examined in mice treated with the combination of LPS and Lentinan, which exhibited a marked

antitumor effect. On the 15th day after transplantation of the tumor, ear-thickness of the tumor-bearing control mice decreased to 0.0175 ± 0.0025 mm. On the other hand, that of the mice treated with the combination of LPS and Lentinan increased to 0.0352 ± 0.0024 mm ($p < 0.01$) (Table 6).

DISCUSSION

When LPS was administered to mice with a tumor, hemorrhagic necrosis occurred in the center of the tumor within 48 h, and the growth of the tumor was inhibited. Parr *et al.* found complete tumor regression in 4 of 5 5178 Y lymphoma bearing DBA/2 mice treated with LPS (4). Berendt *et al.* also found complete tumor regression in all SA-1 sarcoma-bearing $AB6F_1$ mice and Meth A sarcoma-bearing $CB6F_1$ mice treated with LPS on the 7th day after transplantation. However, in CaD2 tumor-bearing $AB6F_1$ mice and BP3 sarcoma-bearing $B6D2F_1$ mice, complete regression was not recognized in spite of hemorrhagic tumor necrosis (5). In the present experiments, both C3H/He mice and BALB/c mice showed hemorrhagic tumor necrosis, but complete regression was not seen in all BACB/c mice, and seen in only about 10 % of the C3H/He mice. These results suggest that the antitumor effect of LPS differs not only among strains of mice but also types of tumors.

Though the mechanism of the antitumor effect of LPS has not been fully clarified, the antitumor effect is generally considered to be a host mediated reaction. Some workers have found a cytotoxic action of LPS against tumor cells (6). Carswell *et al.* (1) reported that the tumor necrosis factor (TNF) derived from macrophages activated by LPS led to hemorrhagic necrosis of tumors. TNF was obtained from the sera of BCG sensitized rabbits or mice by administrating LPS. Forty-four of 51 Meth A sarcoma-bearing (BALB/c $\times$ C57BL/6) F_1 mice treated with TNF showed hemorrhagic tumor necrosis. In the present study, TNF, obtained by the same method as that described Carswell *et al.*, induced hemorrhagic tumor necrosis in four of six C3H/He mice and four of ten BALB/c mice. These results suggest that the antitumor effect of TNF is similar to that of LPS.

According to Matthews *et al.*, rabbit TNF is cytotoxic to mouse tumor cells *in vitro*, (7) and serum containing TNF has interferon like activity (8, 9). TNF-generating cells are reported to be mononuclear phagocytes. When LPS was administered to rabbits sensitized by BCG, a large amount of TNF was produced by the BCG-activated mononuclear phagocytes (10). In addition to its antitumor effect, LPS was found to accelerate the production of antibodies by B cells and to have various immunological activities, such as having mitogen-like effects, having an adjuvant effect on T cells and activating macrophages as well as components (11, 12).

Various antitumor mechanisms of Lentinan such as direct killing of tumor cells through complements, and antibody dependent cell mediated cytotoxicity (ADCC) have been suggested (13). In this experiment it was confirmed that the

combination of LPS and Lentinan exhibits a marked antitumor effect in mice. Some reports concerning LPS have already shown that LPS exhibited an antitumor effect in the presence of T cells *in vivo* (5), and that LPS was effective with helper T cells (14). Considering these reports and our result that reduction in cellular immunity was prevented by the combination of Lentinan and LPS, LPS seems to work much more effectively with helper T cells activated by Lentinan in the tumor-bearing mice. It is also thought that TNF production is stimulated in macrophages activated by the administration of Lentinan.

Mizuno *et al.* (15) reported that immunomodulators could be classified into three types according to the serum protein component LB. They also indicated a close relationship between an increase in LB and antitumor activity, pointing out the clinical usefulness of combinations of various immunomodulators. According to Mizuno LPS and Lentinan are different types of immunomodulators, and, the combination of both agents seems to bring a marked antitumor effect.

A comparative study of LPS and TNF in combination with Lentinan is underway. It is thought, however, that it may be more effective to administer TNF to cancer patients than to administer LPS.

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