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Fractionation of Mycobacterial Cell Wall

Isolation of Arabinose Mycolate and Arabinogalactan from Cell Wall Fraction of *Mycobacterium tuberculosis* Strain Aoyama B

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In previous papers, we reported the isolation and characterization of arabinose mycolate from firmly bound lipids (7; Azuma and Yamamura, *in preparation*) and wax D (3) fractions of *Mycobacterium tuberculosis* and other mycobacterial strains. The chemical structure of arabinose mycolate has also been established as D-arabinose-5-mycolate (7). The serologically active polysaccharide, arabinogalactan, was purified from defatted cells (2), culture filtrate (4), and wax D fraction (5) of *M. tuberculosis* strain Aoyama B in our laboratory. We have suggested that arabinose mycolate and arabinogalactan were the principal components of mycobacterial wax *D* and defatted cells. In this note, we describe fractionation of lipid fractions from the cell wall and isolation of arabinose mycolate and arabinogalactan from the cell wall of *M. tuberculosis*. The chemical structure of the mycobacterial cell wall is discussed.

The cell wall fraction was prepared by the following procedure. Acetone-dried *M. tuberculosis* cells were suspended in cold saline to a concentration of 100 mg (dry weight) per ml. This suspension was then processed in a Sorvall Ribi refrigerated cell fractionator at 25,000 lb/in^2 at 5 to 10 C. The effluent was centrifuged at 800 $\times$ *g* to remove unbroken whole cells. The supernatant fluid was centrifuged at 15,000 $\times$ *g* for 60 min to obtain a supernatant fluid containing most of the soluble cytoplasm and a sediment consisting predominantly of cell wall. The supernatant fluid was recentrifuged at 40,000 $\times$ *g* for 2 hr to obtain a sediment consisting of reddish particles. The supernatant fluid was recentrifuged at 100,000 $\times$ *g* for 2 hr to yield a small amount of sediment consisting of smaller particles. The crude cell wall fraction was suspended in water and centrifuged at 800 $\times$ *g* to remove unbroken whole cells again. After washing in water three times, the cell wall fraction was lyophilized. The yield of the lyophilized cell wall fraction was approximately 10% of starting cells. The cell wall fraction had a characteristic surface structure of paired fibrous prominence by electron micrographic examination.

An 8-g amount of cell wall fraction thus purified was fractionated into 11 lipid subfractions by the method of Anderson (1), with some modifications in the extraction of firmly bound lipids (6). Table 1 shows the results of lipid fractions from the cell wall and intact cells. Larger amounts of lipid were contained in the cell wall fraction than intact cells. Tuberculin-active protein was also obtained from the cell wall of human tubercle bacilli. The chemical and immunological properties of tuberculin-active protein will be reported in a later paper.

Arabinose mycolate was purified from the firmly bound lipid B fraction of the cell wall of *M. tuberculosis* by the column chromatographic procedure. A 700-mg amount of the firmly bound lipid B fraction, dissolved in *n*-hexane, was loaded on a column of 50 g of silicic acid-Celite (2:1) and eluted with chloroform and chloroform-methanol (95:5, 9:1, and 8:2). A 400-mg amount of the second fraction eluted with chloroform-methanol (95:5) in chromatogram I was rechromatographed on a column of 20 g of Florisil-Celite (2:1) and eluted with ether and ether-methanol (95:5, 9:1, and 8:2). The second fraction, eluted with ether-methanol (95:5) in chromatogram II, was dissolved in ether and precipitated with methanol, and 210 mg of white powdered lipid fraction was obtained. The physicochemical properties of this lipid fraction are shown in Table 2. The infrared spectrum of this lipid fraction was almost identical with that of D-arabinose-5-mycolate established previously (7). By acid or alkaline hydrolysis with a 5% HCl or 1% NaOH solution, D-arabinose and mycolic acid were obtained. Mycoloyl alcohol was obtained by the reductive cleavage with $LiAlH_4$. Mycolic acid and mycoloyl alcohol were determined by infrared spectroscopy and elementary analysis. D-Arabinose was determined by gas-liquid chromatography (2). By the quantitative determination of arabinose after acid hydrolysis

by the methods of Dubois et al. (8), 10.1% of arabinose was determined. These data were almost in agreement with those of D-arabinose-5-mycolate obtained from defatted cells of *M. tuberculosis* (7).

Purification of arabinogalactan from the cell wall of *M. tuberculosis* was carried out by the following methods. A 300-mg amount of the cell wall fraction of human tubercle bacilli, which was defatted by repeated extraction with ether-ethyl alcohol (1:1), chloroform, and chloroform-methanol (2:1), was extracted with NaOH at 70 C for 24 hr and centrifuged. The supernatant fluid was neutralized with acetic acid and dialyzed against running water for 72 hr. The inner solution was concentrated to one-half volume and fractionated with ethyl alcohol. The polysaccharide obtained by the addition of ethyl alcohol to a final concentration of 50 to 80% was purified by column chromatography on diethylaminoethyl cellulose and eluted with water and 0.2 M NaH_2PO_4. The water eluate was recovered by the addition of ethyl alcohol, and 25 mg of purified cell wall polysaccharide was obtained. The chemical and immunological properties of this polysaccharide are shown in Table 2. By the gas-liquid chromatographic analysis of the acid hydrolysate of this cell wall polysaccharide fraction, arabinose and galactose were detected in a molar ratio of 2.6:1. The cell wall polysaccharide, arabinogalactan, purified as described, showed potent antigenic titer by the precipitation test with rabbit antiserum against heat-killed *M. tuberculosis*. By the Ouchterlony (10) method of immunodiffusion analysis, the precipitin line of arabinogalactan of the cell wall fused with those of arabinogalactan purified from defatted cells (2), culture filtrate (4), and wax D fraction (5) of *M. tuberculosis*.

From the above results, we concluded that mycolic acid combined by the ester linkage with the 5-hydroxy group of D-arabinose residue of arabinogalactan in mycobacterial cell wall (Azuma and Yamamura, *in preparation*). Recently, Kanetsuna (9) suggested that the mycolic acid-arabinogalactan-mucopeptide complex may be a common structure of the mycobacterial cell wall. Our present experiments showed the combining site of mycolic acid with arabinogalactan. However, the linkage feature between arabinogalactan and mucopeptide is not yet defined.

TABLE 1. *Fractionation of lipids and tuberculin-active protein from the cell wall and intact cells of M. tuberculosis strain Aoyama B*

Fractions	Cell wall[a] (8 g)		Intact cell (10 g)	
	Wt (mg)	Yield (%)	Wt (mg)	Yield (%)
Total lipids	2,890	36.1	2,764	27.6
Ethyl alcohol-ether-soluble	973	12.1	998	9.9
Acetone-soluble	532	6.6	688	6.8
Wax A	419	5.2	130	1.3
Phospholipid	21	0.2	179	1.7
Chloroform-soluble	438	5.4	438	4.3
Wax B	110	1.3	135	1.3
Wax C	187	2.3	174	1.7
Wax D	140	1.7	128	1.2
Chloroform-methanol (2:1) soluble	115	1.4	247	2.4
Bound lipids	1,364	17.0	1,081	10.8
Fraction A	159	1.9	174	1.7
Fraction B	741	9.2	780	7.8
Fraction C	437	5.4	112	1.1
Fraction D	27	0.3	15	0.1
Tuberculin-active protein	87	1.0	165	1.6

[a] Cell wall was prepared with a Sorvall Ribi refrigerated cell fractionator.

TABLE 2. *Arabinose mycolate and arabinogalactan purified from the cell wall of M. tuberculosis strain Aoyama B*

Fraction	$[\alpha]_D^{25}$	Melting point	Elementary analysis (%)		Chemical composition (molar ratio)	Precipitation reaction (Ag titer)[a]
			C	H		
Arabinose mycolate	+9.8	40–42 C	77.05	12.99	Arabinose, 1; mycolic acid, 1	
Arabinogalactan	+24.0		41.95	6.66	Arabinose, 2.6; galactose, 1.0	1:512,000

[a] Antigen titer was determined by the "ring test" with rabbit antiserum against heat-killed cells of *M. tuberculosis* strain Aoyama B.

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