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Antitumor Mechanisms of *Eubacterium lentum* and Its Components

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SUMMARY **In the present study, some antitumor mechanisms of *Eubacterium lentum* (TYH-11) and bacterial components having antitumor effects were investigated. *E.lentum* induced maximum NK cell activity in C3H/He mice on day 1 after injection (90.6% against 33.9% of control at E:T ratio 50:1) and the activity was kept at a level of 48.6% on day 7. Tumoricidal peritoneal macrophages were induced 9 days after *E.lentum* injection into BALB/c mice (56.2% against 10.1% control at E:T ratio 10:1). Tumoricidal macrophage activity persisted at the same level for at least 11 days. Cytotoxic T lymphocyte (CTL) activity was induced only in tumor bearing mice treated with *E.lentum*, 4 weeks after tumor inoculation. Antitumor activity was observed in the cell wall (CW) and membrane fractions (CM) of *E.lentum*. CW induced NK cell activity; the activity was transient while the kinetics of NK activity by CM showed 2 peaks, on day 1 and day 7. Tumoricidal macrophages were induced by CW and the activity level was the same as that induced by whole body, while that induced by CM was at a lower level. Neither CW nor CM induced CTL in tumor bearing mice.**

The intestinal microflora have several effects on the host and many studies have been performed on the relationship between tumors and intestinal microflora.[1-3] However, no definite correlations have been identified between them, but it is possible that the cooperation of some species of normal flora may affect the promotion or inhibition of tumors in the host. We have studied the antitumor activity of bacteria in the intestinal microflora of humans and animals, and reported antitumor activity of several bacterial species;[4] our report was the first on the antitumor activity of one of them, *Eubacterium lentum*, which is a well-known anaerobic, gram positive, short rod form bacterium and natural component of human intestinal flora. Most studies on antitumor activity of various bacteria have reported that their actions were mediated by the activation of immunological effector cells and production of cytokines in the host after administration.[5-11]

We reported in a previous paper that *E. lentum* (TYH-11) apparently had adjuvant effects and had a strong antitumor effect on 11 strains of experimental tumor cell lines, but showed no direct cytotoxicity, and suggested that *E. lentum* might be a biological response modifier (BRM).[12] Therefore, in this paper, mechanisms of antitumor activity of *E. lentum* were studied and cellular components of the bacillus having an antitumor effect were investigated.

MATERIALS AND METHODS

Animals and tumor cell lines

Six to 8 week old ICR, C57BL/6, BALB/c, DBA/2, and C3H/He male mice were obtained form Sankyo Lab. Service Co., Ltd. (Tokyo, Japan). Tumor cells lines used for experiments were maintained as follows: Ehrlich ascites tumor cells were maintained in ICR mice, L1210 lymphoid leukemia and P815 mastocytoma cells in DBA/2 mice and EL-4 lymphoma cells in C57BL/6 mice by successive ascites

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passage. YAC-1 lymphoma cells, P815 mastocytoma cells and EL-4 lymphoma cells were also cultured *in vitro* in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (RPMI-CFS) (Flow Laboratories. North Ryde, Australia), 100 U penicillin/ml, 100 μg streptomycin/ml, 0.25 μg amphotericin B/ml, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 2 mM glutamine.

Agents

E.lentum (TYH-11) was cultured in GAM broth (Nissui) under anaerobic conditions for 18 hours at 37°C, killed with 0.3% formalin and washed with sterilized saline. OK-432 was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

Preparation of effector cells

NK cells were separated from spleens of C3H/He mice according to the method of Kumagai *et al.*[13] Single cells of mice given 10^7 cells of formalin killed *E.lentum*, 300 μg cell wall fraction (CW) or 1,000 μg membrane fraction (CM) were suspended in RPMI 1640 culture medium containing 10% of fresh autologous serum and 5-10% of KAC-2 (Silica suspension as monocyte removal agent, Ohtsuka assay, Tokushima, Japan) and incubated for 45 minutes at 37°C. Then, 6 ml of cell suspension was overlaid onto 3 ml of Ficoll Paque (Pharmacia, New Jersey, USA) and centrifuged at 3,000×g for 30 minutes to separate lymphocytes from KAC-2 phagocyting macrophages, and removed the adherent cells by a nylon column.

Macrophages were obtained as follows. Peritoneal exudate cells (PEC) of BALB/c mice administered 3 times with 10^7 cells of formalin killed *E.lentum* were collected by washing the peritoneal cavity with 7 ml Hank's balanced salt solution (HBSS, Nissui). After centrifugation at 3000×g for 7 minutes, PEC were resuspended in RPMI-FCS and were cultured in plastic dish for 2 hours at 37°C in a 5% CO_2 atmosphere. Adherent cells were collected with cell scraper (Greiner, Nurtingen, Germany) and used as macrophages.

Cytotoxic T lymphocytes (CTL)

Mice were injected iv with 10^7 cells of killed *E.lentum* everyday for 7 days. CTL were obtained from spleen of ICR mice inoculated with 10^6 cells of Ehrlich ascites tumor subcutaneously (sc). Besides, C57BL/6 mice (H-2^b) were immunized with 4×10^6 spleen cells of BALB/c mice (H-2^d) intravenously (iv), and injected with 5×10^6 cells of L1210 (H-2^d) intraperitoneally (ip) on day 21. A week later, CTL in spleen of C57BL/6 mice was collected.[14] Plastic adherent cells were removed from single spleen cells and lymphocyte fractions were separated by centrifugation on Ficoll-Paque. Effector cells from 5 mice in all experimental groups were pooled.

Cytotoxicity assay

Cytotoxicity of NK cells was measured using YAC-1 lymphoma cell line as a target. Approximately 10^7 YAC-1 cells in 1 ml RPMI-FCS were incubated with 3.7 MBq of $Na_2{}^{51}CrO_4$ (Daiichi Radioisotope, Tokyo, Japan) for 1 hour at 37°C in 5% CO_2 atmosphere. The cells were washed with RPMI-FCS, and resuspended in 1 ml of RPMI-FCS supplemented with 10% HEPES buffer (Wako Pure Chemical Industries Ltd., Osaka, Japan). One hundred microlitres of effector cell suspensions at different concentrations were placed in round-bottomed wells of microplates (Corning, Iwaki Glass, Tokyo, Japan) in triplicate, followed by addition of 100 μl ^{51}Cr-labeled YAC-1 cells. Plates were incubated for 4 hours at 37°C in a 5% CO_2 atmosphere; maximum ^{51}Cr-release was measured using target cell lysate in HCl. Culture medium was collected by Supernatant Collection System (Skatron. Lier, Norway) to determine ^{51}Cr-release, which was determined by the following formula :

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}}$$

Activated macrophage activity was determined by ^{51}Cr-release from labeled target cells (EL-4 or P815), after effector cells were cultured with target cells for 20-22 hours at 37°C in a 5% CO_2 humidified incubator. CTL activity *in vitro* was measured by the same method as NK activity. Ehrlich ascites tumor cells and L1210 cells were used as specific target cells. In all experiments, spontaneous release was less than 20%. CTL activity *in vivo* against H-2^d was determined by the number of living L1210 cells in the peritoneal cavity of C57BL/6 mice on day 28. Mice were sacrificed under anesthesia and L1210 cells were collected from the peritoneal cavity with 10 ml RPMI-1640. The number of living L1210 cells judged by the trypan blue method was counted by blood cell counter.[14]

Fractionation of *E.lentum* and antitumor activity measurement of fractions.

Fractionation was performed by the method of Azuma *et al.*[15] *E.lentum* was broken up by MINI-LAB (Rannie, Denmark) under 900 bar. By centrifugation at 20,000×g for 1 hour, the crude cell wall fraction and the membrane fraction (Fraction 2, Fr2) were separated. The crude cell wall fraction was resuspended in 0.07 M phosphate buffer pH 7.8 containing 10% trypsin (Wako) and chymotrypsin (Merck, Darmstadt, Germany), and digested at room temperature for 24 hours. After centifugation at 20,000×g for 1 hour, precipitate

was digested again with pronase (Kaken Pharmaceutical Co, Tokyo, Japan), dissolved in 0.01 M tris HCl-buffer pH 7.2 for 24 hours. The sediment was collected as the cell wall fraction (Frl). In this process, 4 subfractions were also obtained ($Frl_{1,2,3,4}$). Each fraction of *E.lentum* was examined for antitumor activity against Ehrlich ascites tumor cells. ICR mice were inoculated sc or ip with 10^6 or 10^5 cells of Ehrlich ascites tumor, respectively. Fractions were injected intratumorally (it), iv or ip everyday for 7 days for ascites and solid form, respectively as indicated in the tables. Tumor weight was calculated by using the following formula : Tumor weight (mg) = [major axis × (minor axis)^2] ÷ 2.

RESULTS

NK activity

C3H/He mice were injected iv with 10^7 cells of *E.lentum* killed by formalin, and the time course of NK activity in the spleen was measured. NK activity in control mice was 33.9% (E:T ratio 50:1) and 24.6% (E:T ratio 25:1). Those in *E.lentum* given were 90.6% and 66.5% on day 1, respectively ($p < 0.01$), and decreased till day 7, but NK activity represented still 48.6% (E:T ratio 50:1), significantly higher than in controls ($p<0.05$) (Fig. 1).

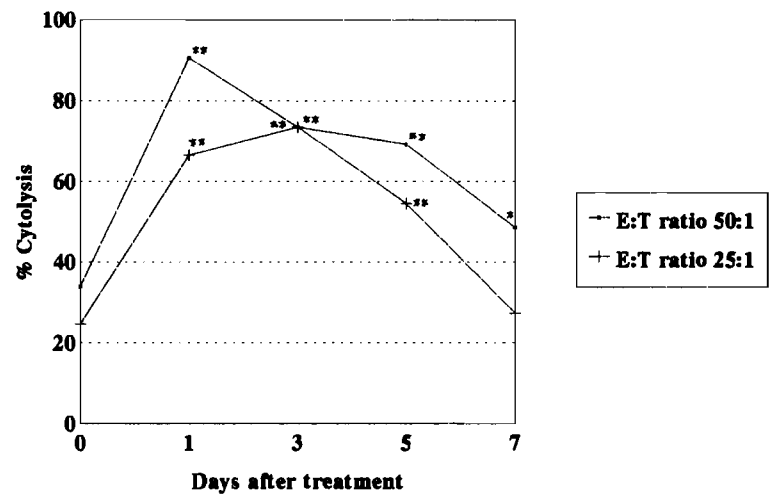

Fig. 1 NK cell activity after iv injection of 10^7 cells killed *E.lentum*. NK activity in spleen cell of C3H/He mice was determined against 2×10^4 cells/well of ^{51}Cr-labeled YAC-1 lymphoma in a 4 hour assay. Each point and bar indicate the mean ±standard error of 3 experiments. *, **: Statistically significant differences from the control group ($p<0.05$ and $p<0.01$, respectively).

Tumoricidal macrophages induced by *E.lentum*

BALB/c mice were ip given 10^7 cells of formalin killed *E.lentum* 3 times every other day for 5 days. As shown in Table 1, tumoricidal macrophages were induced 9 days after the first injection of *E.lentum*. The activity was 56.2% at E:T ratio 10:1 and 55.4% at E:T ratio 5:1 on day 9 as compared to 10.1% and 4.2% in controls ($p<0.01$), respectively. On day 11, similar activity was observed (in Exp 1 and 2) and the same result was also represented by OK-432 (Exp 2).

Table 1. Kinetics of tumoricidal macrophage induced by *E.lentum* in BALB/c mice.[a]

Group	Day after the first treatment	% Cytotoxicity 10 : 1	% Cytotoxicity 5 : 1
Exp 1[b]			
Control		12.3 ±1.1[d]	11.1 ±2.0
E.lentum	1	9.5 ±0.8	9.4 ±1.3
	3	15.8 ±1.6	12.9 ±1.5
	5	14.9 ±2.3	14.4 ±1.8
	7	15.8 ±1.7	11.3 ±2.5
	11	51.3 ±4.8*	48.5 ±3.2*
Exp 2[c]			
Control		10.1 ±1.2	4.2 ±0.9
E.lentum	9	56.2 ±6.8*	55.4 ±0.4*
	11	72.5 ±7.8*	59.3 ±2.5*
OK-432	11	67.5 ±5.3*	46.7 ±2.8*

[a]Mice were injected ip with 10^7 cells of killed *E.lentum* or 2 KE of OK-432 3 times every other day for 5 days.
[b]Cytotoxicity was measured in a 22 hr ^{51}Cr-release assay against 10^4 cells of labeled P815 mastocytoma.
[c]Cytotoxicity was measured against labeled EL4 lymphoma.
[d]Figures indicate the mean ± standard error of 3 experiments.
*$p<0.01$.

tered group was 62.3% in comparison with -3.3% in controls at week 4. At E:T ratio 50:1, the activity was 29.0% in test group and -2.2% in control. CTL activities in the test group were 66.6% and 28.5% at E:T ratio of 100:1 and 50:1, respectively, while those in the control group were -2.4% and -1.1%, respectively, at week 5. As shown in Table 2, on day 28, CTL activity against L1210 was significantly higher in immunized mice given *E.lentum* on day 0, 1, 2 and 21 than in immunized mice without *E.lentum* adminstration ($p < 0.01$).

Antitumor activity of cell fractions

Assessment of each *E.lentum* fraction by weight was approximately 20% in the crude cell wall fraction and 45% in the membrane fraction (Fr2). The cell wall fraction (Fr1), obtained from the crude cell wall fraction, was approximately 12% of *E.lentum* whole body and those of subfractions ($Fr1_{1,2,3,4}$) were 18%, 16%, 1.5% and 1.5%, respectively (data not shown).

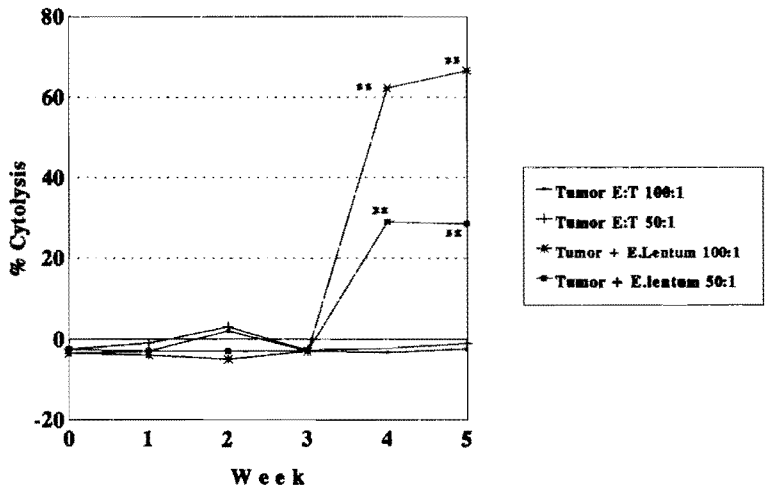

Fig. 2 Effect of *E.lentum* on CTL induction in ICR mice inoculated sc with 10^6 Ehrlich ascites tumor cells. Mice were injected iv with 10^7 cells of killed *E.lentum* for 7 days after tumor inoculation. CTL activity in spleen cells was measured against 2×10^4 cells/well of ^{51}Cr-labeled Ehrlich ascites tumor cells in a 4 hour assay. Each point and bar indicate the mean ± standard error of 3 experiments. **: Statistically significant difference from the normal group at $p < 0.01$.

Table 2. Cytotoxicity against L1210 lymphoid leukemia in C57BL/6 mice.[a]

Group	Injection of *E.lentum*[b]	No. of alive L1210 cells ($\times 10^7$)[c]	% Cytotoxicity[d] 100 : 1	% Cytotoxicity[d] 50 : 1
Control		21.2 ± 1.0	9.6 ± 1.8	7.9 ± 1.5
Immun.	-	8.9 ± 3.2 **	23.8 ± 2.6 **	16.4 ± 1.8 **
Immun.	+	0.3 ± 0.04 **	59.3 ± 5.8 **	41.7 ± 2.8 **

[a] Mice were immunized with 4×10^6 spleen cells of BALB/c mice and injected ip with 5×10^6 cells of L1210 at 3 weeks later.

[b] Mice were injected iv with 10^7 cells of killed *E.lentum* on day 0, 1, 2, and 21.

[c] Number of living cells in peritoneal cavity of C57BL/6 mice on day 28 indicates the mean ± standard error of 15 mice.

[d] Cytotoxic test was performed on day 28 and cytotoxicity was determined by 4-hr ^{51}Cr-release assay against 5×10^3 cells of labeled L1210.

Figures indicate the mean ± standard error of 3 experiments.

*$p < 0.05$, **$p < 0.01$.

Table 3. Effects of components in *E.lentum* on Ehrlich ascites tumor in ICR mice.[a]

Material		MST[b]	No. of survivors/ No. tested	Tumor size on day 21 (g)[d]	Tumor growth T/C (%)
Control		47.7 ±3.2	0/10	5.1 ±0.8	
E.lentum	(800 μg)	ND	1/10	1.1 ±0.3*	21.6
Fr1	(500 μg)	ND	2/10(1)[c]	1.4 ±0.3*	27.5
Fr2	(1,800 μg)	51.7 ±4.5	0/10	2.9 ±0.6*	56.9
Fr1-1	(700 μg)	57.7 ±4.4	0/10	4.0 ±0.5	78.4
Fr1-2	(650 μg)	53.4 ±4.0	0/10	6.5 ±0.9	127.5
Fr1-3	(60 μg)	54.6 ±4.0	0/10	5.5 ±0.7	107.8
Fr1-4	(60 μg)	48.6 ±4.0	0/10	5.0 ±0.8	98.0
Recovery		56.8 ±6.5	0/10	2.1 ±0.5*	41.2

[a] Fractions were injected intratumorally every day for 7 days starting on day 0.
[b] Mean survival time (days) indicates the mean ±standard error.
[c] Figure in parenthesis indicates the number of tumor-bearing animal.
[d] Tumor weight indicates the mean ± standard error.
ND Not determined.
* $p < 0.01$.

Table 4. Effects of various doses of cell wall fraction.[a]

Material		MST[b]	No. of survivors/ No. tested	Tumor size on day 21 (g)[d]	Tumor growth T/C (%)
Exp 1					
Control		57.7 ±1.5	0/10	5.0 ±1.2	
E.lentum	(800 μg)	ND	5/9	0.4 ±0.2*	8.0
Cell wall	(300 μg)	ND	2/10(1)[c]	1.8 ±0.4*	36.0
	(500 μg)	ND	3/10(1)	0.9 ±0.2*	18.0
	(800 μg)	ND	1/ 9	1.0 ±0.3*	20.0
	(1,000 μg)	ND	2/ 9	0.8 ±0.5*	16.0
Exp 2					
Control		32.1 ±3.6	0/20	4.0 ±0.8	
Cell wall	(50 μg)	48.8 ±14.7	0/10	2.1 ±0.7	52.5
	(100 μg)	ND	1/10(1)	1.2 ±0.2*	30.0
	(200 μg)	ND	3/10(2)	1.1 ±0.9*	27.5
	(500 μg)	ND	4/10(2)	1.2 ±0.4*	30.0

[a] Fractions were injected intratumorally every day for 7 days starting on day 0.
[b] Mean survival time (days) indicates the mean ± standard error.
[c] Figure in parenthesis indicates the number of tumor-bearing animal.
[d] Tumor weight indicates the mean ± standard error.
ND:Not determined.
* $p < 0.01$.

Table 5. Effects of various doses of membrane fraction.[a]

Material		MST[b]	No. of survivors/ No. tested	Tumor size on day 21 (g)[d]	Tumor growth T/C(%)
Exp 1					
Control		45.1 ±3.3	0/10	6.1 ±1.0	
Membrane	(1,800 μg)	ND	3/10(1)[c]	6.1 ±0.1*	3.3
	(3,600 μg)	ND	3/10(2)	0.4 ±0.1*	6.6
	(5,400 μg)	ND	4/10(2)	0.9 ±0.3*	14.8
Exp 2					
Control		52.1 ±3.2	0/10	11.9 ±0.7	
Membrane	(180 μg)	56.6 ±2.7	0/10	10.2 ±0.9	85.7
	(360 μg)	54.6 ±2.5	0/10	10.8 ±1.4	90.6
	(720 μg)	ND	1/10	4.0 ±1.2*	35.3
	(1,000 μg)	ND	3/10	2.9 ±0.7*	24.4

[a]Fractions were injected intratumorally every day for 7 days starting on day 0.
[b]Mean survival time (days) indicates the mean ± standard error.
[c]Figure in parenthesis indicates the number of tumor-bearing animal.
[d]Tumor weight indicates the mean ± standard error.
ND Not determined.
* $p < 0.01$.

Antitumor activity of each fraction against Ehrlich ascites tumor is shown in Table 3. Tested dose of each fraction corresponded to the 500 μg of cell wall fraction (Fr1) which was about 5 times the weight of cell of 10^7 *E.lentum* cells. All the mice in untreated group died from on day 34 to 66. Antitumor activity was retained in cell wall fraction (Fr1) and membrane fraction (Fr2), however, the activity was mild compared to the activity of 10^7 cells of *E.lentum* (800 μg).

Dose dependency of cell wall and membrane fraction

As shown in Table 4, when mice were administrated with various doses of cell wall fraction for 7 days, antitumor activities were shown at doses more than 100 μg and a similar activity was recognized within a range of 100 to 1,000 μg. When tumor-bearing mice were injected it with various doses of membrane fraction for 7 days, as shown in Table 5, an antitumor effect was observed at doses more than 720 μg. A maximum effect was observed at the dose of 1,000 to 1,800 μg. Simi lar results of cell wall and membrane fraction were also obtained by intravenous and intraperitoneal injection (data not shown).

Induction of NK activity tumoricidal macrophage and CTL activity by effective component of *E.lentum*

As shown in Fig. 3, NK activity in spleens of C3H/He mice given iv 300 μg of cell wall fraction, elevated to 89.7% (E:T ratio 50:1) and 87.6% (E:T ratio 25:1) on day 1 as compared with 33.3% and 22.5% in control mice, respectively ($p < 0.01$). On and after day 5, NK activity decreased to control level (Fig.3), while that in C3H/He mice injected iv with 1,000 μg of membrane fraction represented the maximum (85.2% and 68.7% at E:T ratio 50:1 and 25:1) on day 1 ($p < 0.01$). Although NK activity decreased to control level on day 5, it was elevated again on day 7 to 49.0% (E:T ratio 50:1) and 31.3% (E:T ratio 25:1), values which were significantly higher than those in controls ($p < 0.01$).

BALB/c mice were given ip 100 μg of cell wall or 720 μg of membrane fraction for 5 days, and on day 9, macrophage mediated cytotoxicity in PEC was determined against EL4 *in vitro*. As shown in

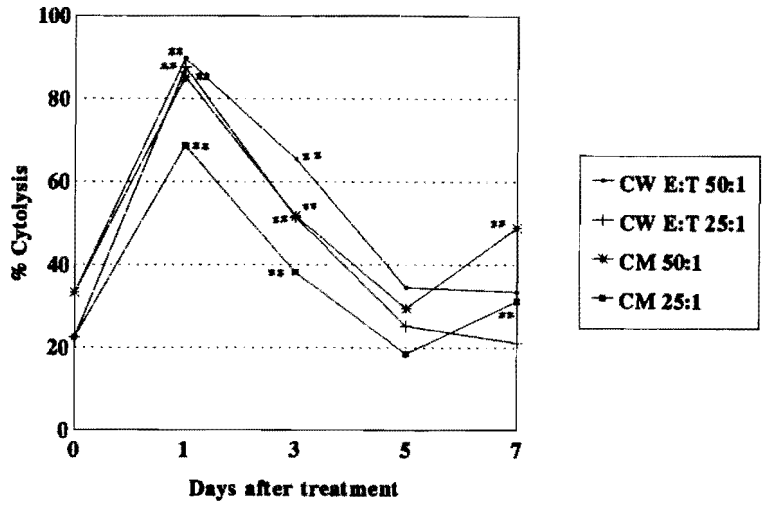

Fig. 3 Kinetics of NK cell activation following iv injection of 300 μg cell wall or 1,000 μg membrane fraction. NK activity in spleen cells of C3 H/He mice was determined against 2×10^4 cells/well of ^{51}Cr-labeled YAC-1 in 4 hour assay. Each point and bar indicate the mean ± standard error of 3 experiments. **: Statistically significant difference from the control group at $p<0.01$.

Table 6. Effects of fractions on tumoricidal macrophage induction in peritoneal exudate cells of BALB/c mice.[a]

Material		% Cytotoxicity[b]	
		10 : 1	5 : 1
Control		10.1 ±2.6[c]	8.5 ±1.5
E.lentum	(800 μg)	53.9 ±3.4**	43.4 ±1.8**
Cell wall	(100 μg)	55.9 ±2.8**	38.5 ±2.0**
Cell membrane	(720 μg)	24.3 ±2.0*	20.3 ±1.5*

[a] Mice were injected ip with *E.lentum* or each fraction every other day for 5 days.
[b] Cytotoxic test was performed at 9 days after the first injection and cytotoxicity was assessed in 20 hr assay against 10^4 cells of labeled EL4.
[c] Figures indicate the mean ± standard error of 3 experiments.
* $p<0.05$, ** $p<0.01$.

Table 6, macrophages from mice treated with *E.lentum* and the cell wall fraction displayed the same level of cytotoxicity ($p< 0.01$). On the other hand, tumoricidal macrophages from mice given the membrane fraction represented only twice the control level ($p < 0.05$). CTL activity was not induced in spleens of ICR mice inoculated with Ehrlich ascites tumor cells by administration of either cell wall fraction or membrane fraction until week 5 (data not shown).

DISCUSSION

E.lentum does not have a direct cytotoxic action against Ehrlich ascites tumor cells and produced a marked increase in plaque-forming cells and humoral antibody against SRBC. Furthermore, *E.lentum* enhanced DTH against SRBC significantly to the control level.[12] Thus, we suggested that *E.lentum* might be BRM and its antitumor effect was exerted via a host-mediated action. In this study, we demonstrated that NK cell activity was enhanced remarkably one day after injection of *E.lentum* and decreased later, but was higher than the control level even on day 7. In cancer patients receiving OK-432, NK cell activity was detectable on day 1, reached a peak on day 3, and returned to the pretreatment level by day 7 and 8.[16,17] Oshimi *et al.*[18] have reported that NK cell activity in PEC of mice given OK-432 peaked on day 3. From these results, NK cell activity was augmented more quickly and durably by administration of *E.lentum* than by that of OK-432. The mechanism of NK cell activation by *E.lentum* has not been clarified. It has been reported that IFN, IL-2 and other lymphokines induced by bacteria might activate NK cells *in vivo*.[9-11,16] Thus, it seems that *E.lentum* may also induce several cytokines and activate NK cells by that mechanism. *E.lentum* induced tumoricidal macrophages in PEC of BALB/c mice on day 9 and its activity remained at the same level at least until day 11. Furukawa *et al.*[19] reported that a mycolic acid derivative containing a high proportion of unsaturated fatty acids rendered macrophages cytotoxic for over 14 days after injection. Furthermore, Keller *et al.*[20] reported that tumoricidal

activity induced by lymphokines was only short lived, while that induced by bacteria persisted and enhanced the secretion of IL-6 and PGE2. Dazord *et al.*[6] reported that activity of tumoricidal macrophages induced by killed *Brucella* reached maximum on day 5 and remained for more than 20 days. The mechanism of induction of tumoricidal macrophage by *E.lentum* is not yet known. Paulanock and Lambert[21] demonstrated that macrophages might be activated in a 2-step mode by IFN gamma and LPS. However, it seems that macrophages may be activated by gram positive bacteria such as *E.lentum* and OK-432 having no LPS with the cooperation of lymphokine, ie IFN gamma induced by the bacteria, and by the bacterium itself. In tumor-bearing mice given *E.lentum*, CTL against the tumor were induced 4 weeks after tumor inoculation, while CTL were not induced in untreated tumor-bearing mice given *E.lentum* was remarkably slower than that in untreated mice 21 days after tumor inoculation. There was a clear correlation between the induction time of CTL and growth or cure of the tumor in *E.lentum*-treated mice. The mechanism of CTL induction by *E.lentum* is also not known. Roberson and Elger[5] have reported that *C.parvum* might induce CTL via the interleukin cascade. Since *E.lentum* induces GM-CSF (unpublished), it seems that CTL induction by *E.lentum* may occur through the same mechanism as that by *C.parvum*.

In this paper, we indicated that cell wall and membrane fractions of *E.lentum* had active antitumor components. Cell wall fractions of *Lactobacillus,*[22] *Bifidobacterium,*[23] *Nocardia,*[7] BCG[24] etc. have been reported to act as BRM.

NK cell activation by the cell wall fraction reached a maximum on day 1 like that by *E.lentum* whole cell, but the activity remained for a short time. On the other hand, NK cell activation by the membrane fraction reached a maximum on day 1 and decreased to the control level on day 5, but increased again on day 7. These results suggest a reason why NK activation by *E.lentum* whole cells persisted for a relatively long time. In contrast to the results in tumor-bearing mice given *E. lentum* whole cell, CTL induction was not observed in mice given cell wall and membrane fractions. These results indicate the reason why *E. lentum* whole cell has a superior antitumor effect than each of its components. Further studies should be carried out on the mechanism of effector cell activation by *E.lentum*.

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