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Antitumor Activity of *Bifidobacterium spp.* Isolated from a Healthy Korean

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The antitumor activity of *Bifidobacterium breve* K-110, and K-111, and *B. infantis* K-525 was investigated. These *Bifidobacterial* cells and their cell wall preparations (WPG) significantly increased the survival rate of mice who had been intraperitoneally implanted with sarcoma 180 cells. Solid tumor growth was inhibited even when the sarcoma 180 cells were implanted into the groins of the mice. However, the *Bifidobacterial* cells did not show *in vitro* cytotoxicity against tumor cell lines. Cell kinetic studies revealed that these WPGs induced neutrophils, which were followed by macrophages, at the site of peritoneal injection. The WPGs directly activated these cells to inhibit the growth of tumor cells in *in vitro* assays. Our results suggest that *Bifidobacterial* WPGs induce and activate nonspecific phagocytes *in situ* to reject growing tumor cells in the mouse peritoneal cavity.

Key words: Bifidobacteria, Antitumor activity, Cell wall, Cytotoxitiy

INTRODUCTION

The antitumor activity of several natural products and synthetic compounds has been well established. It appears that bacterial preparations possess marked inhibitory activity on the growth of syngeneic or autochthonous tumors *in vivo* (Adachi, 1992; Salminen *et al.*, 1984; Simon and Gorbach, 1984). The antitumor activity of these bacterial preparations has been generally considered to be due to a number of host defense mechanisms. In the last 20 years, antitumor activity through macrophage activation has been demonstrated for chemically well-defined bacterial components (Fukui *et al.*, 1987; Hashimoto *et al.*, 1979; Keller *et al.*, 1989; Konenko *et al.*, 1996). The cell wall preparations of *Bifidobacteria* and *Lactobacilli* have a higher efficacy on the regression of established tumors in mice, because they are active stimulators of the host-mediated immune response at the tumor-growing sites (Clemmesen, 1989; Sekine *et al.*, 1985; Sekine *et al.*, 1990). However, the number of immunological studies on *Bifidobacteria*, especially with respect to the activation of antitumor immunity, is quite low when compared to the number of studies on *Lactobacillus* strains. Hashimoto *et al.* (1979) and Sekine *et al.* (1994, 1995) have demonstrated that peritoneal cells collected from both viable and nonviable *B. infantis* injected into BALB/c mice suppressed the growth of Meth A tumor cells. However, the studies related to the many types of *Bifidobacteria* are not complete.

Therefore, we investigated whether *Bifidobacterium breve* K-110 and K-111, and *B. infantis* K-525 isolated from Korean intestinal microflora and their cell wall preparations function as an adjuvant for inducing and enhancing antitumor immunity in tumor bearing animals. We also investigated whether neutrophils and then macrophages could be massively induced at the sites of these *Bifidobacterial* WPG injections.

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MATERIALS AND METHODS

Materials

General anaerobic medium (GAM) was purchased from Nissui Pharm. Co, Ltd., (Japan). Tryptic soy broth (TS) and agar were purchased from Difco, Co., (USA). The other reagents used were of analytical grades. *Bifidobacterium spp.* (*B. breve*, K-110, K-111, and *B. infantis* K-525) were isolated according to a previously utilized method (Park

et al., 1998; Han et al., 1999).

Culture of *Bifidobacteria* and cell wall preparation

The *Bifidobacteria* were subcultured in GAM broth for 24 h. The cultured bacteria were then inoculated into TS broth containing 0.1 % ascorbic acid and 0.01 % sodium thioglycolate and cultured for 24 h at 37°C. Purification of WPG from the cultured bacteria (100g) was performed as described by Sekine et al. (1985). The purified WPG was suspended in saline, and diluted to the indicated concentration before use.

Animals

Forty male mice (ICR 15 g) were purchased from the National Institute of Toxicology Research, Seoul, Korea. They were provided with tap water and a normal diet (Lab Chow, Samyang Co., Ltd., Korea), and housed at 23 °C, 55 ± 10% humidity for ten days before the experiment.

Assay of *in vivo* antitumor activity

Sarcoma 180 cells maintained in the peritoneum of the mice were used for the antitumor activity assay. A mouse was sacrificed on the seventh day after the inoculation of tumor cells, and the ascitic fluid was collected. After the cells were washed three times with ice-cold saline, the cell suspension was diluted to 1×10^7 cells/ml. The mice were injected intraperitoneally with 0.1 ml of these suspended sarcoma 180 tumor cells (1×10^6 cells) in order to examine the effects on life span, and into the left groin to investigate the effects on solid tumors. Each *Bifidobacterium spp.* was administered intraperitoneally for ten days beginning 24 h after the inoculation of tumor cells. Observation was continued for 50 days for the measurement of life span, and for 26 days for the measurement of the inhibition of solid tumor growth.

In vitro cytotoxicity assay

The *in vitro* cytotoxicity was assayed against A549 (human lung cancer cell line), SNU C4 (human colon cancer cell line), P388 (mouse lymphoid neoplasma cell line), L1210 (mouse lymphocytic leukemia cell line) and MA104 (Macacus rhesus monkey kidney cell line) according to the method proposed by Carmichael et al. (1987). Each cultured cell line was harvested, counted, and inoculated in 96-well microtiter plates at the appropriate concentrations (180 μl volume: 4×10^4 cells/ well for P388 and L1210; 3×10^4 cells/ well for A549, SNU C4 and MA104). The P338 and L1210 cell lines were then cultured for two hours. The SNU C4 and MA104 cell lines were cultured for 24 h. The cells were exposed to the test microbe fractions (1 mg/ml) for two days at 37°C. Fifty μl of MTT solution (2 mg/ml in PBS) was added to each well and the plates were incubated for 4 h. After aspiration of the medium, DMSO (100 μl) was added to solubilize the MTT-formazan product. The plates were then read on a microplate reader (540 nm). The 50% inhibitory concentration (IC_{50}) of tumor cell growth was determined.

Preparation of effector cells

Peptone-induced peritoneal exudate cells (P-PEC) were collected from ICR mice who were injected twice i.p. with 2.5 ml of 3% Bacto-peptone, 15 and three hours previously. Thioglycolate-induced peritoneal exudate cells (TG-PEC) were harvested from ICR mice who were injected twice i.p. with 3 ml of 3% NIH thioglycollate broth four or five days previously. WPG-induced peritoneal exudate cells (WPG-PEC) were harvested from ICR mice who were injected i.p. with 1000 μg of WPG 16 h previously. These cells were washed with Hanks' balanced salt solution and suspended in a modified RPMI 1640 medium.

In vitro assay of growth inhibition assay

Growth inhibition was assayed using the method proposed by Sekine et al. (1994), with some modifications. In brief, sarcoma 180 tumor cells were incubated with various concentrations of effector cells in the presence or absence of WPG in a tissue flask at 37°C for four to seven days in a 5% CO_2 incubator. A volume of 0.1 ml was transferred in 1 ml of RPMI 1640 medium containing 5% FBS, in triplicate for five to seven days. The transferred tumor cells were then quantified using cell counter. The direct inhibition of tumor cells by WPG was negligible (<10% inhibition) even at the highest concentration (100 μg/ml).

RESULTS AND DISCUSSION

Antitumor activity of *Bifidobacterium* spp. isolated from a healthy Korean.

We measured the antitumor activity of *Bifidobacterium* spp. (*B. breve* K-110, *B. breve*, K-111, and *B. infantis* K525) isolated from a healthy Korean in the peritoneal cavities of mice bearing sarcoma 180 cells (Table I). *Bifidobacterial* whole cells showed significant prolongation activity at a dose of 100 mg/kg. Among the *Bifidobacteria* tested, *B. breve* K-110 exhibited the strongest activity, It's cure rate was 30% and the rate of survival was 147.3% compared to that of the control group. We measured the antitumor effect of the cell wall fractions of these *Bifidobacteria* on mice bearing sarcoma 180 in the peritoneal cavity, because cell wall fractions have been known to exhibit potent antitumor activity (Table II). None of the sarcoma 180-treated control mice survived. However, when mice bearing sarcoma 180 were treated with cell wall fractions of *B. breve* K-110 and K-111, the cure rate was 20-60%. This was significantly better than the cure rate of mice

Table I. Antitumor activity of *B. breve* K-110, K-111 and *B. infantis* K-525 on mice bearing sarcoma 180

Group[a]	Cure rate (Survivor/total mice)	Mean of survival days (Mean ± SD)	Rate of survival period (%)
Saline control	10/10	>50	
Sarcoma 180 treated control	1/10	18 ± 2[b]	100
B. breve K-110	3/10	26.5 ± 3.1*	147.3
B breve K-111	2/10	24.3 ± 4.0*	135.2
B. infantis K-525	2/10	24.0 ± 5.2	133.2
B. infantis JCM 3126	1/10	22.8 ± 4.4	126.6

a) Sarcoma 180 cells (1×10^6 cell/mouse) were inoculated intraperitoneally into ICR mice and then followed by *Bifidobacteria* administration for ten consecutive days.
b) Mean ± standard deviation.
*Statistically significant compared to control data ($p<0.05$).

Table II. Antitumor activity of the cell wall preparations of *B. breve* K-110 and K-111 on mice bearing sarcoma 180

Group[a]	Dosage (mg)	Cure rate (Survivor/total mice)	Mean of survival days (MeanSD)
Saline control		10/10	
Sarcoma 180 treated control		0/10	31.4 ± 3.8[b]
Sarcoma 180 + K-110 WPG	25	2/0	44.5 ± 5.8*
	100	6/10	-
Sarcoma 180 + K-111 WPG	25	4/10	42.5 ± 4.5*
	100	6/10	-
Sarcoma 180 + K-525 WPG	25	0/10	42.2 ± 38*
	100	0/10	-

a) Sarcoma 180 cells (0.5×10^6 cell/mouse) were inoculated intraperitoneally into ICR mice and then followed by *Bifidobacteria* administration for ten consecutive days.
b) Mean ± standard deviation.
*Statistically significant compared to control data ($p<0.05$).

treated with whole cell fractions.

The inhibitory activity of *Bifidobacterium spp.* on solid tumor growth was measured (Table III). *Bifidobacteria* whole cells significantly inhibited solid tumor growth. The antitumor activity of the cell wall fracitons was similar to that of the whole cell groups.

We investigated the *in vitro* cytotoxicity against tumor cells of the whole cell and WPG of *Bifidobacteria* (Table IV). They did not exhibit cytotoxicity against tumor cell lines.

Our results suggest that the antitumor activity of the *Bifidobacterium* originates from its WPG, and that this bacterium should be an active stimular of the host-mediated immune response at tumor-growing sites.

Table III. Antitumor activity of *Bifidobacteria* isolated from Korean intestinal microflora against solid tumor cells

Group[a]	Dosage (mg)	Weight of solid tumor (g)
Saline control		0
Sarcoma 180 treated control		3.76 ± 1.10[b]
Sarcoma 180 + K-110 whole cell	25	2.03 ± 0.97*
Sarcoma 180 + K-111 whole cell	25	1.97 ± 0.89*
Sarcoma 180 + K-525 whole cell	25	2.35 ± 1.02
Sarcoma 180 + K-110 WPG	25	2.13 ± 1.38
	50	1.87 ± 1.25*
Sarcoma 180 + K-111 WPG	25	2.01 ± 1.44
	50	1.9 ± 1.42*

a) Sarcoma 180 cells (1×10^6 cells/mouse) were subcutaneously injected into the left groin of ICR mice. This was followed by *Bifidobacteria* administration for ten consecutive days.
b) Mean ± standard deviation.
*Statistically significant compared to saline control data ($p<0.05$).

Kinetics of peritoneal and thoracic exudate cells after WPG injection

The antitumor effects of WPG on the peritoneal cavity may be mediated by immune cells induced and activated at the injection site. Therefore, we measured the kinetics of exudate immune cells in the peritoneal cavities of ICR mice after a single 200 μg WPG injection (Table V). The average number of resident cells (day 0) in the peritoneal cavity are approximately 1.5×10^6 cells/mouse. These cells consisted mainly of lymphocytes as determined by Giemsa's stain (data not shown). However, by administering these WPGs i.p., the number of neutrophils and macrophages was significantly increased. Sixteen hours after WPG injec-

Table IV. *In vitro* cytotoxicity of *Bifidobacteria* against tumor cell lines

Microbe[a]	Inhibition (%)							
	L1210	P388	SNU1	Sarcoma 180	SNU C4	A549	HepG2	MA104
Intact cell								
Bifidobacterium K-110	-	-	-	-	-	-	-	-
Bifidobacterium K-111	-	-	-	-	-	-	-	-
Bifidobacterium K-525	-	-	-	-	-	-	-	-
Cytosolic fraction								
Bifidobacterium K-110	-	-	-	-	-	-	-	-
Bifidobacterium K-111	-	-	-	-	-	-	14	-
Bifidobacterium K-525	-	-	-	-	-	-	-	-
Membrane fraction								
Bifidobacterium K-110	-	-	-	-	-	-	-	-
Bifidobacterium K-111	-	-	-	-	-	-	-	-
Bifidobacterium K-525	-	19	-	-	-	-	-	-

[a]The final concentration of each microbe was 1 mg/ml.

Table V. Number of peritoneal exudate cells after WPG injection in mice

Group	Number of total cells (ml)	Neutrophil (%)	Macrophage (%)	The others (%)
Saline	1.5×10^6	13.3	16.7	70
Bacto-peptone	4.0×10^6	63.3	23.3	13.4
Thioglycolate	4.8×10^6	20.0	66.7	13.3
B. breve K-110	1.2×10^7	73.3	16.7	10
B breve K-111	3.9×10^6	66.7	23.3	10
B. infantis K-525	1.2×10^7	63.3	23.3	13.4

tion, neutrophils, macrophages and lymphocytes accounted for 63.3-73.3%, 16.7-23.3% and 10-13.4% of the peritoneal exudate cells, respectively. However, these neutrophis disappeared quickly and the number of macrophages gradually increased.

Direct stimulation of the antitumor activity of immune cells with WPG *in vitro* culture

Injection of WPG into the peritoneal cavity induced neutrophil and macrophage production at the injection site. We tested whether WPG can stimulate the antitumor activity of these cells directly *in vitro* (Fig. 1). In this study, we first used peptone-induced peritoneal exudate cells (P-PEC) as a stimulation model of PMNs *in vitro*, because approximately 63.3% of P-PEC consists of neutrophils by the stimulation of peptone. When P-PEC was co-cultured with sarcoma 180 in media with various concentrations of WPG, P-PEC dose-dependently inhibited the sarcoma 180 cells. Tumor growth was completely inhibited at a concentration of 100 μg/ml. Next we used thioglycollate-elicited peritoneal exudate cells (TG-PEC) as a model of macrophage stimulation. When TG-PEC was co-cultured with sarcoma 180, TG-PEC efficiently inhibited the growth

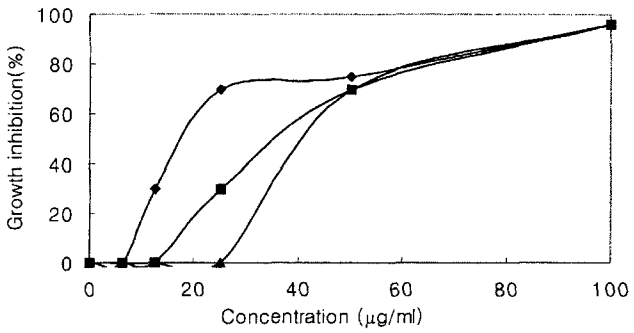

Fig. 1. Sarcoma 180 growth inhibition activity of P-PEC stimulated with various concentrations of WPG and bacteria. Symbols indicate the following: ◆, *B. breve* K-110; ■, *B. breve* K-111; ▲, *B. infantis* K-525.

of sarcoma 180 cells (Fig. 2). We also measured the antitumor activity of WPG-induced peritoneal exudate cells (WPG-PEC) in the same system (Table VI). When these WPG-PECs were co-cultured with sarcoma 180 in media containing WPG, they weakly inhibited the sarcoma 180 cells.

WPG significantly activated and enhanced these effector cells in the peritoneal cavity. Peritoneal exudate cells in

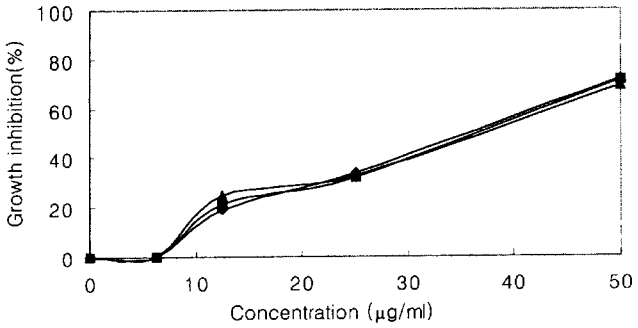

Fig. 2. Sarcoma 180 growth inhibition activity of TG-PEC with WPG and bacteria. Symbols indicate the following: ◆, *B. breve* K-110; ■, *B. breve* K-111; ▲, *B. infantis* K-525.

Table VI. Sarcoma 180 growth inhibitory activity of WPG-PEC

E:T	Inhibition			
	Saline	*B. breve* K-110	*B. breve* K-111	*B. infantis* K-525
0	-	-	-	-
12.5	-	-	-	-
25	-	-	-	-
50	-	+	+	±

-, not inhibited; ±, weakly inhibited; +, potently inhibited

vivo stimulated with WPG also demonstrated antitumor activity against Sarcoma 180. However, the antitumor activity of WPG-PEC was weak compared to the antitumor activity of TG-PEC stimulated with WPG *in vitro*. Similar results from a microbial preparation of *Lactobacillus casei* in mice have been reported. Furthermore, many types of bacteria reportedly activate a panel of antitumor mechanisms, including cytotoxic cytokines and natural killer cells. We also demonstrated that the *Bifidobacteria* cell wall, WPG, could induce and enhance antitumor activity *in vitro* and *in vivo*. Accordingly, we suggest that intestinal *Bifidobacterium breve* in human intestines may play a role in the prophylaxis of some cancers, such as colon cancer.

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