Chemical Analyses and Antitumor Activity of Hydrosoluble Substances from *Mycobacterium bovis*, Strain BCG

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SATO, H., ARAI, H., NAGAI, H., ITO, M., SATOH, K., KUMANO, N., MOTOMIYA, M. and KONNO, K. *Chemical Analyses and Antitumor Activity of Hydrosoluble Substances from Mycobacterium bovis, Strain BCG*. Tohoku J. exp. Med., 1983, **140** (1), 35-43 — Hydrosoluble substances from BCG were prepared by cold water extraction and by hot phenol-water extraction. Chemical analyses revealed that both of them were derived from cytoplasm. The cold water extract (CWE) was effective in the treatment of C3H/He mice which had received an intraperitoneal inoculation of a syngeneic ascites hepatoma, MH134. The growth of a graft in footpad of mastocytoma P815 in CDF₁ mice was retarded by intraperitoneal injections of CWE. A peptidoglycan from cell wall prepared by digestion with lysozyme exerted no antitumor activity in the same experimental condition as for the evaluation of antitumor effect of CWE. These results indicate that the antitumor activity of CWE was not due to the presence of a cell wall component. ——— hydrosoluble substance; BCG; antitumor activity; mastocytoma P815

There are a number of publications dealing with the effects of mycobacteria on immune response and on antitumor activity. Several fractions of mycobacteria have been investigated in order to identify the chemical constituents which are responsible for these activities and as a result cell wall has been found to have these biological activities.

A hot water extract from delipidated BCG, which has been found to be derived from cytoplasm, is adjuvant-active and exhibits weak but significant antitumor activity (Sato et al. 1976, 1978, 1979).

In this study, a cold water extract from delipidated BCG and a phenol-water extract were prepared to compare their antitumor effects with those of a water soluble substance from cell wall isolated by an enzymatic treatment of BCG.

This report deals with the chemical analyses and antitumor activity of the above hydrosoluble substances.

MATERIALS AND METHODS

Bacilli

Mycobacterium bovis, strain BCG (Japanese strain, Tokyo No. 172, supplied by the National Institute of Health, Tokyo) subcultured for 4 weeks on a modified Sauton medium (Sato et al. 1969) were used throughout this series of experiments.

Preparation of hydrosoluble substances

The hot water extract (HWE) was prepared from delipidated *Mycobacterium bovis*, strain BCG, as described previously (Sato et al. 1976). The cold water extract (CWE) was obtained in the course of the preparation of HWE. A suspension in distilled water of defatted BCG was stirred at 4°C for an hour and filtered through a Millipore Filter (0.45 µm, Millipore Filter Corporation, Massachusetts, USA). The precipitate formed by an addition of 3 volumes of acetone was dialysed against running water and then lyophilized.

The phenol-water extract (PWE) was prepared by the method of Cantrell and Wheat (1979) which had been used to obtain phenol-water extract from *Corynebacterium parvum*. Briefly, 10 g of dried BCG were suspended in 70 ml of a 1:1 mixture of distilled water and phenol. The bacillary suspension was stirred for 30 min at 65°C and then cooled at 4°C overnight. The aqueous phase after centrifugation at $2,000 \times g$ for 30 min at 4°C was concentrated. Ten volumes of ethanol were added to this concentrate. The resulting precipitate was dialysed against distilled water and lyophilized.

The neo-WSA from BCG was extracted according to the method of Adam et al. (1973). BCG (10 g, dry weight) was delipidated in a Soxhlet apparatus for 24 hr with acetone, ethanol, chloroform and chloroform:methanol (87:13) in the order described. The defatted cells were washed with 100 times their weight of distilled water containing 0.1 M ammonium acetate, pH 6.2. The washed cells, suspended in 500 ml of 0.1 M ammonium acetate containing 0.01% egg white lysozyme (Sigma Chemical Co., St. Louis, USA) were incubated for 18 hr at 37°C. The residue after filtration was reincubated with lysozyme under the same condition and was filtered again. The two filtrates were united and lyophilized to remove ammonium acetate. The white powder thus obtained was designated crude neo-WSA. This crude neo-WSA was fractionated on a column of a Sephadex G-75. Fraction B (Fig. 1) was designated neo-WSA-BCG.

Analytical methods

Neutral sugars were identified by paper chromatography in the solvent systems of ethyl acetate-acetic acid-formic acid-water (90:15:5:20) and n-butanol-pyridine-water (6:4:3), respectively. Neutral sugar was quantitated by Roe's method (1955) and by phenol-sulfuric acid reagent. Pentose was quantitated according to the direction of Dische (1962). Aminosugar was measured by the method of Elson-Morgan as modified by Blix (1948). Ninhydrin-positive substance was estimated as described by Yemm and Cocking (1955). Quantification of nucleic acid was made by the method of Schmidt and Thannhauser (1945) and Schneider (1946).

Antitumor activity

Animals. Mice for the experiments were purchased from Funabashi Animal Breeder, Shizuoka. The mice were 6 to 8 weeks old at the start of the experiments.

Tumor cells. Sarcoma 180 (S180) and Ehrlich carcinoma (EC) have been maintained in ddY mice by 2-weekly intraperitoneal passage. MH 134, originally induced with carbon tetrachloride in C3H mice and converted into ascites form (Sato et al. 1956), has been maintained in C3H/He mice by intraperitoneal inoculations. Mastocytoma P815, originally isolated by Dunn and Potter (1957) in DBA/2 mice, has been maintained in ascites by serial intraperitoneal transplantations in histocompatible CDF₁ (BALB/c DBA/2) mice.

Tumor cells were harvested from the abdominal cavity and washed with Hanks' balanced saline solution. Viability, checked by the trypan blue exclusion test, was over 95%.

Hydrosoluble substances to be tested were dissolved in saline immediately before injection and 0.25 mg each of which in 0.2 ml of saline was injected intraperitoneally for 14 consecutive days from day 1 after inoculation of tumor cells.

Male ddY mice were inoculated intraperitoneally with 5×10^5 cells of S180 or 3×10^5 cells of EC. PWE, CWE and HWE were injected to mice of different groups. The number of survivors and survival days were recorded.

Male C3H/He mice received an intraperitoneal inoculation of MH134 cells and the survival days were compared. In three experiments to compare the antitumor activity of PWE, CWE and HWE, 5×10^3 , 8×10^3 , 1×10^5 cells respectively were inoculated. For the evaluation of the effect of fractions of crude neo-WSA-BCG, 5×10^3 cells were inoculated.

Male CDF₁ mice which had received a subcutaneous transplantation of 2×10^4 cells of mastocytoma P815 in the left hind footpad were injected intraperitoneally with PWE, CWE or HWE. The thickness of footpad was compared between experimental and control groups.

Student's *t*-test was used in all statistical evaluations.

RESULTS

Chemical analyses

Table 1 shows the results of chemical analyses of PWE, CWE and HWE. Less than 2% of ninhydrin-positive substance and only a trace of nucleic acids were found in PWE. The amounts of ninhydrin-positive substance and nucleic acids in CWE were less than those in HWE. Paper chromatography revealed that neutral sugars in these three substances consisted mainly of glucose and mannose.

The elution profile of the crude neo-WSA-BCG from a column of Sephadex G-75 is illustrated in Fig. 1. The results of chemical analyses of fractions thus obtained are summarized in Table 2. Fraction D is rich in ninhydrin-positive substance presumably due to the presence of lysozyme in this fraction. Paper chromatography of neutral sugars of these fractions indicated the presence of galactose and arabinose in Fraction B, which suggests that this fraction had been derived from cell wall. Thus it is likely that Fraction B is essentially equivalent to neo-WSA described by Adam et al. (1973).

TABLE 1. *Quantitative analyses of hydrosoluble substances*

	Neutral sugar* (%)	Amino sugar† (%)	Ninhydrin-positive‡ substance (%)	Nucleic acids§ (%)
PWE	68.5	0.6	1.0	0.2
CWE	72.5	0.8	5.6	6.2
HWE	52.8	0.8	12.5	10.8

PWE, phenol-water extract; CWE, cold water extract; HEW, hot water extract. The numerals indicate the mean of three experiments.

Determination: * by Roe's method, † by a modified Elson-Morgan's method, ‡ by Yemm and Cocking method, and § by the method of Schmidt and Thannhauser, and of Schneider.

Fig. 1. Elution pattern of 800 mg of crude neo-WSA-BCG from a 2.5×60 cm column of Sephadex G-75. Elution was effected with 0.1 N acetic acid. Fractions (4 ml) were analysed for neutral sugar by phenol-sulfuric acid reagent and for ninhydrin-positive substance by the method of Yemm and Cocking (calculated as glycine). —, neutral sugar; ----, ninhydrin-positive substance.

TABLE 2. *Quantitative analyses of fractions of crude neo-WSA-BCG*

Fraction	Neutral sugar* (%)	Pentose† (%)	Amino sugar‡ (%)	Ninhydrin-positive§ substance (%)
Crude neo-WSA	32.0	11.0	2.6	27.5
A	50.0	4.5	2.3	18.0
B	60.5	25.0	2.6	12.4
C	57.5	15.3	3.3	15.2
D	21.4	4.2	1.7	58.3

The numerals refer to the means of three experiments. Determination: * by Roe's method, † by Dische's method, ‡ by a modified Elson-Morgan's method, § by method of Yemm and Cocking.

Antitumor activity

Antitumor activity on Sarcoma 180 and on Ehrlich carcinoma. As shown in Tables 3 and 4, PWE was not effective under these experimental conditions. The antitumor effect of CWE on Sarcoma 180 was not very strong. However, in the experiment on Ehrlich carcinoma, prolongation of survival time in CWE-injected group was significant. It is apparent that HWE exerted an antitumor activity on Sarcoma 180, whereas there was no difference in survival time between the control group and HWE-treated group in the experiment on Ehrlich carcinoma.

Antitumor activity of neo-WSA-BCG on Sarcoma 180 was compared with that of CWE and HWE (Table 5). Neo-WSA-BCG and HWE were found to be effective. In contrast, CWE was ineffective.

Antitumor activity on mastocytoma P815. As indicated in Table 6, the footpad was thinner in CWE-injected group than in control with statistical significance.

TABLE 3. *Antitumor activity on Sarcoma 180*

Treated with	Survivors/Number of mice, at days			
	20	30	40	50
Saline	5/20	0/20	0/20	0/20
PWE	6/20	1/20	1/20	0/20
CWE	8/40	4/40	4/40	2/40
HWE	10/20	8/20	8/20	8/20

PWE, phenol water extract; CWE, cold water extract; HWE, hot water extract.

Male ddY mice were inoculated intraperitoneally with 5×10^5 cells of Sarcoma 180. From day 1 after inoculation of tumor cells, 0.25 mg of each substance in saline was injected intraperitoneally for 14 consecutive days. The number of survivors was recorded.

TABLE 4. *Antitumor activity on Ehrlich carcinoma*

Group	Number of mice	Treated with	Survival days (mean $\pm$ s.d.)
1	10	Saline	22.7 $\pm$ 3.6
2	10	PWE	23.3 $\pm$ 2.0
3	20	CWE	30.7 $\pm$ 11.9*
4	10	HWE	24.2 $\pm$ 5.8

PWE, phenol water extract; CWE, cold water extract; HWE, hot water extract.

Male ddY mice were inoculated intraperitoneally with 5×10^5 cells of Ehrlich carcinoma. From day 1 after inoculation of tumor cells, 0.25 mg of each substance was injected intraperitoneally for 14 consecutive days. Survival time in days of each group was compared.

* $p < 0.01$.

TABLE 5. *Antitumor activity on Sarcoma 180*

Group	Treated with	Survivors/Number of mice, at days			
		20	30	40	50
1	Saline	4/10	0/10	0/10	0/10
2	Neo-WSA-BCG	5/10	4/10	4/10	3/10
3	CWE	3/10	1/10	1/10	0/10
4	HWE	6/10	5/10	5/10	5/10

CWE, cold water extract; HWE, hot water extract.

Male ddY mice received an intraperitoneal inoculation of 5×10^5 cells of Sarcoma 180. From day 1 after inoculation of tumor cells, 0.25 mg in saline of each substance was injected intraperitoneally for 14 consecutive days. The number of survivors was recorded.

At day 25, no difference in thickness of footpad was observed between PWE-injected and control groups.

Antitumor activity on MH134. The results of three experiments on MH134 are

TABLE 6. *Antitumor activity on mastocytoma P815*

Group	Number of mice	Treated with	Increase of thickness of footpad (mm), at days		
			15	20	25
1	10	Saline	1.66±0.38	2.53±0.31	3.20±0.62
2	10	PWE	1.33±0.26*	1.93±0.61*	2.94±0.37
3	10	CWE	1.36±0.19	1.71±0.44†	1.93±0.37†
4	10	HWE	1.33±0.42	2.04±0.20‡	2.30±0.67‡

PWE, phenol water extract; CWE, cold water extract; HWE, hot water extract.

Male CDF₁ mice were inoculated with 2×10^4 cells of mastocytoma P815 in the left hind footpad. From day 1 after inoculation of tumor cells, 0.25 mg of each substance in saline was injected intraperitoneally for 14 consecutive days. Thickness of footpad was measured at days 15, 20 and 25.

* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.02$.

TABLE 7. *Antitumor activity on MH134*

Treated with	Experiment 1		Experiment 2		Experiment 3	
	Number of mice	Survival days (mean±s.d.)	Number of mice	Survival days (mean±s.d.)	Number of mice	Survival days (mean±s.d.)
Saline	10	21.0±3.9	10	17.2±1.0	10	16.2±0.4
PWE	10	25.9±7.1	10	20.8±1.8†	20	18.1±4.6
CWE	20	26.4±4.5†	20	22.8±5.9†	20	16.4±4.4
HWE	10	25.0±3.5*	10	19.7±2.1†	10	18.4±5.7

PWE, phenol water extract; CWE, cold water extract; HWE, hot water extract.

* $p < 0.05$, † $p < 0.01$.

Male C3H/He mice were inoculated intraperitoneally with 5×10^3 cells of MH134 in Experiment 1, with 8×10^3 cells in Experiment 2 and with 1×10^5 cells in Experiment 3. From day 1 after inoculation of tumor cells, 0.25 mg of each substance in saline was injected intraperitoneally for 14 consecutive days. Survival time in days of each group was compared.

TABLE 8. *Antitumor activity on MH134 of the fractions from crude neo-WSA-BCG*

Group	Number of mice	Treated with	Survival days (mean±s.d.)
1	10	Saline	20.4±4.2
2	10	Crude	27.7±6.5*
3	10	Neo-WSA-BCG Fraction B (Neo-WSA-BCG)	20.7±2.5
4	10	Fraction D	19.5±2.9

* $p < 0.02$ as compared with group 1.

Male C3H/He mice were inoculated intraperitoneally with 8×10^3 cells of MH134. From 1 day after inoculation of tumor cells, 0.25 mg of each fraction was injected intraperitoneally for 14 consecutive days. Survival time in days of each group was compared.

summarized in Table 7. In two experiments in which 5×10^3 and 8×10^3 cells of MH134 respectively had been inoculated, significant prolongation of survival time was observed in CWE-treated and in HWE-treated groups. In PWE-injected group, survival time was prolonged only in one experiment. When 1×10^5 cells had been inoculated, none of the three substances was effective.

The antitumor activity on MH134 of neo-WSA-BCG and Fraction D as well as crude neo-WSA-BCG was evaluated (Table 8). The survival time of the group which had been injected with crude neo-WSA-BCG was prolonged. No prolongation of survival time, however, was observed in mice that had received neo-WSA-BCG or Fraction D.

DISCUSSION

Mycobacteria and taxonomically related microorganisms such as corynebacteria and nocardia have been used in immunotherapy on experimental neoplasms. Since 1972, several adjuvant-active, hydrosoluble substances have been isolated from these microorganisms. However, only a few publications are available concerning the in vivo antitumor effect of the water-soluble substances. Mathé et al. (1973, 1975) reported that a water soluble substance from BCG described by Hiu (1972) was not effective in an attempt of immunoprophylaxis against murine neoplasms. Cantrell and Wheat (1979) reported that inoculation into mice of a mixture of tumor cells and a hot phenol-water extract from *Corynebacterium parvum* provided no significant protection. On the other hand, regression of tumor was observed in rat by an intralesional injection of a hydrosoluble product from *Nocardia opaca* (Barot-Ciorbaru 1981).

The hot water extract (HWE) from delipidated BCG has been found to be adjuvant-active and effective in the treatment of murine ascites tumors (Sato et al. 1976, 1978, 1979). Also evidence for host-mediated antitumor activity of HWE has been presented (Sato et al. 1982).

In this study, a cold water extract (CWE) from delipidated BCG and a phenol-water extract (PWE) were prepared. The fraction of neutral sugars of CWE and PWE was found to consist of glucose and mannose, which indicates that these substances were derived from cytoplasm. The antitumor activity of CWE was observed in mice which had been inoculated with syngeneic tumor cells, whereas no significant antitumor effect was observed in the treatment of ddY mice in which allogeneic tumor cells of Sarcoma 180 had been transplanted. The biological activities of mycobacteria and of related microorganisms have been ascribed mainly to the fraction of cell wall. Thus it was necessary to investigate the antitumor activity of the substance obtained from cell wall fraction. A peptidoglycan was isolated from BCG by an enzymatic treatment which had been used by Adam et al. (1973) for the preparation of a water soluble adjuvant from *Mycobacterium smegmatis*. In this series of experiments a fraction obtained by column chromatography was found to be derived from cell wall and designated neo-WSA-BCG. When neo-WSA-BCG was injected in the same manner as for

the evaluation of antitumor effect of CWE and HWE, no prolongation of survival time was observed in C3H/He mice which had been inoculated intraperitoneally with MH134 cells. Thus, it seems unlikely that the antitumor effect of CWE as well as of HWE is due to the participation of cell wall component.

Efficacy of an RNA fraction from *Mycobacterium tuberculosis* H37Ra and *Corynebacterium parvum* in the treatment of murine experimental cancer has been reported (Millman et al. 1974, 1976, 1977). CWE and HWE which contain nucleic acids are effective, whereas PWE and neo-WSA-BCG which are devoid of nucleic acids are ineffective. Motomiya et al. (1981) found no significant antitumor effect of a subfraction of HWE which contained only a trace of nucleic acids.

Thus it would be of importance to investigate whether nucleic acids contribute to the antitumor activity of hydrosoluble substances. Experiments are now carried out along this line.

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