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Immunopotentiating Activity of *Clostridium butyricum* in Mice

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ABSTRACT

Bacterial vaccine, as generated by heat-inactivated *Clostridium butyricum* cells, displayed antitumor activity against sarcoma 180 in DDY mice and antimetastatic activity against B16-F10 melanoma in BDF1 mice. According to our results, the vaccine has no direct growth inhibitory effect toward the tumor cell lines tested in this study. The vaccine increased gamma-interferon production, elicited delayed type hypersensitivity reaction, and enhanced IgM antibody formation and mitogenicity. The phagocytic activity of macrophage and killing activity of NK cells from mice were enhanced in a dose-dependent manner by stimulating with the heat-inactivated vaccine. Among those responses in the mice treated with CB, elevated NK cell activity may play a prominent role in manifesting antitumor activity in the B16-F10 metastasis experiment.

Key Words: *Clostridium butyricum*; vaccine; antitumor activity; immunopotentiating activity.

I. Introduction

In 1935, Miyairi first reported the activity of *Clostridium butyricum* M II 588 against putrefactive microorganisms. Since then, Asian people have used live bacterial spores to clinically prevent disturbances of microbial flora in intestine. Only administering the bacterial spore has also been reported to lessen clinical symptoms such as diarrhea and others (Kobayashi *et al.*, 1968).

During our studies on elucidating the mechanism by which *C. butyricum* has anti-putrefactive activity, those results indicate that the heat-inactivated bacterium has interesting immuno-activities both *in vitro* and *in vivo*.

This paper demonstrates that antitumor effects and various immunomodulating activities can be induced by the inactivated bacterium. The possible effects involved to induce the system's antitumor activities are also discussed.

II. Materials and Methods

1. Animals

Specific-pathogen-free male Balb/c, BDF1, C57BL/6, DDY and ICR mice, -ages from 6 to 10 weeks old , were purchased from the Shizuoka Agricultural Cooperative for Experimental Animals, Hamamatsu, Japan and used in this study.

2. Preparation of CB Vaccine

Clostridium butyricum M II 588 was cultured at 37°C for 24 hours in PYG medium (Maeda *et al.*, 1986). After cultivation, the culture was washed several times

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with saline and resuspended in saline. The cell suspensions were treated with heat for 30 min at 100°C and lyophilized. The lyophilized sample can be reconstituted with saline to administer 20 mg/ml for experimental use.

3. Media and Cell Lines

YAC-1 lymphoma cells of A/Sn origin were maintained in RPMI 1640 medium (Nissui Seiyaku Co. Ltd., Tokyo) supplemented with 5 to 10% heat-inactivated fetal bovine serum (Flow laboratories, Mclean, Virginia, U.S.A.) containing 40 μg/ml of gentamycin, and 3 μg/ml of glutamine. Eagle's minimum essential medium (MEM, Nissui Seiyaku Co. Ltd., Tokyo) supplemented with 10% FCS, 60 μg/ml of kanamycin and 3 μg/ml of glutamine was used to culture Mouse fibroblast cell line L929 and B16 melanoma F10 sub-line (kindly given by Dr. T. Tsuruo, Japanese Foundation of Cancer Research, Tokyo). All cell cultures were carried out at 37°C in a humidified atmosphere with 5% CO_2. Sarcoma 180 and EL-4 leukemia cells were maintained in the peritoneal cavities of ICR and C57BL/6 mice, respectively, by weekly sequential transplantation. The medium and CB vaccine were routinely tested for endotoxin contamination. Only batches containing endotoxins less than 0.01 ηg/ ml (measured by Limulus ameobocyte lysate assay using the endotoxin detection Pre-gel reagents from Seikagaku Kogyo Ltd., Japan) were used for the study.

4. Antitumor Assay

5×10^5 of sarcoma 180 cells were intraperitoneally injected into DDY mice. After 24 hours, CB was administered by the same route. Antitumor activity was determined by comparing the survival time from the treated groups with that of the control group.

Antimetastatic activity was determined in the following manner: before the injecting tumor cells, mice were given intraperitoneal injections of CB on days -3, -2, and -1. B16-F10 cells (2×10^4), a highly metastatic cell line, in 0.2 ml of MEM were inoculated into the vein of the treated mice on day 0. The animals were sacrificed on day 20 after tumor inoculation. The lungs were removed, rinsed in distilled water containing heparin (1 unit/ml) and then fixed overnight in Bouin's solution. The number of tumor foci was determined by counting nodules on the cell surface.

5. *In Vitro* Assay of Growth Inhibition of Tumor Cells

The growth rates of EL-4 and L1210 cells are faster than sarcoma 180 and B16-F10 cells. Therefore they were chosen for growth inhibition test. 5×10^2 of EL-4 and L1210 leukemia cells, the target cells, suspended in RPMI-1640 medium were separately seeded into each well of a microtest plate . Various dosages of CB were added to each well and the plate was then incubated in CO_2 incubator at 37°C for 3 days. Inhibition of tumor cell growth was determined by measuring the rate of ^{3}H-thymidine incorporation of each well. Briefly, 4 hours before the end of incubation, 1 μCi of ^{3}H-thymidine was added to each well. The tumor cells were then collected onto a glass filter using a Labo-Mash multiple cell harvester. In addition, the radioactivity of ^{3}H-thymidine incorporated into these cells was determined by a liquid scintillation counter.

6. PFC Assay

To examine the effects of CB on antibody formation, the number of hemolytic plaque-forming cells (PFC) in the spleen was calculated on the basis of the Cunningham and Szenberg's method (1968).

7. Mitogenicity

Mitogenicity was determined by the method of Kumazawa *et al.* (1982), with slight modificatio. Briefly, spleen and thymus cell suspensions were prepared by gently tapping, passed through a 200-gauge stainless steel sieve, and then centrifuged. The cell pellets were treated with ammonium chloride tris buffer to lyse red cells, and washed three times with Eagle's MEM. The cells were cultured at a density of 1×10^6 cells/ml with different dosages of CB in a total volume of 0.2 ml. After incubating at 37°C for 2 days in a humidified atmosphere, ^{3}H-thymidine was added to each culture and further incubated for 4 hours. Cells were collected on glass fiber filters using a multiple cell harvester. Incorporation of ^{3}H-thymidine was determined by liquid scintillation counter. Lipopolysaccharide (LPS, Difco Lab., Detroit, U.S.A.) and concanavalin A (Wako Pure Chemical Ind. Ltd., Japan) were used as controls.

8. Delayed-type Footpad Reaction

This reaction was performed by the method described by Yoshikai *et al.* (1979). Ten-week old DDY mice were intraperitoneally injected with 2×10^6 sheep red blood cells (SRBC) and different dosages of CB simultaneously for 3 consecutive days. Four days later, 1×10^8 SRBC was administered and 24 hours footpad swelling was measured.

9. Interferon (IFN) Assay and Induction *in Vivo*

Mice were intraperitoneally administered 1 mg of CB in 0.2 ml of saline. A group of 5 mice were bled by cardiac puncturing to accumulate blood for serum preparation. The sera were frozen at -80°C until assaying for IFN content (Kumazawa *et al.*, 1982). OK-432, a streptococcal whole cell preparation, (Chugai Seiyalu Co. Ltd., Tokyo) was used as a stimulator for producting IFN in the control group. The IFN titer was assayed as antiviral activity of the mouse serum by determining the phage plaque reduction on L-929 monolayer of mouse cell cultures(Armstrong, 1971). Vesicular stomatitis virus (VSV) of Indiana strain was kindly provided by Dr. N. Maehara, Kitasato Institute, Tokyo. IFN titer was expressed in units per milliliter as the reciprocal of the dilution that reduced the cytopathic effect (CPE) by 50% more than the virus control. IFN (A/D), kindly provided by Dr. A. Fuse, Department of Microbiology, School of Medicine, Chiba University, was used as a standard.

10. Neutralization Test

Anti-IFN (α/β) serum, a rabbit antiserum against mouse L-cell IFN, was purchased from Lee Biomolecular Research, San Diego, CA, U.S.A. Neutralization tests (Kawada, 1980.) were performed by Yamamoto and Kawada's method (1980).

11. Cytotoxic Activity Test

Short terms of ^{51}Cr-release assays on macrophage or NK cell activity were performed with APEC (adherent peritoneal exude cells) or with fresh murine spleen cells as the effector cells, respectively. 5×10^6 of target cells in RPMI-1640 medium were labeled for 40 min at 37°C with 100 μCi of $Na_2{}^{51}CrO_4$ (Kumazawa *et al.*, 1982). The labeled B16-F10 melanoma and YAC-1 cells were washed three times with PBS and adjusted to 2×10^5 cells/ml in RPMI-1640 medium. 5×10^6 of target cells in a volume of 100 μl were seeded in round bottom tissue culture plates (Costar). Appropriate concentrations of spleen cells or macrophages in a 100 μl volume were added to the plates. Next, the cells were removed and centrifuged for 5 min at 1,000 rpm then incubated at 37°C in a CO_2 incubator for 4 hours before centrifuged again for 4 min at 1,000 rpm. 100 μl of each supernatant was harvested. Radioactivity was counted in a gamma counter (LKB). The percentage of specific ^{51}Cr-release was calculated according to the formula:

$$\text{Specific lysis (\%)} = \frac{(\text{experimental } {}^{51}\text{Cr-release} - \text{spontaneous } {}^{51}\text{Cr-release})}{(\text{maximum } {}^{51}\text{Cr-release} - \text{spontaneous } {}^{51}\text{Cr-release})} \times 100$$

The spontaneous ^{51}Cr-release from target cells incubated in the medium alone was less than 10% of the total ^{51}Cr. Time courses experiment of increased cytolytic activity of NK cells and macrophages from BDF1 mice treated with CB were performed by the same ^{51}Cr-release assay. Tumor-bearing mice, B16-F10 cells (2×10^4) were intravenously inoculated into the vein of BDF1 mice tail on day 0 and CB was intraperitoneally injected on days -11, -12, -13. On day 20, macrophages were accumulated and their cytotoxic activities were determined.

12. Phagocytic Activity against Opsonized SRBC

SRBC and anti-SRBC IgG were purchased from Nippon Bio-Test Laboratories, Tokyo and from Japan Immunoresearch Laboratories Co. Ltd., respectively. The phagocytic activity was determined by the method of Kato *et al.* (1983). The number of phagocytized SRBC by peritoneal macrophages was counted under microscope and phagocytic activity was determined as the number of phagocytized SRBC by 100 macrophages.

13. Statistical Analysis

The difference between experimental and control groups was assessed by a Student's t test; p value was calculated as well.

III. Results

1. Antitumor Activity

To examine the therapeutic activities of CB against ascitic form of sarcoma 180 in DDY mice, CB was intraperitoneally administered one day after inoculating intraperitoneally of 5×10^5 cells of sarcoma 180 into mice. As Table 1 reveals, administering of CB in which dosages ranged from 0.5 to 3.0 mg have antitumor activity and the mean of survival ratios greater than 125% were observed in those dosages tested herein. Modest therapeutic activity of CB was first reported in this experiment in addition to the prophylactic activity of CB against sarcoma 180 (data not shown).

2. Antimetastatic Activity

Most bacterial BRMs (biological response modifiers) have been reported to be the much more effective in prophylactic models than in therapeutic models. Thus, prophylactic antimetastatic activity of CB

Table 1. Antitumor Activity of CB in Ascitic form of Sarcoma 180 in DDY Mice

Treatment regimen[a] (CB, mg/mouse)	MST±S.D.[b] (day)	T/C(%)[c]	*p* Value[e]
saline	17.3 ± 4.9		
0.5	23.8 ± 6.2	138	< 0.1
1.0	22.0 ± 5.4	127	< 0.1
2.5	24.0 ± 3.0	139	< 0.05
3.0	24.3 ± 3.3	139	< 0.05
5.0	19.0 ± 5.3	110	n.s.[d]

[a] Six mice were used in each group. Sarcoma 180 (5×10^5 cells) was intraperitoneally injected on day 0. CB was intraperitoneally injected with the indicated doses on day 1.
[b] MST: Median survival time ± standard deviation.
[c] T/C: Treated group/control group.
[d] n.s.: not significant.
[e] Values are statistically different from control at the indicated *p* value.

Table 2. Inhibition of Pulmonary Metastasis of B16-F10 Melanoma in BDF Mice by CB Treatment

Treatment. regimena (CB, mg/mouse)	Mean no. of lung tumor Colonies ± S.D. (range)[b]	*p* valuec
saline (control)	132 ± 15 (105 – 147)	
1.0	25 ± 9 (10 – 39)	< 0.001
2.5	81 ± 52 (21 – 153)	< 0.025

[a] Saline or indicated doses of CB were intraperitoneally injected into BDF1 mice for 3 consecutive days (days -3, -2 and -1) prior to intravenous injection of 2×10^4 B16-F10 cells on day 0.
[b] The numbers of lung tumor colonies were on day 20 after melanoma cells injection. Mean number of lung colonies ± standard deviation.
[c] Statistically different from control at the indicated *p* value.

Table 3. Effects of CB on the ^{3}H-Thymidine Incorporation of Tumor Cells *in Vitro*

Target cell	CB dose (μg/ml)	[^{3}H] Thymidine uptake[a] (Mean cpm ± S.D.)
EL-4	0	1029 ± 124
	0.1	1388 ± 243
	0.5	1552 ± 358
	5.0	1112 ± 41
	10.0	1155 ± 518
L1210	0	275610 ± 6054
	0.1	275945 ± 1855
	0.5	270233 ± 4831
	5.0	224954 ± 2453
	10.0	243169 ± 9120

EL-4 or L1210 (500 cells/well) leukemia cells were incubated with various dosages of CB in RPM1-1640 medium containing 10% fetal calf serum for 3 days at 37°C with 5% CO_2 in air. [^{3}H] Thymidine (1 μCi/well) was added to the culture and incubated for 4 h, then incorporation rate of the thymidine was determined.
[a] Mean cpm ± standard deviation.

was examined. BDF1 mice were intraperitoneally administered with CB as a premeditation for three consecutive days before intravenous inoculation with B16-F10 melanoma cells. As Table 2 indicates, in the treated groups receiving 1.0 and 2.5 mg of CB on days -3, -2 and -1 resulted in 25.4 and 81.6 foci respectively. Whereas the numbers of foci in the control were 132.7. Treating 1.0 mg CB reduced the foci number to one fifth when compared to the control group.

3. Direct Growth Inhibitory Effect of Tumor Cell

To obtain information on whether CB inhibits tumor cell growth *in vitro*, a direct growth inhibition test was performed using L1210 and EL4 leukemia as target cells. According to Table 3, ^{3}H-thymidine incorporation was not inhibited with the effective dosages of CB used in our previous experiment.

4. Antibody Formation

To characterize CB as one of the BRMs, various immunomodulating activities were tested. First, the effects of CB on antibody formation *in vivo* were examined by PFC assay in spleen cells. DDY mice were immunized intraperitoneally with 1×10^7 SRBC and different amounts of CB simultaneously. The number of anti-SRBC IgM forming cells in the spleen was determined four days later. As Table 4 reveals, PFC numbers in the spleen markedly increased in the mice treated with 1.0 to 5.0 mg/mouse of CB. The maximum increase was reached when 2.0 mg/mouse were used and the numbers of PFC slightly declined while treating 5.0 mg/mouse of CB.

When delayed-type footpad swelling was measured after CB treatment, the footpad swelling only modestly increased while treating with 1.0 to 5.0 mg of CB (data not shown).

5. Mitogenicity

CB mitogenic activity *in vitro* against spleen and thymocyte cells was determined by the direct mitogenic response to measure the incorporation of pulse-labeling ^{3}H-thymidine into spleen and thymic cells. Table 5 summarizes those results. Mitogenic responses of CB-treatment were observed in the spleen cells and also found to be dose-dependent. Thymidine uptake reached the maximum level with the stimulation of 5.0 μg CB/0.2 ml per well. However, throughout the studies, mitogenic response of CB was somewhat lower than those of LPS and Con-A. No mitogenic activity was

Table 4. Adjuvant Effects of CB on Antibody Response to SRBC

Stimulant	CB dose (mg/mouse)[a]	Anti-SRBC PFC/spleen[b] (mean PFC ± S.D.)	*p* Value[d]
SRBC only	0	61213 ± 3882	
SRBC + CB	0.5	81082 ± 7226	n.s.[c]
	1.0	190492 ± 1445	< 0.005
	2.0	275569 ± 5446	< 0.005
	5.0	169655 ± 846	< 0.005

[a] Five male DDY mice were intraperitoneally immunized with 1×10^7 SRBC and treated with the indicated dosages of CB at the same time (on day 0). Four days later (on day 4), anti-SRBC antibody forming cells were determined as a hemolytic plaque.
[b] Mean PFC number in the spleen cells ± standard deviation
[c] n. s.: not significant.
[d] Statistically different from control at the indicated *p* values.

Table 5. Mitogenic Responses of CB to Lymphocytes from DDY Mice

Cell cultured[a]	Sample (μg/well)	[^{3}H] Thymidine uptake[b] (Mean cpm ± S D.)	*p* Value[d]
Thymocyte	None	58 ± 113	
	Con A (2.0)	44913 ± 1159	< 0.001
	LPS (10.0)	1089 ± 128	< 0.1
	CB (1.0)	306 ± 42	n.s.[c]
	(5.0)	24 ± 7	< 0.01
	(10.0)	619 ± 101	< 0.01
Splenocyte	None	5517 ± 923	
	Con A (2.0)	31823 ± 881	< 0.005
	LPS (10.0)	39741 ± 1181	< 0.001
	CB (1.0)	9244 ± 1396	n.s.[c]
	(5.0)	17224 ± 1554	< 0.01
	(10.0)	16701 ± 846	< 0.01

[a] Thymocytes or splenocytes were cultured with the indicated doses of Con A, LPS or CB for 2 days in microtiter plates at cell density of 1×10^6 cells/ml in a total volume of 0.2 ml. After 2 days, incorporation of [^{3}H] thymidine was determined.
[b] Mean cpm ± standard deviation
[c] n.s.: not significant.
[d] Statistically different from control at the indicated *p* values.

observed in thymocytes. Time course experiment of mitogenic responses in the spleen cells induced by 5.0 μg of CB was performed (Fig.1). Mitogenic activity induced by CB increased with time and reached the maximum level on day 2 and then decreased. Figure 1 indicates that the profiles of thymidine incorporation were similar to those of LPS.

6. IFN Production

When DDY mice were administered 1.0 to 10 mg/mouse of CB intraperitoneally on day 0, the optimum dose for producting IFN activity was 1 mg/mouse as shown in Fig. 2. IFN activity reached the maximum level to 2,560 units after 24 hours of stimulation. Whereas only 320 units of IFN was detected in positive control of OK-432 (IFN-producer). To differentiate which types of IFN production were induced by CB, various physicochemical properties were compared.

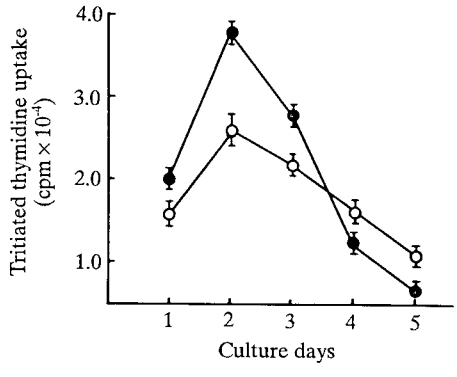

Fig. 1. Kinetics of mitogenic responses of splenocytes to CB. The spleen cells were cultured for 2 days in microtiter plate at a density of 1×10^6 cells/ml with 5 μg of CB(○) or 10 μg of LPS (●). [^{3}H] Thymidine was added to the culture and after 4 h incubation, incorporation rate of the thymidine was determined.

Table 6. Properties of Antiviral Substance (IFN) Induced by CB

Source of serum[a]	IFN titer (unit/ml) after:				
	No treatment	Trypsin	pH 2	Heat	Anti-α/β[b]
CB (1.0 mg/mouse)	2560	<10	<10	<10	2560
OK–432 (0.5 KE/mouse)	320	<10	<10	<10	320

[a] Pooled sera were obtained from 6 to 8 DDY mice 24 h after intraperitoneal injection of 1.0 mg/mouse of CB or 0.5 KE (unit)/mouse of OK-432. Each treated serum was immediately assayed for the antiviral activity.
[b] Result after neutralization by antiserum.

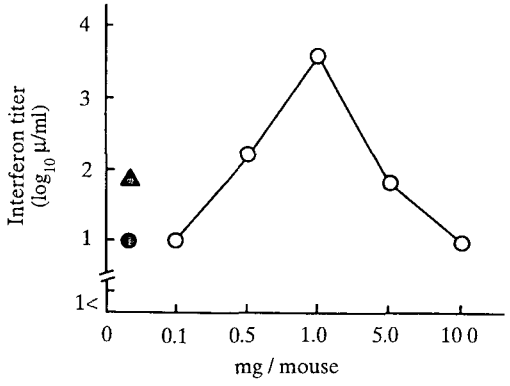

Fig. 2. Production of IFN activity in DDY mice by various dosages of CB. Pooled sera were obtained from DDY mice 24 h after intraperitoneal injection of various doses of CB(○). OK-432(▲) was used as a reference. Five mice were used in each dose.

Table 6 summarizes those results. IFN activity was completely diminished by dialysis against 0.1 M glycine-HCl buffer (pH 2.0) for 24 hours at 4°C or incubation at 56°C for 60 min. The IFN activity did not disappear by treating with anti-IFN (α/β) serum; however, treating with trypsin caused a complete loss of IFN activity.

7. Time Course of NK Cell and Macrophage Activity

BDF1 mice were intraperitoneally received CB on day 0. The cytotoxic activities of NK cells and macrophages were then assayed by using spleen cells and peritoneal macrophages from the mice against YAC-1 cells, respectively. As Table 7 indicates, intraperitoneal administering CB enhanced cytotoxic activity of both NK cell and macrophage. Maximum NK cell activity was detected on day 3 and then decreased, while the activity of macrophages gradually increased and reached the maximum level on day 9 after the CB treatment.

Table 7. Increased Cytotoxic Activity of NK Cells and Macrophages in BDF Mouse by CB Treatment

	% Cytotoxic activity against YAC-1 cells		
	NK cells		Macrophages
Days after treatment[a]	0:1[b]	100:1	20:1
0	1.3 ± 0.6	2.2 ± 0.9	3.3 ± 1.0
1	1.9 ± 0.7	2.8 ± 1.3	3.2 ± 1.7
2	5.6 ± 1.4	7.9 ± 1.8	NT[c]
3	9.5 ± 2.3	15.3 ± 2.1	8.3 ± 0.8
5	4.0 ± 1.2	4.9 ± 0.9	17.7 ± 2.1
9	2.7 ± 1.3	4.9 ± 2.8	21.3 ± 2.6
11	NT	NT	20.8 ± 3.1

Statistically different from control on day 0 ($p < 0.1, p < 0.01$).
[a] CB (1 mg/mouse) was intraperitoneally injected on day 0. Four mice in each day were used. Cytotoxic activity was determined by short term ^{51}Cr release assay.
[b] Effector to target cell ratio.
[c] NT: Not tested.

8. Cytotoxic Activity of Macrophage against B16-F10 Melanoma

Since strong antimetastatic activity arose against B16-F10 melanoma cells in BDF1 mice, macrophage activity against B16-F10 melanoma cells was also studied in normal and B16-F10 tumor-bearing BDF1 mice models. As Table 8 reveals, macrophage activity similarly enhanced on B16-F10 melanoma bearing mice in addition to cytotoxic activity of macrophages significantly increasing from normal mice treated with CB. NK cell activity was also elevated against B16-F10 cells in B16-F10 tumor bearing mice (data not shown).

9. Phagocytic Activity of Macrophages

Phagocytic activity was measured as an index of macrophage activation for further characterization. When DDY mice were administered an intraperitoneal injection of 1 mg/mouse of CB on day 0, phagocytic activity of macrophages was significantly enhanced

Table 8. Effects of CB on Macrophage Activity in Normal and B16-F10 Tumor-bearing BDF1 Mice

Macrophage source	CB[a] treatment	% Cytotoxic activity against B16-10 melanoma (E/T ratio, 50:1) (Mean ± S. D.)[b]
Expt. 1		
Normal Mice[a]	-	0
	+	6.4 ± 2.1[c]
Expt. 2		
Tumor-bearing mice[a]	-	0
	+	10.9 ± 1.4[c]

[a] CB was treated for 3 consecutive days (days -3, -2 and -1). Six mice were given intravenous injections of 2×10^4 B16-F10 melanoma cells on day 0. After 9 days (on day 9), peritoneal macrophages (PEC) were harvested and their activity were tested .
[b] Mean cytotoxic activity ± standard deviation.
[c] Statistically different from control at $p < 0.005$.

Table 9. Phagocytic Activity of Macrophages from DDY Mice Treated with CB

Treatment regimen[a]	% Macrophage engulfing SRBC (Mean ± S.D.)[c]	SRBC/macrophage[b] (Mean ± S.D.)[c]
Control	44 ± 5	151 ± 28
CB (1.0 mg/mouse × 3 days)	80 ± 6[d]	421 ± 80[d]

[a] CB was intraperitoneally injected for three consecutive days (days -3, -2 and -1), and after 2 days (on day 2), peritoneal macrophages (PEC) were harvested. Sheep erythrocytes (SRBC) coated with anti-SRBC antiserum were used.
[b] Number of SRBC found in 100 macrophages including both engulfing and non-engulfing cells.
[c] Mean number ± standard deviation.
[d] Statistically different from control at $p < 0.05$.

(Table 9) and found to be three times higher than that of control.

IV. Discussion

Bacterial BRMs such as Bacillus Calmette-Guerin (BCG), Nocardia rubra, *C. parvum*, and OK-432 have been used to treat certain types of animal and human tumors (Yamaguchi *et al.*, 1984). However, as reported earlier, some subtle differences arise in immunomodulating activities among these bacterial preparations (Talmadge and Herberman, 1986; Foon, 1989).

From the perspective of cell-wall chemistry among the bacterial BRMs, CB shows a slight similarity with *C. parvum*, although they are rather different taxonomically. Therefore, we compared the immunomodulating activities of CB to *C. parvum*. The most striking difference TNF activity was undetected in DDY mice treated with CB. Whereas a high titer of TNF activity was observed in the mice treated with *C. parvum* (data not shown). Therefore, our studies on CB provide further insight into bacterial immunomodulators.

Administration of 0.5 to 3 mg but not 5 mg of CB having the antitumor activity is owing to that the BRM is not like the chemotherapeutic action which is generally in a dose-dependent manner. Notably, different individuals have different immune responses. Since it has been reported that cytokine is one of the BRMs and some bacterial BRMs have the ability to stimulate IFN production both *in vitro* and *in vivo* (Borden et al., 1990; Aizawa, 1995), identifying the antiviral ability of CB in mice is of primary concern here.

Results obtained herein demonstrate that the antiviral activity is species-related. Based on our findings, the IFN activity did not disappear by treating with anti-IFN (α / β) serum. Whereas the major IFN activity is γ-IFN not α or -βIFN , i.e. similar to the report by Falcoff (1972). This result corresponds to the report by Yamaguchi *et al.* (1984). They reported that TH69, a whole cell preparation of *Streptococcus faecalis* (ATCC 31663), is a potent producer of γ-IFN. IFNs have been reported to exhibit different immunomodulating activities on immunocompetent cells such as NK cells and macrophages. Therefore, the immunomodulating activity of CB may be attributed to IFN production.

CB also has adjuvant-like activity. As reported earlier, BRM can be classified into two groups based on their effects on macrophages or T cells. For instance, poly ICLC and MVE-2 belong to the group having the ability to activate macrophage. The other group, e.g. thymosin fraction 5, apparently has a direct effect on T cells (Talmadge and Herberman, 1986). According to this definition, CB might belong to the former group because it exhibited a weak delayed-type hypersensitivity and a mitogenic response in thymocytes.

We used heat-killed *C. butyricum* (CB) cells, obtaining consistent experimental results throughout this study except for the results obtained while treating with formalin-killed CB which were in consistent (data not shown). These different activities in formalin-killed CB

may be attributed to the balance between production of "suppressors and activators" by the formalin-killed CB. The optimum dosages for CB to be function *in vivo* and *in vitro* were 1.0 to 2.0 mg/mouse and 5 to 10 (g/ml, respectively. Although most biological activities of CB *in vivo* were studied with one injection of CB, three consecutive days of treatments were found to be more effective in some experiments. Therefore, some experiments, e.g. tumor metastasis, were treated with CB for 3 days.

In the lung-metastasis model using B16-F10 cells, our results demonstrated strong prophylactic activity although no significant therapeutic activity of CB was observed. In addition, 1mg of CB treatment apparently works better than 2.5 mg of CB. However, both dosages (1mg and 2.5mg) are still within the effective range (0.5 to 3 mg) and their effectiveness do not necessarily correspond to the previous action against tumor. As long as they are working, we can regard as not having deviated too much intrinscially from our expectation. Since some BRMs including *C. parvum* have been reported to mediate their antitumor effects through modulation of organ-specific immune responses (Zhang *et al.*, 1986; Kalland *et al.*, 1985), the observed antimetastatic activity of CB may be due to the activation of either alveolar macrophages or NK-cells in some organs. Time course experiments on the stimulation of macrophage and NK cell (Table 7) indicated that NK cell activity was detected on day 3 accompanied with IFN production. Whereas macrophage activation was not detectable until 9 or 10 days of stimulation. In the metastasis experiments, B16-F10 melanoma cells were intravenously inoculated when the maximum level of NK cell activity was reached on day 3. Therefore, we can conclude that sensitized NK cells play a more prominent role than macrophages in eliminating the B16-F10 cells in CB treated mice. Our previous data also confirmed the hypothesis that CB induces NK cells activity to eliminate B16- F10 tumor cells entering the blood stream (Chen *et al.*, 1993). However, our preliminary experiment indicated that cytotoxicity of NK cells against B16-F10 melanoma cells is highly induced only in BDF1 and C57BL/6 mice treated with CB (Chen *et al.*, 1993). The mechanism enhancing NK cell cytotoxicity by CB in the system of B16-F10 cells and BDF1 or C57BL/6 mice remain unclear. Therefore, further study is deemed necessary to elucidate the mechanism of B16 melanoma metastasis.

Live spores of *C. butyricum*, M II 588 have been clinically used in Japan as an intestinal regulator over the past four decades. In addition, synthetic immunomodulators which enhance NK cell activity have been extensively studied recently (Wiltrout *et al.*, 1985). Thus , more experiments on the oral efficacy of the live spores or determination of the active mechanism of CB are currently underway.

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酪酸菌對老鼠免疫系的增強反應

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摘　要

老鼠以熱殺死的酪酸菌處理後，在DDY品系老鼠具有抗sarcoma 180的效果，而在BDF_1品系老鼠則具有抑制黑色素瘤轉移的效果。酪酸菌在體外無法抑制這些癌細胞的增殖，因此可能與免疫增強有關。經本實驗分析結果發現，酪酸菌死菌在老鼠體內可促進抗體的產生、干擾素的分泌、增強巨噬細胞及NK細胞之殺細胞功能，因此酪酸菌在老鼠體內具抑癌增強效果，可能與老鼠的免疫系統被活化有關。