Chemical and Biological Properties of Hot Water Extract from Delipidated Cells of *Mycobacterium bovis* Strain BCG

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SATO, H., YOKOSAWA, A., ARAI, H., NAGAI, H., KURITA, K., KUMANO, N., MOTOMIYA, M. and KONNO, K. *Chemical and Biological Properties of Hot Water Extract from Delipidated Cells of Mycobacterium bovis Strain BCG*. Tohoku J. exp. Med., 1976, 120 (1), 75-81 — A water-soluble fraction was isolated from delipidated cells of *Mycobacterium bovis* strain BCG by extraction with hot water. Chemical analyses revealed that the above fraction presumably consisted of a peptidoglycan containing 5-10% of nucleic acids. When it was injected into guinea pigs with Freund's incomplete adjuvant plus egg white albumin as antigen, an increase of circulating antibody was observed as shown by the augmented titers of precipitin and hemagglutinin. The results of skin test and corneal reaction indicated that the fraction mentioned above induced delayed hypersensitivity to egg white albumin. Footpad reaction in mice demonstrated that the above fraction induced delayed hypersensitivity to sheep red blood cells. It was confirmed in addition that the adjuvant activity of this fraction was not due to the presence of nucleic acids. This adjuvant-active fraction was designated as HSA (hot-water soluble adjuvant). ——— extract from BCG; adjuvant activity

Freund and McDermott (1942) demonstrated that an injection of killed mycobacteria with an antigen emulsified in mineral oil increased the titer of circulating antibody and caused delayed hypersensitivity against the antigen. Since then, water-in-oil emulsion containing mycobacteria has been used as potent adjuvant. Raffel and Cal (1950) ascribed the adjuvant activity of mycobacteria to wax D. The wax D, a fraction insoluble in hot acetone of the chloroform extract from mycobacteria, is a peptidoglycolipid and is known to be the main component of the cell wall of mycobacteria.

Recently, water-soluble substances which are adjuvant-active have been isolated from delipidated mycobacteria and from cell wall by means of hydrogenation (Hiu 1972), enzymatic treatment (Adam et al. 1972, 1973) and ultrasonication (Migliore-Samour and Jollès 1972, 1973). This report deals with the isolation of a water-soluble fraction from delipidated *Mycobacterium bovis* strain BCG by extraction with hot water. The fraction thus prepared was found to be adjuvant-active as revealed by the results of the tests for biological activities in guinea pigs.

This fraction, hereafter referred to as HSA (hot-water soluble adjuvant), may be useful as adjuvant in place of whole cells of mycobacteria. The differences in chemical composition of HSA from that of other water-soluble adjuvants was also discussed.

MATERIALS AND METHODS

Preparation of HSA

Mycobacterium bovis strain BCG (Japanese strain, Tokyo No. 172, supplied by National Institute of Health, Tokyo, Japan), subcultured for 4 weeks on a modified Sauton medium (Sato et al. 1969) was filtered and washed with distilled water. It was delipidated with a 1:1 mixture of ether and ethanol, chloroform and then with a 2:1 mixture of chloroform and methanol. The defatted bacilli were pulverized in a mortar, then washed thoroughly with 3 changes of water at room temperature. The bacillary suspension in water was heated at 100°C for 15 min, passed through a sheet of filter paper, and then through a Millipore Filter (0.45 μ m) (Millipore Filter Corporation, Massachusetts, U.S.A.) for the removal of cell debris and was concentrated in vacuo. The precipitate prepared by the addition of 3 volumes of acetone to the above concentrate was separated by centrifugation. The resulting supernatant was concentrated and then was mixed with 3 volumes of acetone. The precipitate thus obtained was dialyzed against distilled water for 48 hr and the retentate was lyophilized.

Analytical methods

Neutral sugars were identified by paper chromatography on Toyoroshi filter paper No. 51 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. Quantitative determination of hexose was made by Roe's method (1955) and by phenol-sulfuric acid reagent. In gas-liquid-chromatographic analysis of neutral sugars, a use was made of a column of 3% ECNSS-M on Gaschrom Q. Amino sugars were identified after hydrolysis for 4 hr at 110°C in 4 N HCl by paper chromatography in the solvent systems of ethyl acetate-pyridine-n-butanol-butyric acid-water (10:10:5:1:5) and ethyl acetate-pyridine-water-acetic acid (5:5:1:3). Quantitative estimation of amino sugar was carried out by the method of Elson-Morgan modified by Blix (1948). A column of 15% Apiezon M on Chromosorb CLH was employed in gas-liquid-chromatographic analysis of amino sugars. Amino acids were analyzed also by an amino acid autoanalyzer, JLC-5AH. Ninhydrin-positive substance was measured as described by Yemm and Cocking (1955). For the detection of nucleic acid, absorbance in an aqueous solution was recorded from 260 nm to 320 nm. Quantification of nucleic acid was carried out as described by Schmidt and Thannhauser (1945), and by Schneider (1946).

In the next experiment HSA was applied on a 2.5 $\times$ 60 cm column of Sephadex-G 75 and was eluted with 0.1 N acetic acid. An aliquot from 4 ml-portions in each tube was monitored by quantitative determination of neutral sugar and ninhydrin-positive substance. As illustrated in Fig. 1, there were two separate peaks. The second peak, referred to as Fr-II, was analyzed by chemical and biological methods in a similar manner as HSA.

Biological activity

Male albino guinea pigs weighing 250–300 g were sensitized by an injection of 0.1 ml of water-in-oil emulsion made of an equal part each of Freund's incomplete adjuvant and the saline containing 2 mg of egg white albumin plus 0.2 mg of HSA, or BCG cells. On the 21st day after sensitization, 0.1 mg and 0.01 mg respectively of egg white albumin in 0.1 ml of saline were injected intradermally at different sites of the back. The corneal test according to Stewart-Tull's method (Stewart-Tull and White 1967) was done on the same day. The diameter of induration and the turbidity of the cornea were measured at 24th and 48th hr. Four weeks after sensitization blood was taken by heart puncture

for the quantitative determination of the precipitin titer (Kabat and Mayer 1961) and for passive hemagglutination test (Middlebrook and Dubos 1948).

Male ddI mice received an intraperitoneal or intradermal injection of HSA, Fr-II and BCG respectively 12 or 4 days before sensitization. As sensitizing antigen 10^8 sheep red blood cells (SRBC) were injected intraperitoneally. After 4 days, 10^8 SRBC were injected intradermally on the right hind footpad and the thickness was compared with that of the non-injected footpad after 24 hr.

RESULTS

Physical and chemical properties

From 50 g of the dry delipidated BCG approximately 1.5 g (3%) of HSA was obtained. HSA, when lyophilized, was a whitish powder. It was easily soluble in water and the resulting aqueous solution was slightly opalescent.

The result of fractionation by a column of Sephadex-G 75, as illustrated in Fig. 1, showed two separate peaks. In the aqueous solution, an absorption maximum was observed at 260 nm, but none at 280 nm. The quantitative data showed that the HSA consisted of 50–60% of neutral sugar, 1–3% of amino sugar, 15–20% of ninhydrin-positive substance and 5–10% of nucleic acid.

Fig. 1. Elution pattern of 400 mg of HSA from a 2.5×60 cm column of Sephadex-G 75. Elution was effected with 0.1 N acetic acid. Fractions (4 ml) were analyzed for neutral sugar by phenol-sulfuric acid reagent and for ninhydrin-positive substance by the method of Yemm and Cocking (calculated as alanine).

●—●, neutral sugar; ○—○, ninhydrin-positive substance.

Fr-II was found to contain 60–70% of neutral sugar, 2–3% of amino sugar, 15–20% of ninhydrin-positive substance and less than 1% of nucleic acid.

Paper-chromatography and gas-liquid-chromatography revealed the presence of ribose, arabinose, galactose, mannose and glucose. The quantity of the latter

two sugars, which were the main components of neutral sugars, constituted 90% of the total quantity of neutral sugars in HSA as well as in Fr-II.

On paper-chromatograms of amino sugars, glucosamine and muramic acid were detected. As shown in Table 1, not only Ala, Glu and diaminopimelic acid, but some other amino acids were also detected.

TABLE 1. *Amino acid constituents*

Amino acid	Molar ratio		Amino acid	Molar ratio	
	HSA	Fr-II		HSA	Fr-II
Asp	43.1	97.0	Met	0	0
Thr	25.6	43.2	DAP	1.0	1.0
Ser	20.2	41.7	Ile	15.9	16.8
Glu	53.6	162.5	Leu	32.2	38.8
Pro	35.2	58.2	Tyr	3.2	5.2
Gly	62.6	142.0	Phe	8.9	8.8
Ala	55.7	98.8	Lys	22.1	100.7
Cys	0	0	His	5.2	9.2
Val	31.6	42.8	Arg	21.0	45.5

DAP: diaminopimelic acid.

Biological activity

Table 2 summarizes the results of experiments on humoral antibody response to egg white albumin. In the guinea pigs sensitized with Freund's incomplete adjuvant containing HSA, the titer of precipitin and that of hemagglutinin were significantly augmented ($p < 0.001$). As shown in Table 3, the results of skin test and corneal reaction 3 weeks after sensitization were positive in the animals which had received either HSA or Fr-II in Freund's incomplete adjuvant.

Table 4 summarizes the results of the footpad reaction. An increase in thickness of footpad was found also in the mice which had been injected with HSA and Fr-II.

TABLE 2. *Humoral antibody response to egg white albumin*

Number of animals	Injected with	$\mu\text{g N/ml serum}^*$ mean $\pm$ S.D.	PHA mean $\pm$ S.D.
5	Saline	28 $\pm$ 17	4 $\pm$ 0
5	FIA	283 $\pm$ 157	282 $\pm$ 140
10	FIA + HSA	722 $\pm$ 208	1024 $\pm$ 591
10	FIA + Fr-II	339 $\pm$ 163	486 $\pm$ 244
5	FIA + BCG	1642 $\pm$ 541	2253 $\pm$ 1122

* Determined by quantitative precipitation test.

PHA: passive hemagglutinin, reciprocal titer.

FIA: Freund's incomplete adjuvant (Difco).

TABLE 3. *Delayed hypersensitivity to egg white albumin*

Number of animals	Injected with	Skin test positive	Corneal reaction			
			-	+	‡	‡‡
5	Saline	0/5	5			
5	FIA	0/5	5			
10	FIA+HSA	9/10	5	2	1	1
10	FIA+Fr-II	8/10	4	3	1	2
5	FIA+BCG	5/5		3	2	

-: absent-turbidity, +: slight-turbidity, ‡: moderate-turbidity, ‡‡: marked-turbidity with chemosis

TABLE 4. *Footpad reaction*

Number of mice	Injected with	Route	Day	Increase in footpad thickness 10^{-1} mm
20	Saline	i.p.	- 4	1.14±0.69
10	HSA	i.p.	- 4	2.34±0.90
10	HSA	i.d.	- 4	1.71±0.89
10	HSA	i.d.	-12	3.00±0.90
10	Fr-II	i.d.	- 4	3.61±1.18
10	Fr-II	i.d.	- 4	2.27±1.10
10	Fr-II	i.d.	-12	2.73±1.04
10	BCG	i.p.	- 4	5.60±2.01
10	BCG	i.d.	-12	3.01±1.16

Sensitizing dose: 10^8 SRBC i.p. Eliciting dose: 10^8 SRBC.

i.p., intraperitoneal; i.d., intradermal. -4, injection 4 days before sensitization; -12, injection 12 days before sensitization.

DISCUSSION

Not only live or killed mycobacteria, but also mycobacterial preparations such as cell wall (Lederer 1971) and methanol extracted residue (Shlomo et al. 1973) have been reported to be adjuvant-active. In addition, adjuvant activity of several water-soluble substances from delipidated mycobacteria has been reported. In the present study, a hot water extract from delipidated BCG cell was adjuvant-active and was designated as HSA (hot-water soluble adjuvant). However, the chemical composition of HSA was somewhat different from that of other water-soluble adjuvants.

In the present investigation the presence of ribose was confirmed by paper-chromatography and by gas-liquid-chromatography. There was an absorption maximum at 260 nm. Thus the presence of nucleic acid in HSA was suggested. Other water-soluble adjuvants so far reported contain no nucleic acid. On the other hand, Casavant and Youmans (1975) found that 0.2 mg of mycobacterial RNA injected together with Freund's incomplete adjuvant was adjuvant-active in guinea pigs.

There was a possibility that the adjuvant activity of HSA was due to the presence of nucleic acid. However, judging from the content of nucleic acid in Fr-II (less than 1%), it is unlikely that the adjuvant activity of HSA is due to

nucleic acid. In the water-soluble adjuvant described by Hiu (1972) and Adam et al. (1972), the main component of neutral sugars was arabinose and galactose. Thus they thought that arabinogalactan from cell wall was extracted. In the case of HSA only traces of arabinose and galactose were detected. Mannose and glucose were the main components of neutral sugars when evaluated by gas-liquid-chromatography. In addition, the difference in composition of neutral sugars and the very low content of DAP (diaminopimelic acid) suggested that a substance other than arabinogalactan-mucopeptide from cell wall is responsible for adjuvant activity of HSA.

The content of amino sugar was not more than 3% after hydrolysis under various conditions.

Chedid et al. (1972) investigated the biological activity of the water-soluble adjuvant prepared by Adam and found no toxic effect inherent to whole cells, when it was administered with saline. The biological activities other than adjuvant effect of HSA are now under investigation.

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