Chemical and Biological Properties of Subfractions of Hot-Water Extract from Delipidated BCG

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MOTOMIYA, M., SATO, H., ARAI, H., NAGAI, H., ITO, M., SATOH, K., KUMANO, N. and KONNO, K. *Chemical and Biological Properties of Subfractions of Hot-Water Extract from Delipidated BCG*. Tohoku J. exp. Med., 1981, 133 (3), 293-300 — A hot-water extract from delipidated *Mycobacterium bovis* strain BCG was fractionated by precipitation with ethanol. The chemical and biological properties of the subfractions thus obtained were investigated. A subfraction precipitated at 20% ethanol contained less neutral sugar and more ninhydrin-positive substance than other subfractions. Only the guinea pigs that were treated with 20% ethanol-precipitate showed no humoral antibody response to egg white albumin, despite positive skin reaction. The subfractions precipitated at low concentrations of ethanol exhibited a significant antitumor activity in ddY and C3H/He mice inoculated intraperitoneally with tumor cells. These results suggest that the hot-water extract from delipidated BCG consists of subfractions with different chemical and biological properties. ——— immunotherapy; *Mycobacterium bovis* BCG; adjuvant activity

A hot-water extract from delipidated BCG was shown to be adjuvant-active and to have antitumor activity (Sato et al. 1976). Several water-soluble, adjuvant-active substances isolated prior to this hot-water extract were of cell wall origin and the principal sugars in these adjuvants were arabinose and galactose. Chemical analyses of the above hot-water extract revealed that it included constituents from cytoplasm, since glucose and mannose were the principal neutral sugar components. However, small amounts of arabinose and trace of galactose have also been detected in the hot-water extract (Yokosawa 1978) and there remains a possibility that the adjuvant activity was due to the presence of cell wall fragments.

Thus, the procedure of ethanol fractionation was applied in the present study in order to find out the principal structure responsible for adjuvant activity.

The hot-water extract from BCG contained 50-60% of neutral sugar, 15-20% of ninhydrin-positive substance and about 10% of nucleic acid. Then the hot-water extract was fractionated into six subfractions by the method of fractional precipitation with ethanol which had been employed for the purification of polysaccharide of tubercle bacilli (Azuma et al. 1968).

This report deals with the chemical analyses and adjuvant activity of subfractions thus obtained. The antitumor activity of these subfractions on Sarcoma 180, Ehrlich carcinoma and on a syngeneic tumor system was also investigated. The relationship between adjuvant effect and antitumor activity was discussed.

MATERIALS AND METHODS

The hot-water extract (hereafter HWE) was prepared essentially in the same manner as reported previously (Sato et al. 1976). The method of delipidation was slightly modified in this study. For delipidation, a 1:1 mixture of ether and ethanol, chloroform and then mixtures of chloroform and methanol (9:1, 8:2, 7:3, 6:4 and 5:5 respectively) were used in the order described.

Fractionation of HWE by ethanol

One g of HWE, dissolved in 100 ml of distilled water, received dropwise addition of 25 ml of ethanol. The opalescent solution thus obtained was centrifuged at $10,000 \times g$ for 60 min at 4°C. After removal of the precipitate (Fr20), the clear supernatant received an addition of ethanol to a concentration of 35%. The resulting precipitate (Fr35) was separated by centrifugation ($10,000 \times g$, 60 min, at 4°C). Then ethanol was added further to this supernatant to obtain the saturation required, procedure of centrifugation repeated, and thus Fr50, Fr65 and Fr80 were obtained in a similar manner. The supernatant separated from 80%-ethanol precipitate by centrifugation was designated Fr80S. The fractions thus obtained were dialyzed against tap water and lyophilized. (Fr50: precipitate at a concentration of 50% ethanol, Fr65: precipitate at a concentration of 65% ethanol and Fr80: precipitate at a concentration of 80% ethanol).

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. Amino sugar 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).

Adjuvant activity

Experimental animals (male albino guinea pigs) were sensitized by an injection into both hind footpads of 0.1 ml of water-in-oil emulsion of an equal part each of Freund's incomplete adjuvant (FIA) and the saline containing 1.5 mg of egg white albumin plus 0.15 mg of HWE or the subfractions prepared as above. A group of guinea pigs which received an injection of egg white albumin plus FIA was used as a control. On the 21st day after sensitization, 0.1 mg of egg white albumin in 0.1 ml of saline was injected at different sites of the back. The diameter of induration was measured at 48th hr. Four weeks after sensitization, blood was taken by heart puncture and the titer of antibody to egg white albumin was quantitated as described by Kabat and Mayer (1961).

Antitumor activity

Mice for this experiment were purchased from the Funabashi Animal Breeder, Funabashi, Japan.

1) Male ddY mice were inoculated intraperitoneally with 5×10^5 ascitic cells of Sarcoma 180 which had been maintained by weekly inoculations. The mice were divided into equal groups of 10. From day 1 after inoculation, 0.25 mg of each subfraction in

0.2 ml of saline was injected intraperitoneally for 14 consecutive days and the number of survivors at day 40 was compared.

2) Sixty male ddY mice inoculated intraperitoneally with 4×10^5 cells of Ehrlich carcinoma which had been maintained in ascites by intraperitoneal passage. The experimental animals were divided into 6 equal groups of 10. They were given 15 consecutive intraperitoneal injections of 0.25 mg in 0.2 ml of saline of each subfraction to be tested from day 1 after inoculation of tumor cells. A group of animals which received injections of saline alone was used as a control. The survival days were compared among 6 groups.

3) Age-matched inbred C3H/He mice were used. Fifty adult male mice, 6-week-old, received an intraperitoneal inoculation of 5×10^3 cells of syngeneic MH134 tumor and were divided into 5 equal groups of 10.

The MH134 (mouse hepatoma) was induced in C3H mice by administration of carbon tetrachloride and converted into ascites form (Sato et al. 1956) and have been maintained in C3H/He mice by intraperitoneal inoculation. From day 1 after inoculation of tumor cells, 0.3 mg of Fr20, Fr35, Fr50, and HWE in 0.2 ml of saline respectively were injected intraperitoneally for 14 consecutive days in 4 experimental groups. A group of mice which received injections of saline alone was used as control. The mean survival days were compared with those of a control group.

Statistical analysis

The Student *t*-test was used in all statistical evaluations.

RESULTS

Chemical analyses

The mean yield of subfractions obtained in three experiments of ethanol fractionation is shown in Table 1. The supernatant after separation by centrifugation of 80% ethanol solution (Fr80S) constituted approximately 30% of the starting material by weight. The subfractions obtained at lower concentrations of ethanol (Fr20 and Fr35) were less soluble than HWE in distilled water and in saline.

Paper chromatography showed glucose and ribose in Fr20 and Fr35. In Fr50 and Fr65 mannose was detected in addition to glucose and ribose. However, the ratio of mannose to glucose was higher in Fr65. Trace of arabinose and galactose was found in Fr80S. The results of quantitative determination are summarized in Table 2. Fr20 contained more ninhydrin-positive substance than other subfractions, whereas the content of neutral sugar was next to the lowest of the subfractions tested. The quantitative value of nucleic acids was highest in Fr50.

TABLE 1. *Fractionation of hot-water extract by ethanol*

Fraction	Ethanol added (%)	Yield (mg)	Percent
Fr20	0-20	83	11.0
Fr35	20-35	131	17.4
Fr50	35-50	63	8.4
Fr65	50-65	155	20.6
Fr80	65-80	93	12.2
Fr80S	Supernatant	227	30.4
Total		752	100.0

TABLE 2. *Quantitative analyses of hot-water extract and subfractions*

Fraction	Neutral sugar* (%)	Amino sugar† (%)	Ninhydrin-positive substance‡ (%)	Nucleic acids§ (%)
Fr20	30.6±3.4	2.7	20.3±4.2	11.7±2.2
Fr35	52.1±6.7	1.6	15.2±2.5	16.9±1.8
Fr50	28.6±6.5	1.2	13.0±3.0	33.2±3.1
Fr65	44.0±5.6	1.3	6.0±1.4	21.5±3.3
Fr80	39.7±5.4	1.1	7.4±5.5	10.7±2.0
Fr80S	45.7±8.7	2.5	12.5±6.8	0.9±0.2
HWE	42.2±7.6	1.4	13.6±4.4	10.2±2.1

HWE: hot-water extract.

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.

TABLE 3. *Humoral antibody response and delayed hypersensitivity to egg white albumin*

Injected with	Antibody response* (μ g N/ml serum) (mean±S.D.)	Skin test* (diameter of induration in mm)
FIA	167±40	8, 6, 0, 0, 0
FIA+Fr20	247±173	13, 12, 12, 12, 10
FIA+Fr35	326±109	18, 14, 13, 12, 12
FIA+Fr50	538±181	16, 15, 13, 13, 11
FIA+Fr65	446±114	13, 13, 12, 12, 12
FIA+Fr80	454±327	13, 12, 11, 10, 10
FIA+Fr80S	473±272	12, 12, 11, 11, 10
FIA+HWE	566±248	14, 14, 12, 12, 10

FIA: Freund's incomplete adjuvant. HWE: hot-water extract.

Guinea pigs were sensitized by an injection into both hind footpads of 0.1 ml of water-in-oil emulsion containing 1.5 mg of egg white albumin and FIA alone or FIA plus 0.15 mg of subfractions. Humoral antibody was measured by the method of quantitative precipitation at day 28. The skin test was made at day 21 and the diameter of induration was measured at 48 hr.

* Results observed in 5 guinea pigs

Adjuvant activity

Table 3 summarizes the results of experiments on humoral antibody response and delayed hypersensitivity to egg white albumin in guinea pigs. It was only in the group which had received Fr20 that there was no increase of humoral antibody. The most remarkable increase of antibody response was found in the group which had been injected with Fr50. Skin reaction was positive in all the groups. However, there was no significant inter-group difference in intensity of the reaction.

Antitumor activity

The results of three separate experiments on Sarcoma 180 are summarized in Table 4. There were no survivors at day 21 in the group which had been treated

TABLE 4. *Antitumor activity on Sarcoma 180*

Treated with	Survivors/number of mice			
	Experiment 1	Experiment 2	Experiment 3	Total
Saline	0/10	0/10	0/10	0/30
Fr20	6/10	4/10		10/20
Fr35	7/10	4/10		11/20
Fr50		3/10	4/10	7/20
Fr65		2/10	2/10	4/20
Fr80	5/10	1/10	2/10	8/30
Fr80S	2/10		1/10	3/20
HWE	6/10	5/10	4/10	15/30

HWE: hot-water extract.

In each experiment ddY mice were inoculated intraperitoneally with 5×10^5 cells of Sarcoma 180 and were divided into equal groups of 10. From day 1 after inoculation of tumor cells, 0.25 mg of each subfraction in saline was injected intraperitoneally for 14 consecutive days. The number of survivors at day 40 was recorded.

TABLE 5. *Antitumor activity on Ehrlich carcinoma*

Group	Treated with	Survival days (mean $\pm$ s.d.)	Difference*
1	Saline	27.7 $\pm$ 4.9	
2	Fr20	38.6 $\pm$ 8.0	$p < 0.01$
3	Fr35	36.6 $\pm$ 8.8	$p < 0.02$
4	Fr50	35.0 $\pm$ 8.7	$p < 0.05$
5	Fr80S	28.49 $\pm$ 9.1	$p > 0.5$
6	HWE	34.0 $\pm$ 6.8	$p < 0.05$

* Compared with group 1. HWE: hot-water extract.

Sixty male ddY mice were inoculated intraperitoneally with 4×10^5 cells of Ehrlich carcinoma and divided into 6 groups of 10. From day 1 after inoculation of tumor cells, 0.25 mg of each subfraction in saline was injected intraperitoneally for 14 consecutive days. Survival time in days of each group was compared.

TABLE 6. *Antitumor activity on MH134*

Group	Number of mice	Treated with	Survival days (mean $\pm$ s.d.)	Difference*
A	10	Saline	24.0 $\pm$ 4.4	
B	10	Fr20	32.1 $\pm$ 3.8	$p < 0.01$
C	10	Fr35	28.8 $\pm$ 5.1	$p < 0.1$
D	10	Fr50	29.2 $\pm$ 5.5	$p < 0.05$
E	10	HWE	30.9 $\pm$ 7.6	$p < 0.05$

* Compared with group A. HWE: hot-water extract.

Fifty male C3H/He mice were inoculated intraperitoneally with 5×10^3 cells of MH134 and divided into 5 equal groups of 10. From day 1 after inoculation of tumor cells, 0.3 mg of each subfraction in saline was injected intraperitoneally for 14 consecutive days. Survival time in days was compared among 5 groups.

with saline alone. A remarkable antitumor activity was observed in the groups which had received injections of Fr 20 and Fr35 respectively. On the other hand, only a slight antitumor effect was found in the groups which had received Fr65, Fr80 and Fr80S respectively. The results of experiments on antitumor activity on Ehrlich carcinoma are shown in Table 5. The survival time in mice which had received Fr20 was prolonged. No difference in survival time in days was found between the control group and the group which had been treated with Fr 80S. The antitumor effect in syngeneic tumor system was not so remarkable as in allogeneic system. Table 6 shows the antitumor effect on MH134. The survival time was prolonged in the group which had been injected with Fr50 and HWE respectively ($p < 0.05$). Also in this experiment a significant increase of survival time was observed in the group which had received Fr20.

DISCUSSION

Since the report of Freund and McDermott (1942), tubercle bacilli have been employed as adjuvant in order to increase antibody titer and to induce delayed hypersensitivity reaction to a given antigen. Also various fractions from tubercle bacilli have been used in place of whole bacteria. It had been accepted that the fraction responsible for adjuvant effect contained peptido-glycolipid portions. In recent times water-soluble, adjuvant-active substances have been isolated from mycobacteria by various methods (Adam et al. 1972, 1973; Hiu 1972; Migliore-Samour and Jollès 1972, 1973; Stewart-Tull et al. 1975) and evidence has been presented suggesting that these substances were derived from cell wall. Subsequent studies have revealed that the minimal essential structure for adjuvant activity was muramyl dipeptide in cell wall (Ellouz et al. 1974; Adam et al. 1975; Kotani et al. 1975; Azuma et al. 1976).

The hot-water extract from BCG was adjuvant-active, of which the major portion was derived from cytoplasm (Sato et al. 1976). In the present study a subfraction which contained glucose alone as neutral sugar was adjuvant-active. Thus, a possibility that the adjuvant activity of hot-water extract is due to the concomitant presence of cell wall fragment in this subfraction could be ruled out.

Water-soluble adjuvants have been extracted also from nocardia which is in close taxonomical relation to mycobacteria and adjuvant activity was detected even in a subfraction which was gel-filtered and was devoid of cell wall fragment (Ciorbaru et al. 1975). Therefore it seems unlikely that the cell wall component is the only constituent responsible for adjuvant activity.

An association of adjuvant effect with antitumor activity has been suggested. However, not all the adjuvant-active substances have in vivo antitumor activity. For example, muramyl dipeptide is not effective in the treatment of neoplasma in vivo when they are administered without partner substance.

In the present investigation, a subfraction (Fr80S) obtained from the supernatant after centrifugation of 80%-ethanol precipitate was adjuvant-active, but had little antitumor activity. This subfraction contained less than 1% of

nucleic acids. Though mycobacterial RNA has been reported to be effective in the treatment of murine tumor systems (Millman et al. 1974, 1976), decreased antitumor activity of this fraction cannot be directly ascribed to the low concentration of nucleic acids, because antitumor effect on Sarcoma 180 was found even after removal of nucleic acids by streptomycin sulfate in the previous study (Sato et al. 1978).

There have been numerous publications on immunotherapy using BCG, corynebacteria, and their cell wall fractions which exert significant antitumor activity when administered singly. On the other hand, only limited information is available concerning the antitumor effect of cytoplasm and water-soluble bacterial substances. Butler and Nowotny (1979) reported that polysaccharide-rich hydrolytic product from lipopolysaccharide showed weak, but significant antitumor activity in mice inoculated intraperitoneally with tumor cells. Millman et al. (1977) found antitumor activity in cytoplasmic fraction of *Corynebacterium parvum*.

It is well known that immunotherapy is less effective in syngeneic than in allogeneic tumor systems. Also it has been reported that the results of treatment with immunoadjuvants depend on the number of cells inoculated. When over 10^4 cells of MH134 were inoculated intraperitoneally, no antitumor effect was observed in C3H/He mice which had been treated by the hot-water extract in the same manner as in the present experiment. In an experiment on ddY mice which were inoculated with Sarcoma 180, attempts were made to find out the dose of hot-water extract and the duration of treatment that were most satisfactory from view points of therapeutic results (Sato et al. 1979). Similar experiments would be required to obtain more satisfactory results in syngeneic tumor systems.

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