Protection Activity Induced by the Bacterial Vaccine, Heat-Killed *Clostridium butyricum* Against *Candida albicans* Infections in Mice

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The bacterial vaccine, heat-killed *Clostridium butyricum* exhibits various immunomodulating activities including a strong protection activity against *Candida albicans* infection in mice. The analyses of immunomodulating activities induced by the vaccine showed that the vaccine stimulated macrophage and natural killer cell activity. Stimulation of delayed type hypersensitivity, IgM antibody formation and induction of γ -type interferon were observed. The vaccine was also mitogenic for B-cell lymphocytes. Among these immunomodulating activities, interferon was considered to be one of the important factors in the manifestation of *in vivo* anticandida activity. Further studies using recombinant interferon (IFN α -A/D type interferon) confirmed the active role of interferon in the manifestation of the protection. The interferon production by the vaccine was also found to be dependent on the mouse strain used and was most active in DDY mice.

Key words: *Clostridium butyricum*, vaccine, anti-candida activity, immunomodulating activity, interferon

A butyric acid bacterium *Clostridium butyricum* MII 588 has been used clinically to prevent disturbances of microbial flora in intestine in Japan¹⁾. It has also been reported that oral administration of the bacterial spore improves clinical symptoms, such as diarrhea, constipation and abdominal distension²⁾. During our studies on the mechanism of *C. butyricum* anti-putrefactive action, we found that the bacterium showed a strong antimetastatic activity against B16-melanoma in mice, although it showed no direct cytotoxic effect on various tumor cell lines, such as L1210 and EL 4 leukemias (unpublished data by Chen *et al.*). These data indicate that the antitumor activity induced by the bacterium is host mediated and that the bacterium itself may induce various immunomodulating activities.

In vivo protection activity against various microbial infections by BRMs (biological response modifiers), PS-K, MDP, LPS and OK-432 (Kaneda *et al.*, manuscript in

preparation) has been reported. Since systemic candidiasis is a significant complication of immunodeficiency syndromes, surgical procedures, and immunosuppressive medical therapies³⁾, we are interested in the immunomodulating activities of the vaccine.

In this paper, we report various immunomodulating activities induced by the bacterium including protection activity against *Candida albicans* infection in mice. We also discuss the possible effectors which play an important role in the manifestation of the anti-candida activities.

MATERIALS AND METHODS

Animals: Male Balb/c, BDF₁, C3H/He, DBA/2, DDY and ICR mice, 4 to 10 weeks old were purchased from Shizuoka Laboratory Animal Corporation Ltd., Hamamatsu, Japan and used throughout the study. For systemic *Candida* infection, 4 weeks old DDY mice were used.

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Preparation of CB vaccine: *Clostridium butyricum* MII 588 was cultured for 24 hr at 37°C in PYG medium⁴⁾. *C. butyricum* was then harvested, washed several times with saline and suspended in saline. The cell suspension was heated for 1 hr at 100°C and lyophilized. CB, a lyophilized whole cell preparation of the *C. butyricum* MII 588 cells, was reconstituted with saline to give 20 mg/ml of the cells. MCB (methanol extract of CB) fraction was prepared as follows: lyophilized whole cell preparation of CB (160 g) was extracted with methanol and the solvent extract was evaporated *in vacuo* to dryness. The dried residue was dissolved in a small amount of methanol and chromatographed on Sephadex LH-20 (Pharmacia, Sweden) column with methanol. The fast moving pale yellowish colored fraction was combined and evaporated to dryness. The dried residues thus obtained (2 g) were used as MCB fractions.

Media and cell lines: RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) was supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, Virginia, USA), 40 µg/ml of gentamicin, and 3 µg/ml of glutamine. Eagle's minimum essential medium (MEM, Nissui Seiyaku) supplemented with 10% FCS, 60 µg/ml of kanamycin and 3 µg/ml of glutamine was also used. All cell cultures were carried out at 37°C in a humidified atmosphere with 5% CO₂. YAC-1 lymphoma cells of A/Sn origin were maintained in RPMI 1640 medium. EL-4 leukemic cells were maintained in the peritoneal cavities of C57BL/6 mice by weekly sequential transplantation. Mouse fibroblast cell line L929 was grown in MEM.

Systemic infection with *C. albicans* in mice: A highly virulent *C. albicans* IFM 40009 (ATCC 48130) strain was grown at 37°C on Sabouraud dextrose agar (SDA, Difco Lab., Detroit, USA) for 18 hr. Mice were infected with an intravenous injection of 5×10^5 blastospores of *C. albicans* IFM 40009.

Effect of CB, MCB or interferon (IFN) on *C. albicans* infection: *In vivo* anti-candida activity was evaluated by the comparison of survival time with that of saline treated controls. Before *C. albicans* infection, mice were given an intraperitoneal injection of CB on days -3, -2, and -1. *C. albicans* cells (5×10^5 cells) in 0.2 ml of saline were inoculated into the tail vein of mice on day 0.

In cases of recombinant IFN α -A/D (lot. no. RG-A/D-02) (kindly given by Nippon Roche, Co., Ltd., Japan), 1×10^4 U/day was administered intravenously on day -1.

Mitogenicity: Mitogenicity was determined by a slight modification of the method described by Kumazawa *et al.*⁵⁾. Briefly, spleen and thymus cell suspensions were prepared by gentle teasing, passed through a 200-gauge stainless steel sieve, and centrifuged. The packed cells were treated with ammonium

chloride tris buffer to lyse red cells, and washed three times with MEM. The cells were cultured at a density of 1×10^6 cells/ml in a total volume of 0.2 ml including appropriate concentrations of CB. After incubation at 37°C in a humidified atmosphere with 5% CO₂, ³H-thymidine (1 µCi) was added to each culture and further incubated for 4 hr. Cells were collected on glass fiber filters using a multiple cell harvester. Incorporation of ³H-thymidine as determined by liquid scintillation counter. Lipopolysaccharide (LPS, Difco) and concanavalin A (Wako Pure Chemical Ind. Ltd., Japan) were used as controls.

Delayed-type footpad reaction: This reaction was determined by the method described by Yoshikai *et al.*⁶⁾. Ten-week old DDY mice were injected intraperitoneally with 2×10^6 sheep red blood cells (SRBC) and at the same time with various doses of CB by the same route. Four days later 1×10^8 SRBC was given and 24 hr footpad swelling was measured.

PFC assay: Numbers of hemolytic plaque-forming cells (PFC) in the spleen were determined to study the effect of CB on antibody formation according to the method of Cunningham and Szenberg⁷⁾.

IFN assay and its induction by CB treatment: Mice were intraperitoneally given CB in 0.2 ml of saline. At intervals, groups of 5 mice were bled by cardiac puncture, the blood was pooled and sera were separated by centrifugation. The sera were frozen at -80°C until testing for IFN contents⁸⁾. OK-432 (0.5 KE/mouse), a streptococcal whole cell preparation (Chugai Seiyaku Co. Ltd., Tokyo) was used as control IFN producer. The antiviral activity of the mouse serum (IFN titer) was determined using the plaque reduction method on mouse L-929 monolayer cell cultures by the method described previously⁹⁾. IFN titer was expressed in units per milliliter as the reciprocal of the dilution that reduced cytopathic effect (CPE) by 50% as compared with the virus control.

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 tests¹⁰⁾ were performed by the method described by Yamamoto and Kawada¹¹⁾.

Natural killer (NK) cell activity assay: The activity was determined by cytotoxic activity against YAC-1 target cells. Short term ⁵¹Cr-release assay of NK cell activity was performed with fresh murine spleen cells used as the effector cells. Briefly, YAC-1 target cells were labeled for 40 min at 37°C with 100 µCi of Na₂⁵¹CrO₄⁵⁾. In all assays, 2×10^4 target cells in a volume of 100 µl were seeded in roundbottom tissue culture plates (Costar). Appropriate concentrations of spleen cells in a 100 µl volume were added. Radioactivity was counted in a gamma counter (LKB). The percent

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 medium alone was less than 10% of the total ^{51}Cr .

Macrophage activity: The activity was also determined by the cytostatic activity of macrophages against EL-4 leukemia. Macrophage fraction was prepared from peritoneal exudate cells (PEC) of CB (1 mg) treated mice by the plastic adhering method. The *in vitro* cytostatic activity of macrophages was determined using the ^3H -thymidine incorporation inhibition method described by Kato *et al.*¹²⁾. EL-4 leukemia cells were used as target cells. Results obtained as average counts per minute were expressed as percent incorporation calculated by the formula:

$$\frac{[\text{cpm (tumor + effector cell)} - \text{cpm (effector cell alone)}]}{\text{cpm (tumor cell alone)}} \times 100(\%).$$

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. Assay method was the same as that of Kato *et al.*¹²⁾. The number of SRBC phagocytized by peritoneal macrophages was counted microscopically and phagocytic activity was also determined as the number of SRBC phagocytized per 100 macrophages.

Statistical analysis: The difference between experimental and control groups was evaluated statistically with a student's *t*-test and the probability (*p*) was calculated.

RESULTS

Protection induced by CB against systemic *C. albicans* infection in mice:

When CB was administered intraperitoneally to DDY mice as a premedication, *i.e.*, for three consecutive days before intravenous infection with *C. albicans*, significant increase in survival time was observed at all doses of CB tested (Fig. 1). Optimum dose was around 0.5 to 1.0 mg/mouse and 40% of the group survived after 30 days. These results indicate that CB can induce strong host mediated anti-candida activity. MCB, the methanol extract of CB, was also found to be active against the infection. Seventy percent of the mice were still surviving after 30 days at the dose of 0.5 mg/mouse of MCB. These results indicate that active principles of CB were extractable by the solvent, although they have not yet been purified.

Fig. 1. Protection activity induced by CB and MCB against systemic *Candida albicans* infection in mice. Ten DDY mice were used in each group. CB: (— control, ■—■ 0.1 mg/mouse, ●—● 0.5 mg/mouse, ▲—▲ 1.0 mg/mouse) MCB: (— control, □—□ 0.1 mg/mouse, ○—○ 0.5 mg/mouse, △—△ 1.0 mg/mouse)

Mitogenicity of CB: Since CB was found to be one of the active BRMs against the infection, various immunomodulating activities of CB were studied. First, mitogenic responses to CB were determined by the enumeration of ^3H -thymidine incorporation into cultured spleen and thymic cells by pulse labeling. Both cell suspensions prepared from DDY mice were cultured with the indicated amount of CB as compared with LPS and Con A. The results are shown in Table 1. Stimulation of mitogenic responses in splenocytes was observed to be dose dependent. Maximum stimulation of thymidine uptake was seen with $5.0 \mu\text{g}$ of CB per 0.2 ml well. However, throughout the studies, the mitogenic response with CB was somewhat lower than those of LPS and Con A. On the other hand, no mitogenic activity with thymocytes was observed. Time course of mitogenic responses with the spleen cells induced by $5.0 \mu\text{g}$ of CB was studied and compared with that of LPS (Fig. 2). Mitogenic index increased with time, reached maximum at day 2 and then decreased. The incorporation profiles of thymidine were similar to those of LPS. These results indicate that CB has B-cell mitogenicity.

Effect of CB on antibody formation: Antibody formation after CB treatment in mice was determined using a PFC assay method with the spleen cells. DDY mice were immunized intraperitoneally with 1×10^7 SRBC and concomitantly given intraperitoneal injections of 0.5, 1.0, 2.0 and 5.0 mg/mouse of CB. Four days later, the number of anti-SRBC IgM forming cells in the spleen was determined. As shown in Table 2, a marked enhancement of PFC numbers in the spleen was observed in groups of mice treated with 1.0 to 5.0

Table 1. Mitogenic responses of CB to lymphocytes from DDY mice

Cell cultured	Sample(μ g/well)	$[^3\text{H}]$ Thymidine uptake (Mean cpm $\pm$ S.D.)**	Significance(p)**
Thymocyte*	None	585.3 $\pm$ 113.5	
	Con A (2.0)	44913.0 $\pm$ 1159.1	<0.001
	LPS (10.0)	1089.3 $\pm$ 128.2	<0.1
	CB (1.0)	306.3 $\pm$ 42.6	n.s.
	(5.0)	424.3 $\pm$ 7.0	n.s.
	(10.0)	619.0 $\pm$ 101.8	n.s.
Splenocyte*	None	5517.3 $\pm$ 923.5	
	Con A (2.0)	31823.3 $\pm$ 881.1	<0.005
	LPS (10.0)	39741.6 $\pm$ 1181.1	<0.001
	CB (1.0)	9244.0 $\pm$ 1396.4	n.s.
	(5.0)	1722.46 $\pm$ 1554.7	<0.01
	(10.0)	16701.3 $\pm$ 846.3	<0.01

*Thymocytes or splenocytes were cultured with the indicated doses of Con A, LPS and CB for 2 days in microtiter plates at cell density of 1×10^6 cells/ml in a total volume of 0.2ml. After 2 days, incorporation of $[^3\text{H}]$ thymidine was determined.

***Mean cpm $\pm$ standard deviation.

***n.s.: not significant. Statistically different from control at the indicated p values.

Fig. 2. 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 μ g of CB (○) or 10 μ g of LPS (●). $[^3\text{H}]$ Thymidine was added to the culture and after 4 hr incubation, incorporation ratio of the activities were determined.

mg/mouse of CB; the maximum dose was 2.0 mg/mouse and the numbers of PFC declined with the dose of 5.0 mg/mouse.

Delayed-type footpad reaction: When delayed-type footpad swelling was measured with CB doses ranging from 0.1 to 10.0 mg, a slight increase (statistically significant) in swelling was observed with doses from 1.0 to 5.0 (data not shown).

Time course of NK cell and macrophage

activity: Activities of macrophages and NK cell after CB treatment were studied and the results are shown in Fig. 3. Maximum NK cell activity of spleen cells was observed on day 3 and then decreased rapidly. Macrophage activity of PEC, meanwhile, gradually increased and attained maximum on day 9 to 10.

Effect on phagocytic activity by macrophage: To further characterize macrophage activation, phagocytic activity was measured as an index of macrophage activation. As shown in Table 3, when DDY mice were given an intraperitoneal injection of 1 mg/mouse of CB, phagocytic activity of macrophages was significantly enhanced and the increase was three times higher than that of control.

IFN production: In the above experiments, we demonstrated the high stimulation of macrophage and NK cell functions. Since macrophage and NK cell have been reported to be stimulated by IFN, we attempted to determine the IFN inducing activity of CB. When DDY mice were given CB intraperitoneally at doses of 0.1 to 10 mg/mouse, high titers of IFN were observed. Optimum CB dose for the IFN production was 1 mg/mouse as shown in Fig. 4. The maximum IFN activity was 2,560 units, while there were only 320 units of IFN titer with a positive control of OK-432. Time course of IFN production is shown in Fig. 5. IFN production occurred as early as 18 hr after administration of CB and reached a maximum at 24 hr. Thereafter, IFN activity fell rapidly and returned to a normal level within 7 days.

Table 2. Adjuvant effect of CB on antibody response to SRBC

Stimulant	CB dose(mg/mouse)*	Anti-SRBC PFC/spleen (mean PFC $\pm$ S.D.)**	Significance(<i>p</i>)***
SRBC only	0	61213 $\pm$ 3882	
SRBC+CB	0.5	81082 $\pm$ 7226	n.s.
"	1.0	190492 $\pm$ 1445	<0.005
"	2.0	275569 $\pm$ 5336	<0.005
"	5.0	169655 $\pm$ 846	<0.005

*Five male DDY mice were immunized intraperitoneally with 1×10^7 SRBC and treated with the indicated doses 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.

**Mean PFC number in the spleen cells $\pm$ standard deviation.

***n.s.: not significant. Statistically different from control at the indicated *p* values.

Table 3. Phagocytic activity of macrophages from CB treated DDY mice

Treatment regimen*	%Macrophage engulfing SRBC (Mean $\pm$ S.D.)***	SRBC/macrophage** (Mean $\pm$ S.D.)***
Control	44.4 $\pm$ 5.3	151.0 $\pm$ 28.7
CB(1.0mg/mouse $\times$ 3 days)	80.2 $\pm$ 6.7****	421.0 $\pm$ 80.7****

*CB was injected intraperitoneally for 3 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.

**Number of SRBC found in 100 macrophages including both engulfing and non-engulfing cells.

***Mean number $\pm$ standard deviation.

****Statistically different from control at *p*<0.05.

Fig. 3. Time course of NK-cell and macrophage activities after CB treatment. Macrophage (○—○) and NK cell (●—●) activities (mean $\pm$ standard deviation).

Production and characterization of interferon in different mouse strains: In order to determine the response of different mouse strains to CB, 6 strains of mice, C57BL/6, C3H/He, Balb/c, BDF₁, ICR and DDY mice were used. These different mouse strains were

Fig. 4. Production of IFN in DDY mice by various doses of CB. Pooled sera were obtained from DDY mice 24 hr after intraperitoneal injection of various doses of CB (○). OK-432 (▲) was used as a reference. Five mice were used in each dose.

treated with CB and IFN titers at 24 hr were determined. As shown in Table 4, there were significant differences in IFN production among the mouse strains tested. The highest response was observed with DDY and BDF₁, followed by C57BL/6 and ICR, and the lowest response

Table 4. Induction and characterization of IFN induced by CB treatment in various mouse strains

Mouse strain	IFN titers(unit)				
	IFN produced* Control	CB	Residual IFN titer(unit/ml)after tereatment with PH 2	Heat	Anti- α/β **
DDY	<10	2560	<10	<10	2560
C57BL/6	20	1280	<10	<10	NT
C3H/He	40	320	<10	<10	NT
ICR	10	1280	<10	<10	NT
Balb/c	<10	640	<10	<10	NT
BDF1	<10	1280	<10	<10	NT

*Pooled sera were obtained from 6 to 8 DDY mice from each group 24 hr after intraperitoneal injection of 1.0 mg/mouse of CB.

Each treated serum was assayed immediately for the antiviral activity.

**Result after neutralization by antiserum.

Fig. 5. Time course of IFN production in DDY mice. Five mice were used in each experiment.

Fig. 6. Effect of recombinant IFN α -(A/D) on *Candida albicans* infection in mice. IFN (1×10^4 units of IFN α -A/D) were administered intravenously into the tail vein of DDY mice. Control (—) and interferon treated group (---).

was observed with C3H/He and Balb/c mice. The difference was among them more than 4 fold. In order to determine the type of IFN induced by CB, various physicochemical properties were compared. IFN activity was diminished by dialysis against 0.1 M glycine-HCl buffer (pH 2.0) for 24 hr at 4°C or incubation at 56°C for 60 min, and not decreased by treatment of anti-IFN (α/β), indicating that the present major IFN activity is similar to the immune-type interferon, IFN- γ reported by Falcoff¹³.

Effect of recombinant IFN α -(A/D) on *C. albicans* infection in mice: Since it was found that a high titer of IFNs is produced after CB treatment in DDY mice, protection activity of recombinant IFN α -A/D against *Candida* infection was studied. As shown in Fig. 6, IFN α -A/D showed a significant protection activity when administered intravenously. These data suggest the important role of IFNs as effectors in the protection against *Candida* infection in mice.

DISCUSSION

In the present studies, we first confirmed that CB has

various immunomodulating activities which have already been reported in various bacterial BRMs such as bacillus Calmette-Guerin (BCG)¹⁴, PSK¹⁵, MDP¹⁶, LPS¹⁷ and OK-432¹⁸. However, it has been reported that there exist subtle differences in immunomodulating activity among these bacterial preparations¹⁹. Furthermore, living spores of *C. butyricum* have been used clinically over the past 40 years in Japan and their clinical safety has been confirmed². Therefore, CB is considered worth of further evaluation as an active BRM for anti-candida therapy, although detailed studies are necessary for its application.

Most BRMs can be classified into two groups due to their immunomodulating activities: Poly ICLC and MVE-2 belong to one group and have ability to activate macrophage. Thymosin fraction 5 belongs to the other and these immunomodulators have an apparent direct effect on T-cells¹⁹. According to this definition, CB might belong to the former group, because it showed only a weak delayed-type hypersensitivity and mitogenic response with thymocytes.

Throughout the present studies, we used mainly

heat-killed whole cell preparation of *C. butyricum* (CB). However, we were able to demonstrate that the active principles of CB are extractable with organic solvent. MCB fraction is partially purified by Sephadex LH-20 column chromatography. In our preliminary studies, MCB was found to be a potent NK-cell activator and to have B-cell mitogenic activity. Further purification studies are now in progress.

Since some bacterial preparations have been also reported to have IFN inducing activity both *in vitro* and *in vivo*²⁰⁾, we attempted to identify the antiviral principle. The present experiments showed that IFNs produced by CB treatment is mainly γ -type.

With reference to effectors, IFNs were considered to have the most potential as candidates with anti-candida activity in mice. Actually it was found that recombinant IFN α -A/D showed a protection activity when administered intravenously. Although IFN produced by CB was mainly γ -type, production of α - and/or β -type IFN(s) by CB could not be excluded. Recently Brummer *et al.*²¹⁾ and Kagaya *et al.*²²⁾ reported *in vitro* and *in vivo* anti-candida activity of recombinant IFN- γ .

Therefore further detail studies on the role of each type of IFNs against the *Candida* infection are necessary. However, we found that lentinan also exhibits strong protection against *C. albicans* infection (Kaneda *et al.*, manuscript in preparation). As already reported²³⁾, and we also confirmed it, production of IFN by lentinan is very weak or negligible. Therefore, it is reasonable to consider that some other factors such as interleukins, in the present model might be playing a role as effectors to protect mice against the infection.

We also found that IFN production by CB is strain dependent and DDY or BDF₁ mice were high responders in IFN production by CB. Therefore, the experimental model using DDY mice and CB vaccine is considered a suitable tool for analysis of the protection mechanisms induced by CB treatment.

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