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Antitumor Activity of Cell Wall from *Streptoverticillium cinnamoneum*

YOSHIO OKAWA, KAZUHIKO HASHIMOTO, KO SUZUKI, SHIGEO SUZUKI and MASUKO SUZUKI*

Tohoku College of Pharmacy, Sendai, Miyagi 983, Japan

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A cell wall specimen prepared from *Streptoverticillium cinnamoneum*, designated as P-CW, showed a marked antitumor activity towards Ehrlich and Meth A ascites tumors in mice whether given as a pre- or post-treatment. The intracellular levels of acid phosphatase, β-glucuronidase and lysozyme of peritoneal exudate cells in mice given P-CW *i.p.* were elevated as compared with those of the control group. Treatment of mice with P-CW also resulted in a significant increase in chemiluminescence due to the generation of active oxygen species in peritoneal exudate cells.

Keywords——*Streptoverticillium*; cell wall; antitumor activity; macrophage; lysosomal enzyme; chemiluminescence

Many papers have appeared on the antitumor activity of cell wall preparations from microorganisms. In particular, the water-insoluble components of certain microorganisms, *Mycobacterium*,[1-3] *Corynebacterium*[2,4] and *Nocardia*,[2,5,6] so-called cell wall skeletons, were found to display both antitumor and immunoadjuvant effects.

We have conducted a series of studies on the antitumor effect of mycelia of a non-pathogenic microbe belonging to Streptomycetaceae.[7] A previous report showed that there is an anti-infection effect in mice injected with the cell wall from *Streptoverticillum cinnamoneum*.[8] The present paper reports the antitumor activity of the cell wall against allogeneic and syngeneic tumors in ascites form in mice.

Materials and Methods

Strain——*Streptoverticillium* (*Stv.*) *cinnamoneum* IFO 12852 was used.

BCG——Lyophilized *Mycobacterium bovis* BCG was obtained from Japan BCG Laboratory Ltd., Tokyo.

Animals——Male ddY and BALB/c mice, 6 to 8 weeks old, were obtained from Shizuoka Experimental Animal Farm (Shizuoka).

Tumors——Ehrlich carcinoma was from the National Cancer Center Research Institute of Japan. Meth A tumor was kindly donated by Dr. Y. Hashimoto, the Department of Hygienic Chemistry, Pharmaceutical Institute, Tohoku University. These tumors were maintained in our laboratory in an ascites form.

Preparation of Cell Walls——The purified cell walls (P-CW) of *Stv. cinnamoneum* were prepared as described previously.[8]

Assay for Antitumor Activity against Ehrlich and Meth A Ascites Tumor——Mice, 6—18/group, were given intraperitoneal injections of various doses of each sample in 0.1 ml of 0.9% NaCl solution on days 6, 4 and 2 before or after tumor inoculation. Each mouse was given an intraperitoneal injection of tumor cells (Ehrlich tumor cells, 1×10^5/mouse, Meth A tumor cells, 4×10^4 and 4×10^5/mouse). The mice were observed for 60 d.

Preparation of Peritoneal Exudate Cells——Two days after the last P-CW administration, peritoneal exudate cells were obtained by washing the peritoneal cavity with Hanks' BSS containing 5 units of heparin per ml. When the cell types were determined morphologically by Giemsa staining after washing with saline, peritoneal exudate cells of normal mouse were found to be composed of 80—90% macrophages and 10—20% lymphocytes. In the case of BCG and P-CW, the cells were composed of 80—90% macrophages, 10—20% polymorphonuclear leucocytes and less than 3% lymphocytes.

Assay of Enzyme Activity——Enzyme activities of peritoneal exudate cells were determined as described previously.[8,9] Namely, the activities of acid phosphatase and β-glucuronidase were determined by measuring *p*-

nitrophenol liberated from *p*-nitrophenyl phosphate and *p*-nitrophenyl-β-D-glucuronide, respectively, according to the method of Komatsu *et al.*[10] Lysozyme activity was assayed according to the method of Parry *et al.*[11]

Measurement of Chemiluminescence——This was measured as follows: peritoneal exudate cells (2×10^6) were added to a vial containing 1.0 ml of Ca^{2+}-free Krebs–Ringer phosphate buffer (122 mM NaCl, 4.9 mM KCl, 13 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.4), 50 μl of 0.2% luminol in dimethyl sulfoxide (DMSO) and 0.1 ml of 50 mM D-glucose. Each vial was incubated for 10 min at 37 °C, then 7.5 μl of 0.001% phorbol myristate acetate in DMSO was added. Chemiluminescence was measured by means of a Biolumat instrument (LB 9500, Berthold, Wildbad, West Germany).

Results and Discussion

It has been reported that non-viable cell preparations of *Mycobacterium*,[12,13] *Corynebacterium*,[14] or *Streptococcus*[15] are less effective in terms of tumor-suppressive activity than the corresponding viable cell preparations. In the present work, antitumor activity

TABLE I. Effect of P-CW on Growth of Ehrlich Ascites Tumor

Sample	Dose (mg/kg/d)	Injection days	No. of tumor-free mice / No. of mice tested	Cure (%)
Control			0/18	0
P-CW	1	−6−4−2	4/12	33
P-CW	1	+2+4+6	3/12	25
P-CW	5	−6−4−2	14/18	78
P-CW	5	+2+4+6	10/16	63
P-CW	10	−6−4−2	5/6	83
P-CW	10	+2+4+6	4/6	67
P-CW	50	−6−4−2	12/18	67
P-CW	50	+2+4+6	9/16	56
BCG	5	−6−4−2	2/6	33
BCG	5	+2+4+6	3/6	50
BCG	10	−6−4−2	4/6	67
BCG	10	+2+4+6	3/6	50
BCG	50	−6−4−2	4/6	67
BCG	50	+2+4+6	3/6	50

Mice received *i.p.* injections of various doses of each sample on days 6, 4 and 2 before, or 2, 4 and 6 days after tumor grafting. Each mouse was given an *i.p.* injection of 10^5 Ehrlich tumor cells. This table shows the results 60 d after tumor inoculation.

TABLE II. Effect of P-CW on the Growth of Meth A Ascites Tumor

No. of inoculated tumor cells ($\times 10^4$)	Sample	Injection days	No. of tumor-free mice / No. of mice tested	Survival days of mice that died (mean ± S.D.)
4	Control		0/6	26.0 ± 3.0
	P-CW	−6−4−2	3/6	29.5 ± 1.1
	P-CW	−6−4−2, +1—+6	4/6	28, 33
	BCG	−6−4−2, +1—+6	2/6	27.5 ± 5.3
40	Control		0/6	13.5 ± 0.5
	P-CW	−6−4−2, +1—+6	1/6	21.0 ± 3.9
	BCG	−6−4−2, +1—+6	0/6	18.8 ± 2.5

Each mouse was given an *i.p.* injection of Meth A tumor cells. Mice received *i.p.* injections of 10 mg/kg/d of each sample on days 6, 4 and 2 before, or 6, 4 and 2 before and 1 to 6 after tumor grafting. This table shows the results 60 d after tumor inoculation.

TABLE III. Enzyme Activity of Peritoneal Exudate Cells in ddY and BALB/c Mice Treated with P-CW

Sample	Dose (mg/kg)	Acid phosphatase	β-Glucuronidase	Lysozyme
ddY mice				
Control		1.9±0.8	1.1±0.2	3.8±1.5
P-CW	5	11.8±1.2	4.4±0.4	
P-CW	10	9.7±0.6	3.5±0.6	6.8±1.7
P-CW	50	10.5±0.4	3.7±0.3	
BCG	10	10.8±0.9	2.3±0.4	6.0±1.7
BCG	50	9.9±0.5	2.8±0.3	
BALB/c mice				
Control		1.6±0.4	1.1±0.2	
P-CW	10	7.9±2.1	5.6±0.8	
BCG	10	3.2±0.7	2.5±0.3	

The ddY and BALB/c mice were injected intraperitoneally with P-CW or BCG on days 0, 2 and 4. Enzyme activity was measured on day 6. Acid phosphatase and β-glucuronidase activities are expressed in mU/10^6 cells. Lysozyme activity is expressed in μg/10^6 cells. Each value is the mean±S.D. $n=5$.

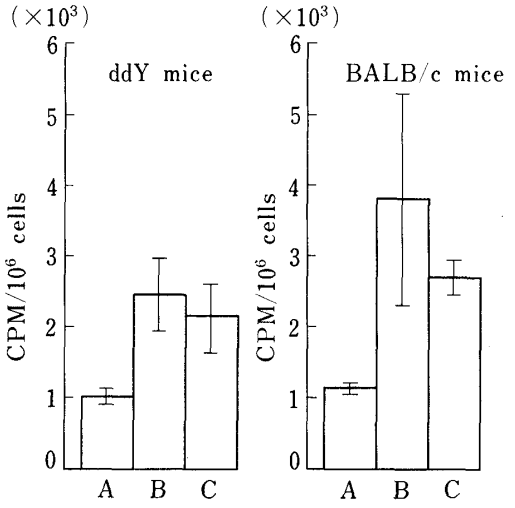

Fig. 1. Chemiluminescence of Peritoneal Exudate Cells in ddY and BALB/c Mice Treated with P-CW and BCG

The ddY and BALB/c mice were injected intraperitoneally with 10 mg/kg/d of P-CW or BCG on days 0, 2 and 4. Chemiluminescence of peritoneal exudate cells was measured on day 6 and is expressed in cpm/10^6 cells (mean±S.D.). $n=5$. A, control; B, P-CW; C, BCG.

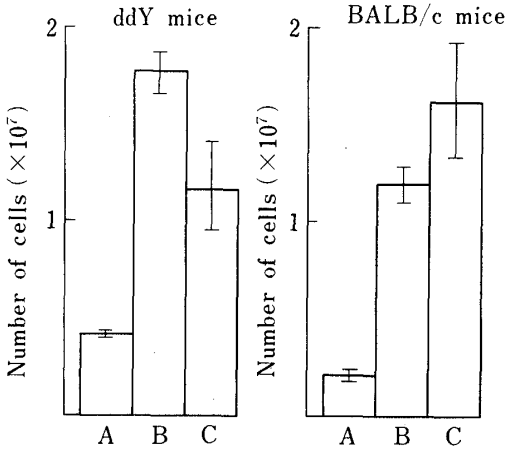

Fig. 2. Number of Peritoneal Exudate Cells in ddY and BALB/c Mice Treated with P-CW and BCG

The ddY and BALB/c mice were injected intraperitoneally with 10 mg/kg/d of P-CW or BCG on days 0, 2 and 4. The number of peritoneal exudate cells was determined on day 6. Bar: mean±S.D. $n=10$. A, control; B, P-CW; C, BCG.

of P-CW was assayed as a pre- or post-treatment with respect to tumor inoculation. Table I shows the results for Ehrlich ascites tumor *in vivo*. The P-CW specimen showed a marked inhibitory effect on the growth of Ehrlich ascites tumor grafted in ddY mice when administered at doses of 5—50 mg/kg/d, 3 times. The growth inhibition in this instance was over 60%. Antitumor effect of P-CW was almost the same as that of BCG under the conditions of pre- and post-treatments used here. On the other hand, injection of P-CW resulted in a marked suppression (about 50% survival) of the growth of Meth A ascites tumor in mice inoculated with 4×10^4 cells (Table II).

Basing on the finding that P-CW has no direct cytotoxicity towards tumor cells (data not shown), the effect of P-CW on lysosomal enzyme activity and chemiluminescence response of the peritoneal exudate cells was examined in order to analyze the activation mechanism (Table III and Fig. 2). It is well-known that cytotoxicity of activated macrophages against tumor cells is mediated by lysosomes and active oxygen species. Namely, Hibbs[16] suggested that lysosomal enzymes of activated macrophage origin might be the final molecular effectors of target-cell destruction in their cytotoxicity system. Recently, Nathan *et al.*[17,18] reported that the peritoneal macrophages of mice treated either with BCG or with *Corynebacterium parvum* were activated to release increased amounts of active oxygens able to lyse extracellular tumor cells in the presence of phorbol myristate acetate. Also, Allen *et al.*[19,20] reported that macrophages and polymorphonuclear leukocytes generate a chemiluminescence following phagocytosis; the chemiluminescence is correlated to metabolic activation of the hexose monophosphate shunt, which is also oxygen-dependent.

As shown in Table III, acid phosphatase and β-glucuronidase activities of peritoneal exudate cells (per 10^6) in ddY mice treated with P-CW (5,10, or 50 mg/kg/d) were increased 3—6 times as compared with those of the normal group. Moreover, the lysozyme activity of the cells was increased to about twice the control value. The acid phosphatase and β-glucuronidase activities of peritoneal exudate cells in BALB/c mice treated with P-CW (10 mg/kg/d) were increased to about 5 times the control value. It has been shown by Yagel *et al.*[21] that injection of the MER fraction of BCG into BALB/c mice by the *i.p.* and *i.v.* routes caused a pronounced increase in the activity of hydrolytic lysosomal enzymes of peritoneal macrophages. These findings suggest that macrophages activated by the cell wall fractions from microorganisms contain abundant cellular lysosomal enzymes. The chemiluminescence response in peritoneal exudate cells (per 10^6) from mice given 10 mg/kg/d of P-CW or BCG is shown in Fig. 1. Chemiluminescence response of peritoneal exudate cells in ddY and BALB/c mice given P-CW and BCG was increased to 2—3 times the control values. The number of peritoneal exudate cells in mice treated with the samples was measured. The ddY and BALB/c mice were injected intraperitoneally with 10 mg/kg/d of P-CW or BCG on days 0, 2 and 4. The number of peritoneal exudate cells was determined on day 6 (Fig. 2). A large increase of the cell number was seen in the mice pretreated with P-CW and BCG (3—8 times). Thus, the total lysosomal enzyme and chemiluminescence response activities of peritoneal exudate cells in mice treated with the two samples were very much higher than those of control mice.

The peritoneal exudate cells in mice treated with P-CW were composed of 80—90% macrophages, 10—20% polymorphonuclear leucocytes and less than 3% lymphocytes. From the results summarized in Table III and Figs. 1 and 2, it can be concluded that the cytotoxicity of activated peritoneal exudate cells towards the tumor cells was mediated by lysosomal enzymes and active oxygens induced by P-CW.

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