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A New Morphologically Characterized Cell Wall Preparation (Whole Peptidoglycan) from *Bifidobacterium infantis* with a Higher Efficacy on the Regression of an Established Tumor in Mice

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ABSTRACT

Three kinds of morphologically distinct cell wall preparations were isolated from heat-killed *Bifidobacterium infantis* and examined for the relative antitumor efficacy with syngeneic Meth A fibrosarcoma in BALB/c mice. Ultrastructural examinations revealed that cell wall skeleton (CWS) did not retain morphologically recognizable cell wall structure but showed fibrous structure. By contrast, a new cell wall preparation, whole peptidoglycan (WPG), which was isolated from whole cells without being subjected to physically destructive methods, completely retained the intact cell wall structure. When WPG was disrupted by sonic treatment, it retained some degree of physical integrity of cell wall structure, as compared with CWS. The results of chemical analysis indicated that the three cell wall preparations had similar chemical properties. A single s.c. injection of either CWS, WPG, or sonicated WPG in a mixture with tumor cells resulted in a significant suppression of the tumor growth. They were of equally high activity. However, when WPG, sonicated WPG, or CWS was injected intralesionally five times into mice bearing 5-day-old tumors, the incidence of complete tumor regression was demonstrated to decrease in the order of 70, 40, and 20%, respectively. The *in vitro* cytotoxicity test excluded the possibility that the tumor cell destruction was the result of direct cytotoxicity of the cell wall preparations. From these findings, it was concluded that WPG was an active stimulator of host-mediated response at the tumor-growing sites.

INTRODUCTION

The ability of several natural products (8, 22, 28) or synthetic compounds (15, 22, 23) to modulate the host resistance to tumor growth has been well established. Among these studies, it has appeared that bacterial preparations, such as whole cells (12, 16, 26, 32), cell walls (27, 30), or other cellular components (10, 13, 30) possess marked inhibitory activity on the growth of syngeneic or autochthonous tumors *in vivo*. The antitumor activity with bacterial preparations has been generally considered due to a number of host defence mechanisms (11, 18, 20, 31). However, the actual processes and mechanisms whereby these preparations lead to tumor cell destruction *in vivo* are still poorly understood. The immediate aim of future studies designed to cast light on their *in vivo* mode of action should be the separation or preparation of the chemically defined active components having a high *in vivo* antitumor activity and being free of direct toxic properties.

In the last 10 years, the *in vitro* antitumor activity through macrophage activation has been demonstrated for chemically well-defined bacterial components, muramyl dipeptide, or its derivatives (3, 9, 25). In general, however, their *in vivo* effect was developed only when they were modified with chemical moieties or enclosed with liposomes (1, 6, 7, 24, 29, 30). Moreover, other chemically defined components, and CWS[2] of *Bacillus Calmette-Guérin* and *Nocardia rubra* CWS also have been reported to show a potent antitumor activity when associated with oil droplets (2, 31, 33). These findings suggest that to be active as an antitumor agent *in vivo*, these active components require to be administered in physically concentrated form.

In the present study, the 3 kinds of chemically defined and morphologically characterized cell wall preparations were isolated as active components from *Bifidobacterium infantis*. They afford an opportunity to study the antitumor effects of these 3 physical forms of cell wall preparations and to correlate the *in vivo* activity with physical structure. Our results provide highly promising evidence that the cell wall preparation having the physical structure integrity can be expected to behave as a highly effective antitumor agent *in vivo*.

MATERIALS AND METHODS

Mice. Male BALB/c mice were obtained at 6 weeks of age from Shizuoka Union for Experimental Animals, Shizuoka, Japan. They were used at 7 to 9 weeks of age in this study.

Tumors. Syngeneic fibrosarcoma, Meth A, was maintained by i.p. inoculation into BALB/c mice at weekly intervals. For antitumor assays *in vivo*, ascitic Meth A cells were washed 3 times with Dulbecco's phosphate-buffered saline (NaCl, 8 g/liter:KCl, 0.2 g/liter:Na_2HPO_4, 1.15 g/liter:KH_2PO_4, 0.2 g/liter, pH 7.4) and suspended to 10^6 viable cells/ml. For direct cytotoxicity tests *in vitro*, 2 tumor cell lines, P388 leukemia which had been chemically induced in DBA/2 mice, and Meth A fibrosarcoma, maintained in culture medium at 37° in a humidified atmosphere of 5% CO_2 in air were used.

Culture Medium. RPMI Medium 1640 (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, $NaHCO_3$ (2 mM), sodium pyruvate (1 mM), 1× nonessential amino acids, penicillin (100 units/ml), and streptomycin (100 μg/ml), were used.

Organisms. *B. infantis* Reuter, ATCC 15697, was obtained from the American Type Culture Collection and maintained in our laboratory. The

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[2] The abbreviations used are: CWS, cell wall skeleton; [^{3}H]dThd, tritiated thymidine; WPG, whole peptidoglycan; WPG-sonicated, sonically disrupted product of WPG; i.l., intralesionally.

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organisms were grown in semisynthetic medium[3] at 37° for 24 hr with slight stirring.

Cellular Fractionation of Heat-killed *B. infantis*. Heat-killed whole cells (at 65° for 40 min) were suspended in distilled water (water) and treated twice with ultrasonic oscillation for 20 min. The homogenate was then centrifuged 2 times at 800 × *g* for 30 min until no intact cells were visible by microscopic examination. This sonically disrupted material was designated as "disrupted-cell product." A part of the disrupted product was then centrifuged at 28,000 × *g* for 1 hr to separate into supernatant fluid and a pellet of insoluble component. The sediment was suspended with a glass homogenizer in 10 mM KH_2PO_4 and 5 mM $MgCl_2$, allowed to stand for 30 min at 4°, and washed 5 times with water by centrifugation. After the upper layer (white amorphous substance) of the sediment was discarded, the lower layer was suspended in water, dialyzed for 3 days, and lyophilized. This insoluble material was designated as "crude cell walls." The supernatant fluid was recentrifuged several times at 28,000 × *g* for 1 hr, and the final opalescent supernatant was 20-fold concentrated by lyophilization. The concentrated fluid was applied to a column of Sephadex G-15, and the fractions that were eluted in the void volume with a molecular weight greater than 1500 were collected and lyophilized. This material was called "supernatant fraction."

Purification of Crude Cell Walls. The crude cell walls (10 g, dry weight) were suspended in 200 ml of 20 mM $CaCl_2$:50 mM Tris-HCl buffer, pH 7.2 (Tris-HCl buffer) containing 10 mM $MgCl_2$, trypsin (1 mg/ml) (type IV; Sigma Chemical, St. Louis, MO), DNase (50 μg/ml) (type I; Sigma), and RNase (150 μg/ml) (type XII-A; Sigma) at 37° for 14 hr with magnetic stirring, 5 drops of toluene being added to prevent contamination. The insoluble material was suspended in Tris-HCl buffer (200 ml) containing trypsin (0.5 mg/ml) and α-chymotrypsin (0.5 mg/ml) (ICN Pharmachemicals, Inc., Cleveland, OH) and stirred at 37° for 14 hr. The digested residue was separated by centrifugation at 20,000 × *g* for 40 min, and the sediment was digested with pepsin (1 mg/ml; P-L Biochemicals, Inc., Milwaukee, WI) in 100 ml of 0.01 N HCl at 37° for 14 hr. The pepsin-digested residue was treated 3 times with Pronase P (1 mg/ml) (Kaken Pharmachemicals, Tokyo, Japan) in 100 ml of Tris-HCl buffer (pH 7.4) containing 3% (v/v) ethanol and then refluxed sequentially with methanol (200 ml), methanol:chloroform (1:1, v/v) (200 ml), and chloroform (200 ml). The preparation thus obtained (1.19 g, dry weight) will be called CWS.

Isolation of WPG and Its Sonicated Material. One hundred fifty g (wet weight) of heat-treated whole cells were treated with 500 ml of 0.5% Triton X-100 in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.0) for 1 hr with continuous stirring at 80–85°. The mixture was immediately cooled and washed thoroughly with water. The removal of detergent from the insoluble residue was achieved by successive washing with methanol:water (2:1, v/v), methanol, and acetone. The residual mass was incubated in 400 ml of Tris-HCl buffer (pH 7.2) containing 10 mM $MgCl_2$, trypsin (1 mg/ml), DNase (100 μg/ml) and RNase (150 μg/ml) at 37° for 14 hr. The insoluble residue was separated by centrifugation at 20,000 × *g* for 40 min and digested with trypsin (0.5 mg/ml) and α-chymotrypsin (0.5 mg/ml) in 400 ml of Tris-HCl buffer at 37° for 14 hr. The residue was treated with pepsin (1 mg/ml) in 200 ml of 0.01 N HCl and then with Pronase P (1 mg/ml) in 200 ml of Tris-HCl buffer (pH 7.4) at 37° for 14 hr. The Pronase-digested residue was delipided by successive refluxing with methanol (400 ml), methanol:chloroform (400 ml) (1:1, v/v), and chloroform (400 ml). The delipided material was digested 3 times with Pronase P (1 mg/ml) in 200 ml of Tris-HCl buffer (pH 7.4) at 37° for 14 hr and then dialyzed against water for 3 days. Finally, the insoluble material was treated with 0.01 N H_2SO_4 (100 ml) at 85–95° for 5 min. The mixture was immediately cooled and centrifuged at 15,000 × *g* for 30 min. The sediment was dialyzed against water for 7 days and lyophilized. This material will be called WPG (4.32 g, dry weight). WPG (0.50 g, dry weight) was suspended in water and physically disrupted 2 times by sonic treatment (20 min). The disrupted mixture was centrifuged at 28,000 × *g* for 1 hr. The resulting supernatant was discarded, and the sediment was dialyzed for 3 days and then lyophilized (0.42 g, dry weight). This preparation was designated as WPG-sonicated.

Analytical Methods. For determinations of amino acids and amino sugars, samples were hydrolyzed in sealed tubes with 6 N HCl for 16 hr at 105° and quantitative analyses were performed with an amino acid analyzer. For reducing sugars, samples were hydrolyzed with 1 N H_2SO_4 for 6 hr at 100 °. The hydrolysate was filtered and then applied to a small column of Amberlite IRA-410 (bicarbonate form). The content of sugars in the neutral eluent was estimated with gas-liquid chromatography by the usual method. For fatty acids, samples were saponified in 2.5% KOH in methanol:benzene (1:1) for 6 hr. The reaction mixture was cooled, acidified with 10% HCl, and extracted 3 times with diethyl ether. The ether extracts were combined, washed with water, and then dried over anhydrous sodium sulfate. The ether was removed by evaporation, and the residue was dried under reduced pressure and weighed. DNA and RNA were determined by the methods of Ceriotti (5).

Electron Micrography. The cell wall preparations were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 hr at room temperature and then washed thoroughly with phosphate buffer, followed by water. The fixed material was dehydrated by successive treatment with 25, 50, 75, and 100% methanol. The cell wall preparations were rotary shadowed with platinum:palladium. A part of the cell wall preparations was also examined by the method of tungstophosphoric acid stain. Electron micrographs were taken with a Hitachi H-600 transmission electron microscope.

Suspensions of Test Substance. The test substances were suspended in 5% mannitol solution (osmotic pressure, 283 to 284 mOsmol/kg), unless otherwise stated. The homogeneous suspension was achieved by ultrasonic oscillation for 15 sec at 4°. In some experiments, they were suspended in 0.85% NaCl solution.

Antitumor Activity *in Vivo*. Antitumor activity was evaluated by measuring the growth rate of tumor in mice after s.c. administration of active substance admixed with tumor cells or 1, 2, or 5 intralesional injections into the tumor-growing site using the Meth A tumor-BALB/c mouse system. In this study, the former protocol was referred to as the tumor suppression test, and the latter was referred to as the tumor regression test, according to the report of Ribi *et al.* (21). In the tumor suppression test, equal volumes of tumor cell suspension (1×10^6 viable cells/ml in phosphate-buffered saline) and the suspension of active substance were mixed just before tumor injection, and a 0.2-ml portion was inoculated s.c. into the right flank of each mouse. Control animals were inoculated with the admixture of the tumor cell suspension and 5% mannitol or 0.85% NaCl solutions. Tumor growth and incidence were measured twice a week with calipers for 30 days after tumor inoculation. In the tumor regression test, mice were inoculated s.c. with 10^5 tumor cells in the right flank. On Day 5 after tumor inoculation, unless otherwise stated, each of the active substances was injected i.l. 5 times into the palpable tumor, every other day. The growth of tumor was measured by the same methods as described above.

Direct Cytotoxicity on Tumor Cells. Meth A cells, 1×10^5, were distributed into each well of a 96-well round-bottomed culture plate (Flow Laboratories, Hamden, CT) in triplicate and were cultured with the desired concentration of test substances for 48 hr. In the final 20 hr of the culture, 0.5 μCi [^{3}H]dThd (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to each well. P388 cells (2×10^4) were distributed into each well and cultured with test substances for 6 hr. The pulsing time of [^{3}H]dThd was 90 min. The cells were harvested, and

[3] T. Toida, K. Sekine, M. Saito, M. Kuboyama, and T. Kawashima, manuscript in preparation.

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incorporated radioactivity was measured by liquid scintillation.

Statistical Analysis. The difference of tumor incidence in the tumor suppression test and the cure rate of tumor in the tumor regression test were analyzed by Fisher's test. The significance of tumor size was estimated by Cochran-Cox *t* test. Survival span of tumor-bearing mice was evaluated by Mann-Whitney *U* test.

RESULTS

Isolation of 3 Cell Wall Preparations from *B. infantis*. Heat-killed whole cells were disrupted by sonic treatment and then separated into opalescent supernatant and insoluble residue (crude cell walls). CWS was prepared by treatment of the crude cell wall fraction with proteolytic enzymes, nucleases, and organic solvents. On the other hand, cell wall preparation which had a nascent physical structure (WPG) was directly isolated from heat-killed whole cells. To maintain the physical integrity of the cell wall structure, this isolation procedure did not make use of any chemical or physical destructive processes. For comparative studies, physically disrupted WPG (WPG-sonicated) was also obtained by sonic treatment of WPG. The chemical properties of the 3 kinds of cell wall preparation, WPG, WPG-sonicated and CWS are shown in Table 1. WPG was composed of 67.3% neutral sugars (galactose:glucose in a M ratio of approximately 2.5:1.0), 8.6% amino sugars (glucosamine and muramic acid in an equimolar ratio), and 14.7% amino acids (alanine:glutamic acid:threonine:serine:ornithine, molar ratio about 3.8:1.0:1.0:1.3:1.0, respectively). The chemical composition of WPG was very similar to that of CWS and WPG-sonicated. Chemical analysis indicates that each of the 3 cell wall preparations constitute a highly purified insoluble complex consisting of polysaccharide and peptidoglycan polymers.

The results of electron microscopic observations of the 3 cell wall preparations are shown in Fig. 1, *A* to *E*, where it can be seen that the physical structure of WPG was morphologically distinct from that of PCW and WPG-sonicated. The physical structure of CWS was completely degraded during the isolation and chemical purification procedures (Fig. 1*B*). By contrast, WPG had the physically intact cell wall structure of the whole cells (Fig. 1*C*). The ultrastructural examination by the tungstophosphoric acid stain method (Fig. 1*E*) also demonstrated that WPG retained whole-cell shape (Fig. 1*A*) completely. The intact structure of WPG was disrupted by sonic treatment, but WPG-sonicated thus obtained maintained the recognizable cell wall structure somewhat, as compared with CWS (Fig. 1*D*). The results obtained in this experiment indicate that these 3 cell wall preparations have a similar chemical composition but apparently differ in physical form.

Antitumor Activity of Cell Wall Fraction of *B. infantis*. Table 2 shows the results of antitumor activity of the cellular fractions of heat-killed whole cells. As can be seen, the crude cell walls showed a high activity in the tumor suppression and the tumor regression tests. In contrast to the effectiveness of crude cell walls, the supernatant fraction had no significant activity. This result suggests that antitumor activity of heat-killed whole cells is mainly attributable to the cell wall portion. Unexpectedly, however, CWS had almost no effect on the progressively growing tumor, whereas other related components were reproducibly effective (Table 3). However, it is worth emphasizing that the possibility of CWS as an active principle structure cannot be excluded. Evidence to support this belief is supplied in Table 4, where it can be seen that CWS exhibited a high capacity to inhibit tumor cell growth, as evaluated in the tumor suppression test, similar to that seen with whole cells. Finally, CWS was

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Table 1

Chemical composition of WPG, WPG-sonicated, and CWS

Constituent	mg/g WPG	mg/g WPG-sonicated	mg/g CWS
Neutral sugars			
Glucose	191 (1061)[a]	177 (983)	186 (1033)
Galactose	482 (2678)	490 (2722)	530 (2944)
Amino sugars			
Glucosamine	37.84 (175.51)	34.50 (160.02)	29.97 (139.01)
Muramic acid	47.72 (189.97)	46.72 (185.99)	41.24 (164.17)
Amino acids			
Glutamic acid	23.41 (159.10)	24.19 (164.52)	23.40 (159.04)
Threonine	19.70 (165.38)	19.94 (167.52)	19.60 (164.53)
Serine	21.50 (204.59)	21.74 (207.00)	23.38 (222.48)
Ornithine	21.24 (160.07)	22.20 (197.96)	19.73 (149.29)
Alanine	54.04 (606.58)	54.76 (615.28)	54.69 (613.87)
Glycine	1.02 (13.59)	0.93 (12.40)	0.85 (11.32)
Lysine	0.95 (6.50)	0.92 (6.28)	0.78 (5.34)
Leucine	1.14 (8.69)	1.37 (10.44)	0.58 (4.22)
Valine	2.68 (22.88)	1.80 (15.28)	2.43 (20.74)
Isoleucine	0.81 (6.18)	0.78 (5.92)	0.45 (3.48)
Aspartic acid	0.53 (3.98)	0.49 (3.64)	0.41 (3.08)
Other amino acids[b]	0	0	0
Fatty acids	18.3	15.1	10.6
DNA	Tr[c]	Tr	Tr
RNA	Tr	Tr	Tr

[a] Numbers in parentheses, μmol/g.
[b] Other amino acids include phenylalanine, arginine, tyrosine, histidine, proline, and methionine.
[c] Tr, trace.

Table 2
Antitumor activity of cellular fractions of heat-killed whole cells

Cellular fraction	Tumor suppression test[a]		Tumor regression test[b]	
	Tumor incidence (tumor take/total no. of mice)	Mean diameter of tumor growth on Day 28 (mm)[c]	No. of cured mice/total no. of mice	Mean diameter of tumor growth on Day 26 (mm)[c]
Control, 5% mannitol	12/12	27.4 ± 2.0[d]	0/15	24.5 ± 2.3
Whole cells	5/12[e,f]	4.7 ± 3.7[f,g]	12/15[h,i]	1.5 ± 1.4[f,g]
Disrupted cell product	6/12[e,j]	6.7 ± 3.7[g,k]	10/15[h,i]	4.2 ± 3.7[g,k]
Crude cell walls	4/12[e,f]	4.7 ± 3.8[f,g]	14/15[h,i]	1.1 ± 2.1[f,g]
Supernatant	9/12[e,k]	12.9 ± 4.5	6/15[h,j]	6.6 ± 2.8

[a] Mice were inoculated s.c. in the right flank on Day 0 with 10^5 tumor cells with or without 100 μg of cellular fraction.
[b] Mice were inoculated s.c. on Day 0 with 10^5 tumor cells, and cellular fraction (100 μg) was injected i.l. 5 times every other day from Day 5.
[c] All animals in each group are included. The diameter of tumor in tumor-free animals was calculated as 0.
[d] Mean ± S.D.
[e] Statistical analysis for tumor incidence with control group.
[f] $p < 0.01$.
[g] Statistical analysis for tumor diameter with supernatant-treated group.
[h] Statistical analysis for cure rate of tumor with control group.
[i] $p < 0.001$.
[j] $p < 0.05$.
[k] Not significant.

Table 3
Effect of i.l. injection of CWS and related cellular fractions on established Meth A tumor

Mice were inoculated s.c. with 10^5 tumor cells on Day 0, and CWS (100 μg) or cellular fraction (100 μg) was injected i.l. 5 times every other day from Day 5.

Test substance	No. of cured mice/total no. of mice	
	Experiment 1	Experiment 2
Control 1, 5% mannitol	0/20	ND[a]
Control 2, 0.85% NaCl solution	ND	0/34
CWS	1/20[b,c]	4/34[c,d]
Whole cells	16/20[b,e]	23/34[d,e]
Disrupted cell product	11/20[b,e]	ND
Crude cell walls	15/20[b,e]	ND

[a] ND, not done.
[b] Statistical analysis for cure rate of tumor with Control 1.
[c] Not significant.
[d] Statistical analysis for cure rate of tumor with Control 2.
[e] $p < 0.001$.

ascertained to have a significant inhibitory effect on the local growth of admixed tumor cells but had no capacity to cause the destruction of already established tumor.

Comparison of Antitumor Activity of WPG, WPG-sonicated, and CWS. The relative antitumor efficacy of 3 cell wall preparations is shown in Table 5. In the tumor suppression test, the tumor growth was significantly inhibited by 100 μg as well as 200 μg of the whole cells. However, the percentage of animals protected from tumor growth was significantly higher in mice given 100 μg of WPG than in mice given the same dose of the whole cells. The suppressive activity of WPG has a dose dependency in a range of 10 to 100 μg. The inhibitory effect of tumor incidence became detectable at a dose of 20 μg (Table 6). Ten μg of WPG had no discernible proportion of tumor incidence, but did have a positive inhibitory effect on the growth rate of the tumor (Chart 1). One hundred μg of CWS also profoundly inhibited the local growth of the tumor. In addition, the marked inhibition of tumor growth also occurred in mice given WPG-sonicated. Thus, the proportions of tumor-free animals in the cell wall-treated groups were approximately equivalent.

In the tumor-regression test, however, remarkable differences among the activity of 3 cell wall preparations were observed. WPG induced complete regression against the growth of tumor in a high proportion of animals. The efficacy of WPG was comparable to that of whole cells. Significant protection was also bestowed by WPG-sonicated. In comparison with WPG, however, this preparation was less effective. Furthermore, CWS, in spite of having a similar chemical composition as WPG, had little regressive activity. Statistical analysis of the proportion of cured mice indicated a significant difference between WPG-treated and CWS-treated groups ($p < 0.01$). Thus, the tumor-regressive activity of cell wall fraction was demonstrated to increase in

Table 4
Suppression of Meth A tumor growth with whole cells or CWS

Mice were inoculated s.c. in the right flank with 10^5 tumor cells with or without 100 μg of test substance on Day 0.

Experiment	0.85% NaCl solution (control)		Whole cells		CWS	
	Tumor incidence (tumor take/total no. of mice)	Mean survival time (days)	Tumor incidence (tumor take/total no. of mice)	Mean survival time (days)	Tumor incidence (tumor take/total no. of mice)	Mean survival time (days)
A	11/11	32.8 ± 2.9[a]	0/8		0/8	
B	11/11	37.5 ± 6.0	2/8	31, 35	0/8	
C	12/12	31.8 ± 2.2	4/12	45.0 ± 2.5	1/12	64
D	9/9	32.0 ± 3.6	1/9	49	0/9	
Total	43/43		7/37[b]		1/37[b]	

[a] Mean ± S.D.
[b] Significantly different from control group, at $p < 0.001$.

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Table 5
Antitumor activity of WPG, WPG-sonicated, and CWS

		Tumor suppression test[a]		Tumor regression test[b]	
Test substance	Dose (μg/mouse)	Tumor incidence (tumor take/total no. of mice)	Mean survival time (days)	No. of cured mice/total no. of mice	Mean diameter of tumor growth on Day 28 (mm)[c]
Control, 5% mannitol		20/20	38.7 ± 3.3[d]	0/40	29.4 ± 2.2
Whole cells	100	7/20[e]	52.4 ± 4.4[f]	16/20[e,g]	2.3 ± 2.7
	200	2/20[e,h]	41, 57[f]	ND[i]	
WPG	100	0/20[e,j]		14/20[e,k]	5.4 ± 4.8
WPG-sonicated	100	0/20[e,j]		8/20[e,l]	8.1 ± 4.1
CWS	100	3/20[e,m]	55.3 ± 2.0[f]	4/20[n]	16.2 ± 5.0

[a] Mice were inoculated s.c. in the right flank with 10^5 tumor cells and desired dose of each test substance on Day 0.
[b] Mice were inoculated s.c. with 10^5 tumor cells on Day 0, and test substance (100 μg) was injected i.l. 5 times every other day from Day 5.
[c] All animals in each group are included. The diameter of tumor in tumor-free animals was calculated as 0.
[d] Mean ± S.D.
[e] $p < 0.001$ compared with control group.
[f] Significantly different from control group at $p < 0.01$.
[g] $p < 0.001$ compared with CWS-treated group.
[h] $p > 0.10$ compared with 100 μg whole-cell-treated group.
[i] ND, not done.
[j] $p < 0.01$ compared with 100 μg whole-cell-treated group.
[k] $p < 0.01$ compared with CWS-treated group.
[l] $p > 0.30$ compared with CWS-treated group.
[m] $p > 0.20$ compared with 100 μg whole-cell-treated group.
[n] $p > 0.20$ compared with control group.

Table 6
Dose-dependent effect of WPG on Meth A tumor growth
Mice were inoculated s.c. in the right flank with 10^5 tumor cells and desired dose of WPG on Day 0.

	Dose (μg/mouse)	Tumor incidence (tumor take/total no. of mice)	Mean diameter of tumor growth on Day 27 (mm)[a]
Control, 5% mannitol		20/20	25.9 ± 1.8[b]
WPG	100	0/20[c,d]	0 (100.0)[e]
	50	3/20[c,d]	1.5 ± 1.9 (94.2)
	25	6/20[c,d]	2.2 ± 1.9 (91.5)
	20	13/20[c,f]	11.8 ± 4.9 (54.3)
	10	20/20	19.5 ± 3.3 (24.5)

[a] All animals in each group are included. The diameter of tumor in tumor-free animals was calculated as 0.
[b] Mean ± S.D.
[c] Statistical analysis for tumor incidence with control group.
[d] $p < 0.001$.
[e] Numbers in parentheses, percentage of inhibition.
[f] $p < 0.01$.

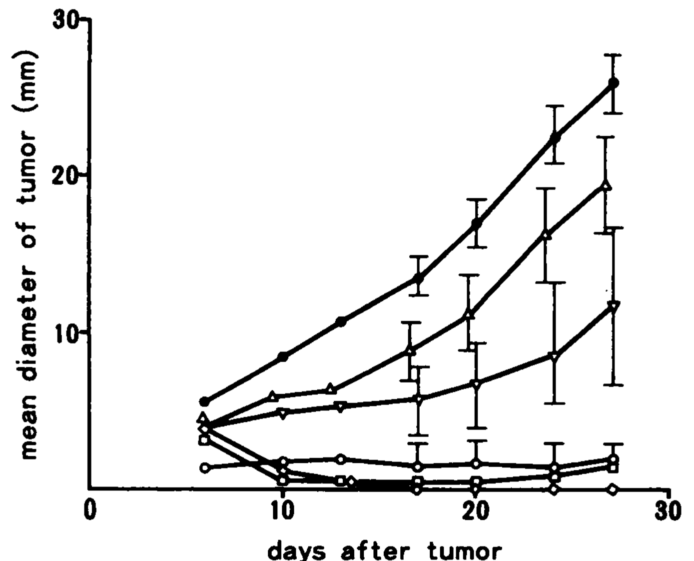

Chart 1. Effect of different doses of WPG on the growth rate of Meth A tumor. Mice were inoculated s.c. (in the right flank) with the mixture of 10^5 Meth A cells and 10 (△), 20 (▽), 25 (○), 50 (□), or 100 μg (◇) of WPG. Controls (●) received the mixture of tumor cell suspension and 5% mannitol solution. *Bars*, S.D.

proportion to the degree of integrity of its physical form.

The therapeutic effect of WPG was also examined using the Meth A-BALB/c system. The mice were inoculated s.c. with Meth A tumor cells (1 × 10^5), and then WPG (500 μg) was injected i.l. on Day 6 (Group 1), on Days 6 and 10 (250 μg each, Group 2), or on Days 6 to 10 (100 μg each, Group 3) after tumor inoculation. As shown in Table 7, a single injection of 500 μg of WPG had almost no effect on the established tumor, whereas multiple injections of WPG induced regressive effect. Particularly, 5 separate injections of 100 μg WPG induced complete regression of the established tumor. The mice cured with WPG showed systemic resistance to reinoculation with Meth A cells.

Direct Influence of Bacterial Preparations on Tumor Cell Growth *In Vitro*. One of the most important problems concerning the antitumor effect of the preparations tested in this study is whether or not these preparations have a direct cytotoxicity on Meth A tumor cells. To test this possibility, the effect of WPG on DNA synthesis of tumor cells was examined. As shown in Table 8, WPG had no inhibitory effect on [^{3}H]dThd uptake of Meth A and P388 cells in a range of 10 to 1000 μg. Whole cells and CWS also showed no effect in the same range. Viable cell counts by the method of trypan blue dye exclusion confirmed this result.

DISCUSSION

We have demonstrated that purified cell walls, which essentially consisted of only 2 hydrophilic polymers of polysaccharide and peptidoglycan, was responsible for the antitumor activity of heat-killed *B. infantis* and also that the cell wall preparation which had physical integrity of cell wall structure (WPG) was much more effective than were physically disrupted cell walls (CWS or WPG-sonicated) against the progressively growing tumor. These results provided highly suggestive evidence that the physical form of cell wall preparation played an important role in the

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Table 7

Effect of injection frequency of WPG-induced regression of Meth A tumor

Mice were inoculated s.c. with 10^5 tumor cells on Day 0, and injections of WPG were made i.l.

Injection frequency	No. of cured mice/total no. of mice	Mean survival days	Tumor take after second tumor challenge/total no. of mice tested[a]
Control (5% mannitol) on Days 6 to 10	0/15	39.9 ± 4.3[b]	[c]
WPG (500 μg) on Day 6	3/15[d, e]	48.6 ± 5.1[f, g]	0/3
WPG (250 μg) on Days 6 and 10	6/15[d, h]	50.8 ± 4.1[f, g]	0/6
WPG (100 μg) on Days 6 to 10	12/15[d, i]	48.3 ± 0.3[f, g]	0/12

[a] On Day 35, cured mice were reinoculated s.c. in the contralateral flank with 5 × 10^5 Meth A cells.
[b] Mean ± S.D.
[c] As control, 5 intact mice were challenged with 5 × 10^5 Meth A cells. All the control mice died with tumor growth.
[d] Statistical analysis for cure rate of tumor with control group.
[e] Not significant.
[f] Statistical analysis for survival days with control group.
[g] $p < 0.01$.
[h] $p < 0.05$.
[i] $p < 0.001$.

Table 8

Effect of WPG, whole cells, and CWS on [³H]dThd uptake in tumor cells

Test substance	Concentration of test substance in culture medium (μg/ml)	Tumor cells ([³H]dThd cpm)		
			P388	
		Meth A	Experiment 1	Experiment 2
0.85% NaCl solution		240 ± 17[a]	8,345 ± 249	7,939 ± 87
WPG	10	549 ± 16	ND[b]	ND
	50	ND	8,550 ± 370	10,930 ± 532
	100	598 ± 50	9,481 ± 341	11,434 ± 0
	500	ND	9,306 ± 111	11,753 ± 419
	1000	347 ± 36	9,762 ± 188	10,940 ± 455
Whole cells	10	279 ± 11	ND	ND
	50	ND	8,614 ± 241	8,125 ± 150
	100	263 ± 16	8,123 ± 249	8,896 ± 220
	500	ND	9,210 ± 53	9,661 ± 747
	1000	268 ± 26	11,030 ± 130	11,903 ± 173
CWS	10	322 ± 14	ND	ND
	50	ND	7,838 ± 236	11,692 ± 302
	100	325 ± 18	9,020 ± 115	11,456 ± 381
	500	ND	9,257 ± 127	10,889 ± 389
	1000	344 ± 6	9,371 ± 211	11,900 ± 149

[a] Mean ± S.D.
[b] ND, not done.

expression of the tumor-regressive activity.

The comparative study on the antitumor activity of the cellular components of whole cells revealed that an active principle structure existed in crude cell wall portion. However, the chemical purification of the crude cell wall fraction markedly reduced its regressive activity on the growing tumor without any detectable loss of its suppressive activity. Moreno *et al.* (19) demonstrated that intratumoral injection of cell walls isolated in pure form from *Corynebacterium parvum* failed to retard tumor growth in mice. They reported that a synergism between, or molecular association of cell wall and non-cell wall components might be required for complete antitumor activity. Certainly the possibility that the molecular interaction of several components in crude cell walls augments the tumor-regressive activity of the active principle cannot be entirely exclusive. Even so, however, the *in vivo* treatment with chemically complex substances, such as crude cell walls or whole cells is considered to induce undesirable side effects, which in turn limits their clinical application for tumor immunotherapy. Therefore, we decided to focus our subsequent studies on the isolation of purified cell walls with a high tumor-regressive activity *in vivo*.

WPG isolated in this study had a physically intact skeleton structure of whole cells and had a chemical composition similar to CWS. Therefore, it would seem reasonable to suggest that WPG has a bag-shaped structure formed by polymerization of active units consisting of polysaccharide and peptidoglycan.

The administration of WPG into BALB/c mice resulted in marked suppression of Meth A growth, and the effect was highly dosage dependent. Furthermore, WPG was clearly more effective than was CWS on the regression of established tumor. WPG-sonicated induced the inhibitory effect to a degree far in excess of that predicted when the structurally disrupted cell wall fraction was administered to established tumors in mice. However, this is not surprising in view of the ultrastructural appearance of WPG-sonicated; namely, the retention partly of the physical cell wall structure in WPG-sonicated would seem to be the most plausible explanation as to why WPG-sonicated could exhibit more potent activity than did CWS. Thus, a clear correlation was present between the induction of elevated tumor-regressive activity of the cell walls and their physical structures. By contrast, the tumor-suppressive activity of the cell wall preparations was not essentially affected by the physical element. Recently, Lepoivre *et al.* (14) demonstrated that trehalose dimycolate particles which have a liposomal structure in aqueous suspension, had a greater antitumor activity to syngeneic tumor, as compared with free trehalose dimycolate. This report confirms that even in the absence of oil or emulsion, active substances show an appreciable antitumor activity if they have a physically recognizable structure.

The *in vivo* role of the physical form of cell wall preparations is a question that might be answered by future experimentation. However, several possibilities can be proposed in this study. (*a*) WPG is able to remain longer than does CWS within the lesional s.c. tissues. This possibility is easily acceptable by a comparison of the physical forms of WPG and CWS with electron microscopic observation. According to this hypothesis, the potent antitumor effect of WPG would be considered to be the consequence of its longer persistence in the tissues. (*b*) WPG has the integrity of the skeletal structure, which has resistance to chemical degradation of the active sites by lysozyme or proteolytic enzymes in the mammalial tissues. In view of the physical form of WPG, polysaccharide polymer appears to screen peptidoglycan matrix from the external environment. By contrast, since the peptidoglycan portion of CWS is physically exposed, peptidoglycan may have a better opportunity to interact with lytic enzymes in the host. (*c*) WPG can be effectively phagocytized by macrophages owing to its physically recognizable structure. Recently, Mehta *et al.* (17) demonstrated that the uptake of liposome-encapsulated muramyl dipeptide derivatives was enhanced 20-fold

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greater than that of the free compounds by human monocytes *in vivo*. At present, studies are in progress in our laboratory to explore these or other possibilities.

The advantages in using chemically purified active substances as molecular probes of host-mediated cellular function are apparent. However, it is equally important to recognize that the physical form of the active substances shares important characteristics with the chemically required structure for the *in vivo* expression of antitumor activity. Cantrell and Wheat (4) also suggested the importance of the integrity of cell wall structure in the *in vivo* antitumoral potency. The importance of the physical element may depend upon the stage of tumor growth. The difference in the antitumor capacity of the physically distinct 3 cell wall preparations as observed in the tumor suppression and tumor regression tests strongly supports this latter possibility.

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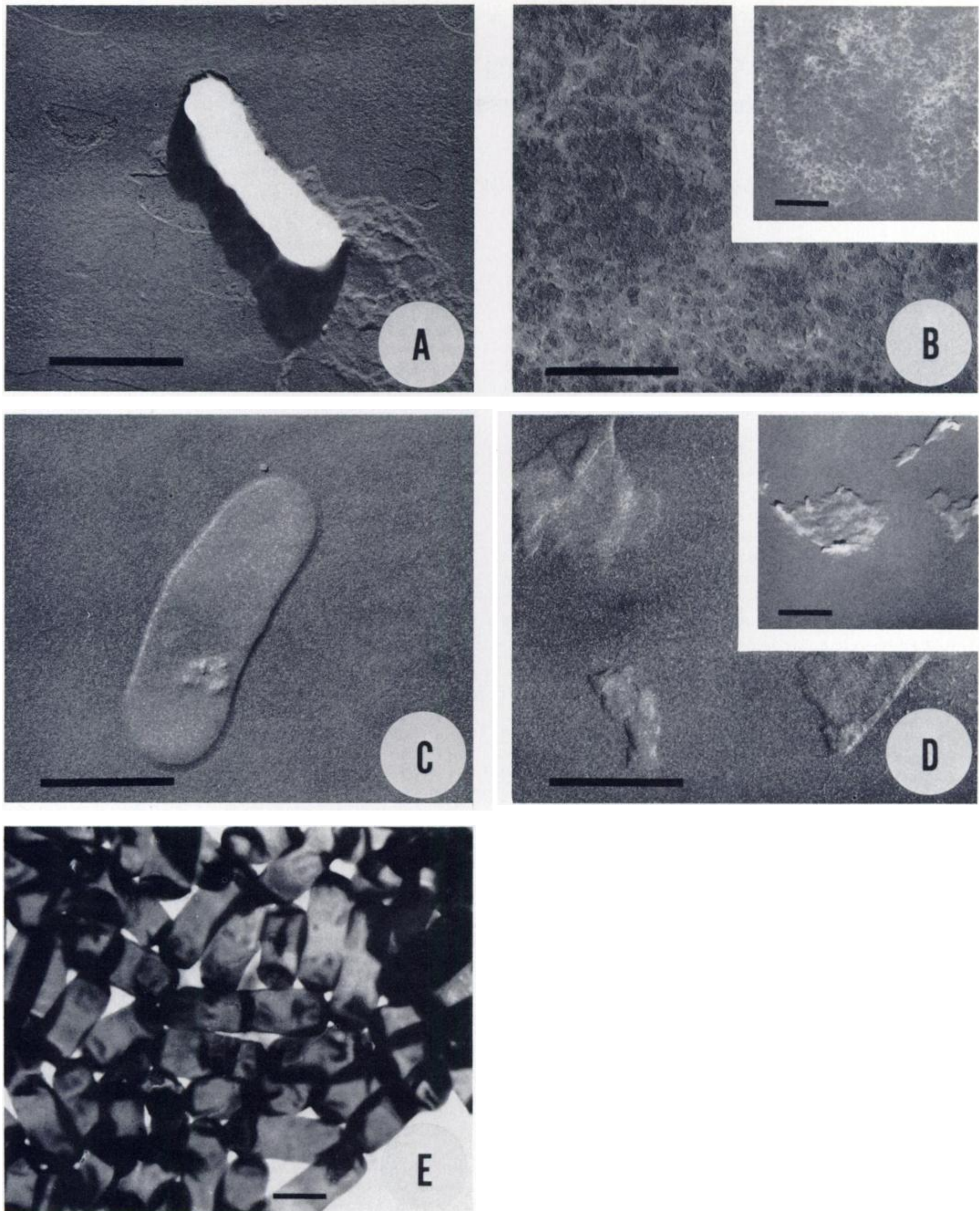

Fig. 1. Electron micrographs of whole cells and cell wall preparations. *A*, whole cells shadowed with platinum:palladium; *B*, CWS shadowed with platinum:palladium; *inset*, a low-magnification view of CWS exhibiting fibrous structure; *C*, WPG shadowed with platinum:palladium; *D*, WPG-sonicated shadowed with platinum:palladium; *inset*, a low-magnification view of WPG-sonicated; *E*, WPG stained with tungstophosphoric acid. All specimens were fixed with phosphate-buffered 2.5% glutaraldehyde (pH 7.2), dehydrated through graded methanol, and then shadowed with platinum:palladium or stained with tungstophosphoric acid. *Bar scale* in all micrographs is 1.0 μm.

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