Antitumour Activity of Purified Arabinogalactan–peptidoglycan Complex of the Cell Wall Skeleton of *Rhodococcus lentifragmentus*

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Antitumour activity of arabinogalactan–peptidoglycan (AP) complex (peptidoglycan and arabinogalactan liberated by an acid or alkaline treatment from *Rhodococcus lentifragmentus* AN-115 cell wall skeleton) was examined in mice and compared with that of the cell wall skeleton. The growth of syngeneic fibrosarcoma Meth A cells after implantation in BALB/c mice was significantly suppressed by AP complex, and also regressed after intratumoral injection of AP complex on days 1, 4 and 7 after tumour implantation. Although the activity of peptidoglycan was less than that of AP complex, peptidoglycan also showed both tumour-suppressive and regressive activities. Arabinogalactan did not show antitumour activity. It is interesting that peptidoglycan has an important role in the effect against tumours.

INTRODUCTION

The cell wall skeleton of *Rhodococcus lentifragmentus* (R-CWS) possesses immunoadjuvant activity for circulating antibody formation (Ohara *et al.*, 1986), and results in induction of cell-mediated immunity and antitumour activity in experimental animals (Azuma *et al.*, 1976; Yamawaki *et al.*, 1978). Cell wall skeletons of various bacteria, such as *Mycobacterium bovis* BCG, *Nocardia asteroides* 131 and *Corynebacterium diphtheriae* PW8, also have adjuvant activity (Azuma *et al.*, 1975). These bacteria have similar cell wall constituents: mycolic acid, arabinogalactan and peptidoglycan (Lederer, 1971; Michel & Bordet, 1976; Barksdale & Kim, 1977). Among these constituents, peptidoglycan is responsible for adjuvant activities of cell walls, causing increased serum antibody levels and inducing delayed-type hypersensitivity to ovalbumin (Azuma *et al.*, 1975). Detailed studies on adjuvant-active units using enzyme-degraded components of cell walls (Azuma *et al.*, 1974; Adam *et al.*, 1976) and synthesized model compounds (Adam *et al.*, 1974) have shown that the *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) fragment of peptidoglycan is the minimum unit needed to display the adjuvant activities. However, antitumour activity has not been demonstrated for MDP in experimental tumour systems. Furthermore, little is known about the antitumour activity of peptidoglycan moieties purified from bacterial cell walls, nor is it known how other constituents, especially arabinogalactan, relate to the antitumour activity of peptidoglycan. This paper describes antitumour activity of R-CWS and its constituents including the arabinogalactan–peptidoglycan complex (AP complex), peptidoglycan and arabinogalactan isolated from R-CWS.

METHODS

R-CWS. This was prepared from '*Nocardia rubra*' No-115 preserved in the Research Laboratories of Fujisawa Pharmaceutical Co. Organisms labelled '*N. rubra*' should properly be considered as members of the species

Abbreviations: AP, arabinogalactan–peptidoglycan; MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; R-CWS, cell wall skeleton of *R. lentifragmentus*.

Rhodococcus lentifragmentus (Tsukamura, 1985). Culture conditions and preparation of cell walls have been described by Fujioka *et al.* (1985).

Mycolic acid. Mycolic acids of R-CWS were obtained according to Koda *et al.* (1986).

AP complex. R-CWS was hydrolysed with 0.5% (w/v) KOH/methanol solution at 37 °C for 48 h under nitrogen. After neutralization with concentrated HCl, the mixture was evaporated to a syrup and then suspended in water. The suspension was washed with diethyl ether, filtered and washed with water, ethanol/diethyl ether (1:1, v/v), chloroform and chloroform/methanol/water (10:10:1, by vol.). The residue was resuspended in water and lyophilized to yield the AP complex.

Arabinogalactan. The AP complex was hydrolysed with 1 M-NaOH at 70 °C for 24 h under nitrogen. After neutralization with acetic acid, the mixture was concentrated by evaporation and successively filtered (pore sizes 3.0, 0.8 and 0.45 µm). The filtrate was dialysed against water for 4 d, and concentrated by evaporation. This solution was applied to a column of Dowex 50W X 8 (H⁺ form) and eluted with water. The eluate was evaporated to a smaller volume and applied to a column of DEAE-cellulose DE52 (OH⁻ form). The arabinogalactan was eluted with water and lyophilized.

Peptidoglycan. The AP complex was hydrolysed with 0.1 M-HCl at 60 °C for 12 h. The mixture was filtered and washed with water to remove HCl. The residue was suspended in water and then lyophilized to yield the peptidoglycan.

Analytical methods for each fraction have been described by Fujioka *et al.* (1985).

Animals and tumours. BALB/c mice (8–12 weeks old) purchased from Shizuoka Jikken-Dobutsu Norkyo (Shizuoka), were given feed (from Oriental Yeast Industries Ltd, Osaka) and water *ad lib*. Mouse fibrosarcoma Meth A cells, which were chemically induced in BALB/c mice with methylcholanthrene, were serially passed in ascites form in syngeneic BALB/c mice.

Tumour suppression test. A single-cell suspension of Meth A fibrosarcoma in Hanks' balanced salt solution was mixed with R-CWS, or each constituent purified from R-CWS, suspended in saline containing a placebo preparation (a lyophilized preparation including squalene, polysorbate 80 and D-mannitol, 4:1:28.2 by wt, mixed thoroughly with the sample and suspended in saline just before use). The mixture of tumour cell suspension and the sample suspension was injected intradermally into syngeneic BALB/c mice and the tumour sizes at the inoculated sites were measured every week.

Tumour regression test. BALB/c mice were injected intradermally with 1×10^5 Meth A cells, and on days 1, 4 and 7 after tumour cell inoculation, R-CWS, or each constituent purified from R-CWS, mixed with the placebo preparation in saline was injected intralesionally. Tumours were dissected out and weighed on day 14.

Statistical analysis. Tumour size and weight were subjected to the Student's *t*-test and Cochran-Cox test, and inhibition rates of tumour take were subjected to Fisher's exact probability test.

RESULTS

Chemical composition of R-CWS and constituents purified from it. R-CWS was mainly composed of mycolic acid, arabinogalactan and peptidoglycan. The latter two constituents were bound together by phosphodiester linkages and glycosidic linkages to form the rigid structure of the AP complex (Fujioka *et al.*, 1985). Mycolic acid was bound to the arabinogalactan moiety of this complex through ester bonds. As shown in Table 1, the arabinogalactan consisted of arabinose and galactose together with small amounts of rhamnose. The peptidoglycan consisted of glucosamine, muramic acid, alanine, glutamic acid and diaminopimelic acid.

Antitumour activities of R-CWS and constituents purified from it. AP complex and R-CWS at doses of 10 and 100 µg suppressed the growth of fibrosarcoma Meth A in BALB/c mice (Table 2). Peptidoglycan also showed tumour-suppressive activity at a dose of 100 µg, but not at a dose of 10 µg. Arabinogalactan did not suppress tumour growth even at a dose of 100 µg.

Regression of the growth of Meth A was evident in BALB/c mice that received doses of 100 and 320 µg of AP complex (Table 3). Peptidoglycan also showed significant tumour-regressive activity at a dose of 320 µg (Table 4). No tumour-regressive activity was observed in the case of arabinogalactan; its effect was similar to that seen in the suppression test (data not shown).

DISCUSSION

Intratumoral administration of the cell wall skeletons of *Mycobacterium bovis* BCG and of *Corynebacterium parvum* can protect experimental animals from proliferation of tumours (Azuma *et al.*, 1974). In the present study, R-CWS also showed strong suppressive and regressive

Table 1. *Chemical composition (percentage by weight) of R-CWS and constituents purified from it*

For R-CWS the mean values of five different preparations are given. Other values were obtained by a single determination.

Component	R-CWS	AP complex	Peptidoglycan	Arabinogalactan
Lipids	27.6 ± 0.7	1.2	0.9	ND
Sugars				
Arabinose	15.2 ± 0.4	27.9	—	67.9
Galactose	8.9 ± 0.3	15.1	—	32.0
Rhamnose	0.92 ± 0.04	1.3	—	2.4
Amino sugars				
Glucosamine	4.8 ± 0.2	8.0	12.7	0.6
Muramic acid	6.2 ± 0.3	11.3	16.8	0.08
Amino acids				
Alanine	8.5 ± 0.3	8.6	15.1	0.03
Glutamic acid	5.8 ± 0.2	7.3	13.3	0.03
Diaminopimelic acid	7.2 ± 0.2	10.4	19.2	0.15
Glycine	1.2 ± 0.1	0.2	0.4	—
Serine	1.7 ± 0.1	0.1	0.1	—
Threonine	1.4 ± 0.2	0.1	0.1	—
Leucine	1.2 ± 0.1	0.2	0.3	—
Phenylalanine	1.5 ± 0.2	0.3	—	—
Phosphorus	0.09 ± 0.01	0.1	ND	ND
Loss on drying	7.2 ± 1.5	11.9	ND	ND

ND, No data; —, not detected.

Table 2. *Suppressive effects of R-CWS and constituents purified from it on fibrosarcoma Meth A in BALB/c mice*

Mice were inoculated intradermally with a mixture of syngeneic Meth A cells (1×10^5) and R-CWS or a constituent purified from R-CWS. The results were obtained on day 21 after the tumour cell implantation, when the mean tumour size in placebo and saline control groups was, respectively, 16.1 ± 1.43 and 16.1 ± 2.10 (expt I), and 15.1 ± 2.81 and 15.5 ± 2.28 mm (expt II).

Sample	Dose (μg per 0.05 ml per mouse)	No. of mice with tumour/ no. of mice used	
		Expt I	Expt II
R-CWS	10	NT	1/10**
	100	0/10**	0/10**
AP complex	10	NT	3/10**
	32	NT	2/10**
	100	0/10**	0/10**
Peptidoglycan	10	NT	8/10
	100	1/10**	0/10**
Arabinogalactan	100	10/10	8/10
Placebo control	0	10/10	10/10
Saline control	0	10/10	10/10

NT, Not tested.

** Significantly different from placebo control ($P < 0.01$).

effects on syngeneic fibrosarcoma Meth A in BALB/c mice. Furthermore, significant antitumour activity of the AP complex and peptidoglycan against the Meth A growth was observed.

The minimal adjuvant-active subunit of BCG cell walls is MDP (Elouz *et al.*, 1974; Kotani *et al.*, 1975; Azuma *et al.*, 1983). Synthetic *N*-acetylmuramyl-dipeptide that had potent adjuvant activity in immune systems, however, proved to be active in experimental tumour systems only if administered admixed with trehalose dimycolate (McLaughlin *et al.*, 1980; Yarconi *et al.*, 1981), or incorporated into liposomes (Fidler *et al.*, 1981). Saiki *et al.* (1982) speculated that the

Table 3. *Regressive effect of AP complex on growth of syngeneic fibrosarcoma Meth A in BALB/c mice*

Meth A cells (1×10^5) were implanted intradermally on day 0 and a sample was injected intratumorally on days 1, 4 and 7. Ten animals were used in each dose and control group. Tumours were dissected out and weighed on day 14. The numbers in parentheses show the tumour growth inhibition rate: $(1 - T/C) \times 100$ (%), where T is the tumour weight of the sample-treated group and C is the tumour weight of the placebo-treated control group.

Sample	Dose (μg per mouse d^{-1})	Mean tumour wt (mg) on day 14, $\pm$ SE		
		Expt I	Expt II	Expt III
AP complex	320	NT	$32 \pm 8^*$ (91)	$144 \pm 44^{*\dagger}$ (73)
	100	$211 \pm 54^{*\dagger}$ (55)	$207 \pm 49^\dagger$ (45)	$280 \pm 62^{*\dagger}$ (48)
	32	$389 \pm 41^\dagger$ (18)	243 ± 46 (35)	NT
	10	$473 \pm 49^\dagger$ (0)	377 ± 33 (-1)	NT
R-CWS	320	NT	$23 \pm 6^*$ (94)	$25 \pm 6^*$ (95)
	100	$34 \pm 8^*$ (93)	$58 \pm 24^*$ (85)	$57 \pm 16^*$ (89)
	32	$93 \pm 22^*$ (80)	216 ± 39 (42)	NT
	10	$201 \pm 29^*$ (58)	329 ± 32 (12)	NT
Placebo control	0	473 ± 51 (0)	375 ± 68 (0)	534 ± 44 (0)
Saline control	0	504 ± 49	387 ± 43	408 ± 55

NT, Not tested.

* Significantly different from placebo-treated control group ($P < 0.05$)

† Significantly different from the same dose group of R-CWS ($P < 0.05$).

Table 4. *Effect of peptidoglycan on growth of syngeneic fibrosarcoma Meth A in BALB/c mice*

Meth A cells (1×10^5) were implanted intradermally on day 0, and a sample was injected intratumorally on days 1, 4 and 7. Ten animals were used in each dose and control group. The tumour growth inhibition rate (%) is shown in parentheses. For other information, see Table 3.

Sample	Dose (μg per mouse d^{-1})	Mean tumour wt (mg) on day 14, $\pm$ SE	
		Expt I	Expt II
Peptidoglycan	320	$240 \pm 51^{*\dagger}$ (46)	$229 \pm 39^{*\dagger}$ (35)
	100	$439 \pm 36^\dagger$ (1)	$338 \pm 46^\dagger$ (5)
	32	$492 \pm 32^\dagger$ (-11)	$401 \pm 52^\dagger$ (-13)
R-CWS	320	$19 \pm 5^*$ (96)	$21 \pm 6^*$ (94)
	100	$46 \pm 15^*$ (90)	$48 \pm 12^*$ (86)
	32	$226 \pm 58^*$ (49)	$131 \pm 46^*$ (63)
Placebo control	0	442 ± 50 (0)	355 ± 37 (0)
Saline control	0	353 ± 38	341 ± 60

lack of antitumour activity of MDP might be due to its low molecular weight and water solubility. *N*-Acetylmuramyl-dipeptide with mycolic acid bound to the 6-*O*-position of its muramic acid residue showed suppressive activity against experimental murine tumour growth in syngeneic mice (Kusumoto *et al.*, 1978). Hrsak *et al.* (1983) reported that intratumorally administered peptidoglycan monomer, isolated from *Brevibacterium divaricatum* cell wall, inhibited the nodule growth of B-16 melanoma at a relatively high dose (1 mg per site). Peptidoglycan from acid-treated R-CWS contained a minute fraction of lipid (see Table 1). Since it seems unlikely that the lipid component is responsible for the antitumour activity of the peptidoglycan fraction, peptidoglycan is considered to be essentially the antitumour-active moiety and needs its polymer structure to display strong antitumour activity.

Saiki *et al.* (1982) and Kamisango *et al.* (1982) reported that peptidoglycan was obtained by mild acid treatment from the cell wall skeleton of *Listeria monocytogenes* without influencing its chemical nature or decreasing its adjuvant and antitumour activities. In the present experiments, however, the antitumour activity of peptidoglycan purified from R-CWS was less than that of the AP complex and R-CWS. Azuma *et al.* (1975) have shown that adjuvant

activities of cell wall skeletons of *M. bovis* BCG and *N. asteroides* after acid treatment (peptidoglycan) significantly decreased because of the drastic acid treatment (0.1 M-HCl, 60 °C, 24 h).

Arabinogalactan itself showed no significant antitumour activity. However, the present results suggest that arabinogalactan enhances the antitumour activity of peptidoglycan, as judged by the difference in activities between peptidoglycan and the AP complex.

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