

Antitumor Activity of *Eubacterium lentum* (TYH - 11) on Various Tumor Cell Lines

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Abstrak

Proliferasi fibrosarkoma Meth-A dan AH60C yang diinokulasi secara intraperitoneal pada mencit, terhambat dengan pemberian *Eubacterium lentum* (TYH-11) intraperitoneal. Proliferasi hepatoma M134 dan leukemia limfoid L1210 juga terhambat pada sebagian mencit dengan pemberian strain bakteri tersebut. Diantara bentuk tumor padat, pemberian strain bakteri ini menghasilkan penyembuhan pada beberapa mencit yang mengandung M134 hepatoma. Waktu hidup mencit yang diinokulasi dengan melanoma B16 secara subkutan memanjang setelah pemberian *Eubacterium lentum*. Pemberian strain ini sebelum inokulasi sel hepatoma M134, leukemia limfoid L1210 dan leukemia P388 menampakkan aktivitas antitumor yang lebih baik daripada pemberian sesudah inokulasi. Strain ini tidak menunjukkan aktivitas antitumor terhadap limfoma EL4 dan karsinoma paru Lewis.

Abstract

The proliferation of Meth-A and AH60C fibrosarcoma inoculated intraperitoneally was suppressed in all mice given *Eubacterium lentum* (TYH-11) intraperitoneally. The proliferation of MH134 hepatoma and L1210 lymphoid leukemia was also suppressed in some of the mice receiving this strain. Among forms of solid tumors, this bacterial strain cured some mice with MH134 hepatoma. The survival times of mice inoculated with B16 melanoma were prolonged by injection of *Eubacterium lentum*. The antitumor activity induced by pre-administration of this strain against MH134 hepatoma, L1210 lymphoid leukemia and P388 leukemia cells was superior to that produced by post-administration. This strain, however, had no antitumor activity against EL4 lymphoma and Lewis lung carcinoma.

Keywords : Antitumor, *Eubacterium lentum*, Tumor Cell lines

In a previous study, we have reported that 11 bacterial strains, which are indigenous in the intestinal tract of humans, guinea pigs and mice, have antitumor activity against Ehrlich ascites tumor.¹ At present, antitumor activities of some bacteria including *Propionibacterium acnes*,² BCG,^{3,4} *Nocardia rubra*,^{5,6,7} *Streptococcus pyogenes* (OK-432),^{8,9,10} *Lactobacillus casei*^{11,12,13} and *Listeria monocytogenes* are being studied by many workers.¹⁴ These bacteria are generally used as biological response modifiers, and have little direct cytotoxicity against tumor cells. Furthermore, these bacteria, except *Lactobacillus casei*, are not normal microorganisms in humans intestinal tract.

The mechanisms of antitumor activity of *Eubacterium lentum* include the improvement of cytotoxic effect of Natural killer (NK) cells, macrophages and cytotoxic T lymphocyte (CTL).^{15,16} We have specially indicated that no other workers had reported the antitumor activity of *Eubacterium lentum*.

In the present study, the antitumor activity of *Eubacterium lentum* (TYH-11), an indigenous bacterium in human intestinal flora, was examined against various experimental tumor cell lines.

METHODS

Animals and tumor cell lines : Six to 8 week-old male mice and 4 to 5 week-old Donryu male rats were obtained from Sankyo Labo. Service Co., Ltd., Japan. They were housed under standard laboratory conditions and given a commercial pallet diet and water *ad libitum*. Tumor cell lines used for experiments were maintained as follows : Meth-A fibrosarcoma in BALB/c mice, MH134 hepatoma in C3H/He mice, L1210 lymphoid leukemia and P388 leukemia in DBA/2 mice and EL4 lymphoma in C57BL/6 mice by successive ascites passages. AH60C was maintained by successive ascites passage in 4 week-old Donryu rats. B16 melanoma and Lewis lung carcinoma were maintained by successive solid-form passage subcutaneously in C57BL/6 mice.

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Bacterial strain : Eubacterium lentum (TYH-11) isolated from a human fecal sample was cultured in GAM broth (Nissui, Japan) under anaerobic conditions. After 24 hours of incubation, it was killed with 0.3% formalin and washed with sterilized saline.

Antitumor activity : Each tumor cell line suspended in RPMI-1640 (Nissui, Japan) supplemented with 10% FCS was inoculated at 10^5 cells/animal intraperitoneally, or 10^6 cells/animal subcutaneously. *Eubacterium lentum* was injected at 10^7 cells/animal intraperitoneally or intravenously for 7 days prior to or after tumor inoculation.

All mice inoculated with ascites form of tumor, received intraperitoneal injection of *Eubacterium lentum* and those inoculated with solid form of tumor, received intravenous injection of *Eubacterium lentum*.

The assessment of survival was made for 42 days for the ascites form, and for 80 to 100 days for the solid form. Tumor weight was determined using the following formula : tumor weight (mg) = {major axis x (minor axis)²}/2.

Statistical analysis : All data were recorded on special forms and were managed with a data base and a statistical software package (Version 6, Epi Info) in ap-

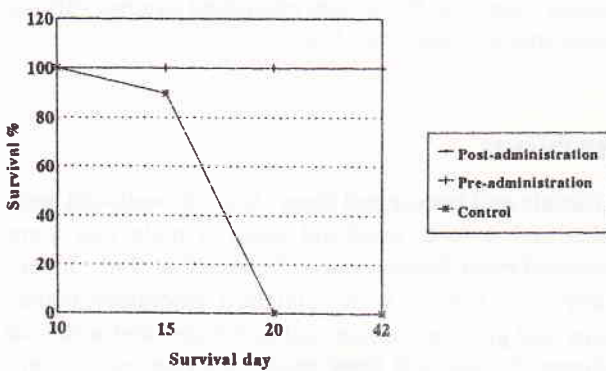
propriate hardware. The comparison of tumor weight and survival time between the treated and control group was determined by applying Student's *t test*. P value of 0.05 was taken as the limit of significance.

RESULTS

Meth-A fibrosarcoma tumor : All mice in the control group (20 animals) died within 20 days after intraperitoneal inoculation of tumor cells (17.2 ± 1.5 days). All 20 mice given *Eubacterium lentum*, both before and after inoculation, did not develop ascites and survived for more than 42 days after inoculation (Figure 1).

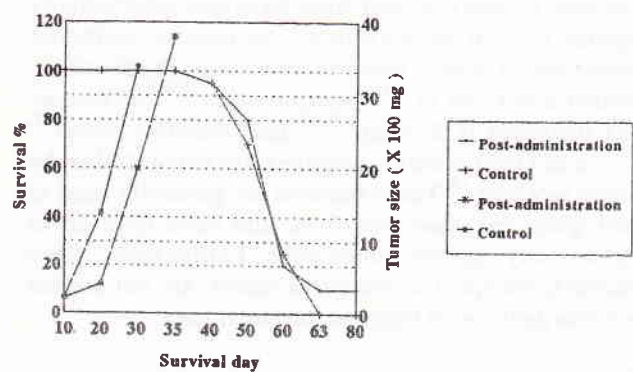
When solid form of tumor was inoculated subcutaneously, all mice in the control group (11 animals) died between day 35 and day 63 (38.7 ± 8.1 days). Only one of 12 mice receiving intravenous *Eubacterium lentum* after tumor inoculation were cured completely, with survival time of more than 80 days. The other 11 treated mice died within same time period as the controls.

Tumor growth in the treated group was also significantly suppressed in comparison to the control group (0.32 ± 0.02 vs 3.83 ± 0.08 gr) measured at day 28 after tumor inoculation ($p < 0.01$) (Figure 2).



a. Mice were inoculated with Meth-A fibrosarcoma cells intraperitoneally
 b. Killed *Eubacterium lentum* given intraperitoneally for 7 days

Figure 1. Effect of *Eubacterium lentum* on Meth-A fibrosarcoma (ascites form) in BALB/C mice



a. Mice were inoculated with Meth-A fibrosarcoma cells subcutaneously
 b. Killed *Eubacterium lentum* given intravenously for 7 days

Figure 2. Effect of *Eubacterium lentum* on Meth-A fibrosarcoma (solid form) in BALB/C mice

MH134 hepatoma : Intraperitoneal inoculation of MH 134 hepatoma lead to the death of all control mice (n : 20) by day 17. No difference of survival time was observed between the post-treated and control group (16.7 ± 2.0 vs 16.2 ± 1.4 days), but 8 of 10 intraperitoneally pre-treated mice survived without tumor growth, and the other 2 mice died on days 28 and 31, respectively (Figure 3).

Among the mice with tumor inoculated subcutaneously, all control mice (13 animals) died between day 22 and day 54 (26.5 ± 9.7 days). In contrast, 2 of 12 intravenously post-treated mice, were cured completely and 2 survived with tumor for more than 80 days after inoculation. The other 8 mice died between days 34 and 60. Tumor weight in post-treated mice showed significant suppression compared to the control group (0.39 ± 0.03 vs 3.08 ± 0.07 gr) at day 21 after tumor inoculation ($p < 0.01$) (Figure 4).

L1210 lymphoid leukemia : All of the control (11 animals) and intraperitoneally post-treated mice (10 animals) died by day 9 and day 10, respectively (8.5 ± 0.6 vs 8.5 ± 0.7 days). On the other hand, 9 of 10 intraperitoneally pre-treated mice died between day 9 and day 11. The remaining one survived for more than 16 days after inoculation and showed no tumor growth (Figure 5).

P388 leukemia : All of the control mice (20 animals) died on day 10 after tumor inoculation (3.9 ± 2.7 days). But all the intraperitoneally pre-treated animals (n:7) died on day 19 (17.7 ± 1.4 days) and the post-treated mice (10 animals) died within 14 (13.6 ± 0.5 days) days after inoculation, but both the treated groups showed significant prolongation of survival time ($p < 0.01$) (Figure 6).

AH60C : Five week-old Donryu rats were inoculated with 5×10^5 cells/animal intraperitoneally, and 5×10^7 cells/animal of *Eubacterium lentum* were injected intraperitoneally before or after inoculation. All of the control mice (10 animals) died between day 11 and day 46 (21.5 ± 12.9 days), but 8 of 10 post-treated and all 10 pre-treated mice showed no tumor growth. Two rats were died in at 46 and 50 after tumor inoculation. (Figure 7).

B16 melanoma : When mice were inoculated intraperitoneally, all of the control mice (10 animals) died between day 15 and day 23 (16.3 ± 2.4 days). Whereas, all of the intraperitoneally pre-treated and post-treated mice (n = 10 each) died between day 22 and 30 (28.6 ± 2.4 days) and between day 18 and 27

(25.3 ± 2.7 days), respectively. These data confirmed the prolongation of survival time in both intraperitoneally treated groups (Figure 8).

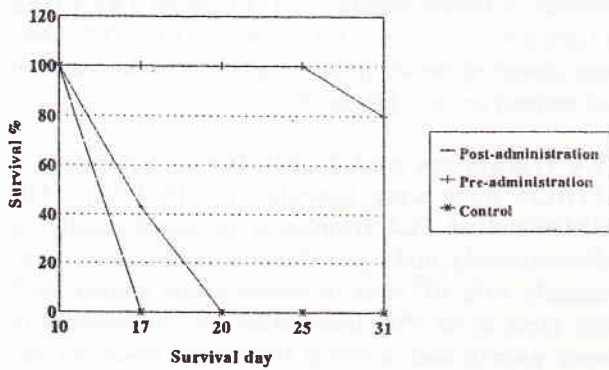
When mice were inoculated subcutaneously, no differences in tumor weight (2.71 ± 0.28 vs 2.85 ± 0.12 gr) and survival time (21.4 ± 3.6 vs 21.6 ± 3.2 days) were observed between the intravenously post-treated and control group. (Figure 9).

EL4 lymphoma and Lewis lung carcinoma : C57BL/6 mice were inoculated with 10^4 to 10^5 cells/animal of EL4 lymphoma intraperitoneally or subcutaneously, and injected intraperitoneally or intravenously with 10^7 cells of *Eubacterium lentum* for 7 days prior to or after inoculation. No differences in tumor growth and survival time were observed between the control and the treated group. Other C57BL/6 mice were inoculated with 5×10^4 to 10^6 cells/animal of Lewis lung carcinoma into the foot pad, and 10^7 cells of *Eubacterium lentum* was administered intravenously for 7 days before or after inoculation. In this experiment, no differences were found between the control and treated groups both in tumor growth and survival time (data not shown).

DISCUSSION

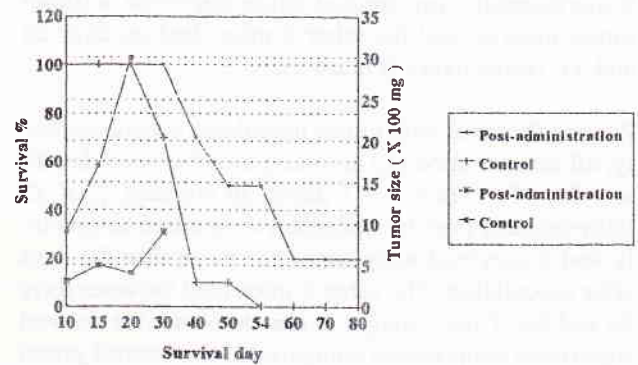
Eubacterium lentum is an anaerobic gram-positive bacterium, short rod form, non-spore-bearing and non-motile. This bacterium possesses an enzyme of the arginine-dehydrolase pathway and its growth is vastly accelerated by the addition of arginine both in vitro and in the intestinal tract.¹⁷ This bacteria can transform the bile salt into their 3 and 7-keto derivatives. These derivatives are transformed to an unsaturated form by *Clostridium paraputrificum*, which has been shown to be present in the feces of bowel cancer patient.¹⁸ Acevedo *et al.*¹⁹ found that *Eubacterium lentum* isolated from rectal tumors has a choriogonadotropin-like immunoreactive substance similar to that produced by "cancer-associated" bacteria. These workers postulated that synthesis of a hormone-like polypeptide by a procaryote, only when associated with eucaryotic cells, suggests the presence of a transferable plasmid. Such a plasmid would have important relationship with the incidence of malignant transformation.

In our previous paper¹, we reported that four species of bacilli, i.e., *Eubacterium lentum*, *Propionibacterium acnes*, *Proteus mirabilis* and *Serratia marcescens*, isolated from the components of normal intestinal microflora, showed antitumor activity against Ehrlich ascites tumor in mice. Among these 4



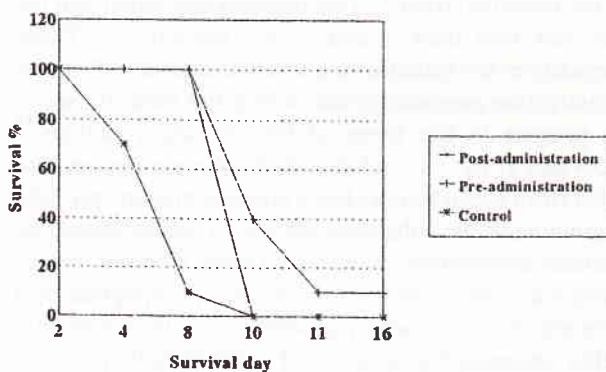
a. Mice were inoculated with MH134 hepatoma cells intraperitoneally
 b. Killed Eubacterium lentum given intraperitoneally for 7 days

Figure 3. Effect of Eubacterium lentum on MH134 hepatoma (ascites form) C3H/He mice.



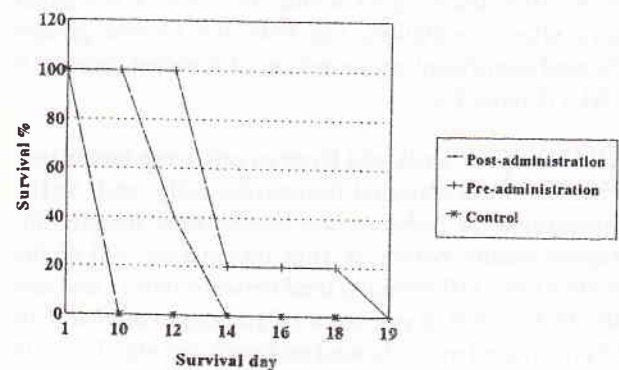
a. Mice were inoculated with MH134 hepatoma cells subcutaneously
 b. Killed Eubacterium lentum given intravenously for 7 days

Figure 4. Effect of Eubacterium lentum on MH134 hepatoma (solid form) in C3H/He mice



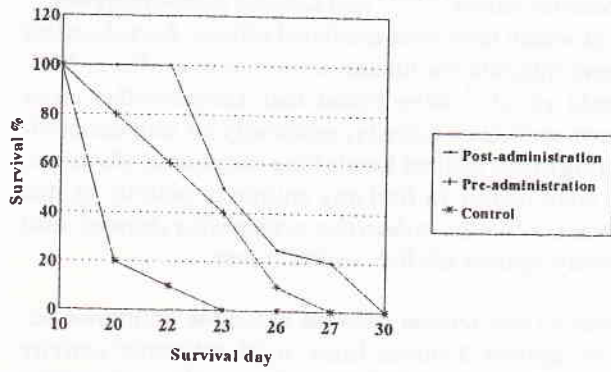
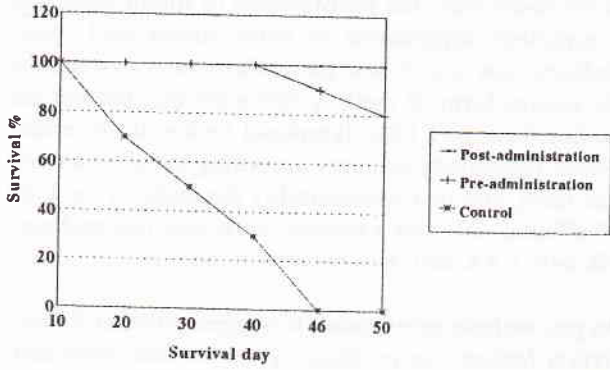
a. Mice were inoculated with L1210 lymphoid leukemia cells intraperitoneally
 b. Killed Eubacterium lentum given intraperitoneally for 7 days

Figure 5. Effect of Eubacterium lentum on L1210 lymphoid leukemia in DBA/2 mice



a. Mice were inoculated with P388 leukemia cells intraperitoneally
 b. Killed Eubacterium lentum given intraperitoneally for 7 days

Figure 6. Effect of Eubacterium lentum on P388 Leukemia in DBA/2 mice

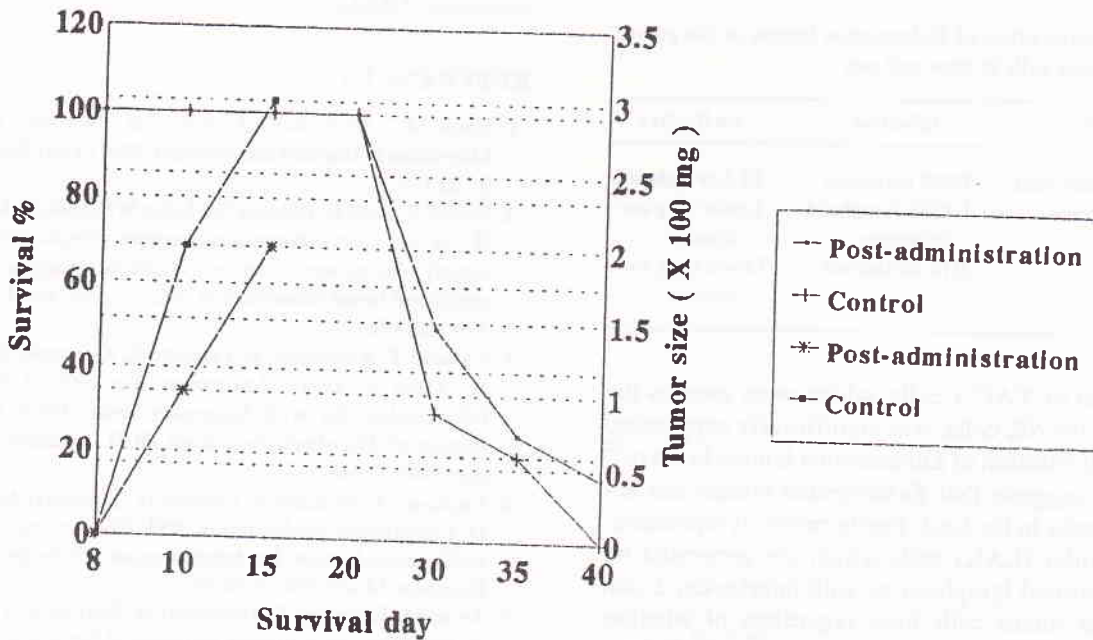


a. Rats were inoculated with AH60C intraperitoneally
 b. Killed *Eubacterium lentum* given intraperitoneally for 7 days

a. Rats were inoculated with B16 melanoma cells intraperitoneally
 b. Killed *Eubacterium lentum* given intraperitoneally for 7 days

Figure 7. Effect of *Eubacterium lentum* on AH60C in Donryu rats

Figure 8. Effect of *Eubacterium lentum* on B16 melanoma (ascites form) in C57BL/6 mice



a. Mice were inoculated with B16 melanoma cells subcutaneously
 b. Killed *Eubacterium lentum* given intravenously for 7 days

Figure 9. Effect of *Eubacterium lentum* on B16 melanoma (solid form) in C57BL/6 mice

species, the antitumor activity of *Eubacterium lentum* had never been reported previously. In the past, many researchers had carried out studies of antitumor activity of bacteria including *Corynebacterium parvum*,² BCG,^{3,4} *Nocardia rubra*,^{5,6,7} OK-432,^{8,9,10} *Lactobacillus casei*^{11,12,13} and *Listeria monocytogenes*,¹⁴ all of which have host-mediated effects. *Lactobacillus casei* inhabits the human intestinal microflora. Matsuzaki *et al.*¹¹ have found that *Lactobacillus casei* shows antitumor activity, especially an immunomodulating effect against Lewis lung carcinoma. However, we were unable to find any antitumor activity of this species, while *Lactobacillus acidophilus* showed mild activity against Ehrlich ascites tumor.

Eubacterium lentum showed noticeable antitumor activity against 3 tumor lines, mild antitumor activity against 3 tumor cell lines and no antitumor activity against EL4 lymphoma and Lewis lung carcinoma (table 1). When *Eubacterium lentum* was injected before inoculation of tumor cells, it exhibited remarkable antitumor activity. It may be due to host-mediated effects such as those shown with *Corynebacterium parvum*, etc. In particular, the growth of leukemia cells such as P388 and L1210, and MH134 hepatoma was suppressed as the result of pre-administration of *Eubacterium lentum*.

Table 1. Inhibitory effect of *Eubacterium lentum* on the growth of tumor cells in mice and rats

Highly effective	Effective	Ineffective
Meth-A fibrosarcoma	P388 leukemia	EL4 lymphoma
MH134 hepatoma	L1210 lymphoid leukemia	Lewis lung carcinoma
AH60C	B16 melanoma	Lewis lung carcinoma

Proliferation of YAC-1 cells, which were used as the target cells for NK cells, was significantly suppressed by the administration of *Eubacterium lentum* *in vivo*.¹⁶ This result suggests that *Eubacterium lentum* can activate NK cells in the host. Furthermore, lymphokine-activated killer (LAK) cells which are generated by culture of normal lymphocytes with interleukin 2, can lyse various tumor cells lines regardless of whether they are NK cell-sensitive or resistant.^{20,21,22} *Eubacterium lentum* showed prologation of survival of tumor-bearing mice and suppression of tumor growth against the solid form of MH 134 hepatoma. This result suggests that this bacterial strain may also activate LAK cells in the host. However, this bacterium did not

show any antitumor activity against EL4 at all. These result may indicate the existence of a relationship between the virulence of a tumor and the capability of effector cells. When the tumor cell line was inoculated intraperitoneally and *Eubacterium lentum* was injected in the same way, the proliferation of tumor cells was completely suppressed in some tumor cell lines. *Eubacterium lentum* was particularly effective against the ascites form of meth-A fibrosarcoma, but not the ascites form of L1210 lymphoid leukemia. In other-words, the activity can vary according to the tumor cell line used, and that susceptibility depends on the kind of effector cell (for example, activated macrophage, NK cell, LAK cell, etc) present in the host.

As yet, we have no evidence to suggest whether *Eubacterium lentum* can produce cytokines and other substances, and what type of effector cells it activates in the host. The mechanisms of antitumor activity of *Eubacterium lentum* remain to be clarified in subsequent studies.

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