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Chemical and Immunological Properties of Polysaccharides of Wax D Extracted from *Mycobacterium tuberculosis* Strain Aoyama B

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Wax D fraction extracted from human tubercle bacilli is a macromolecular peptide-glycolipid consisting of mycolic acid, polysaccharide, and peptide. Wax D fraction obtained from human tubercle bacilli showed characteristic biological activities such as adjuvant activity in the immune response, the induction of adjuvant arthritis in rats, and growth factor activity for pathogenic *Leptospira* when used in place of rabbit serum. Jollès et al. (4) proposed a hypothetical structure; however, the chemical structure of wax D has not been confirmed. Recently, Tanaka and Kitagawa (6) reported the fractionation of wax D by column chromatography on silicic acid after acetylation. In a previous paper (1), we reported the isolation of a new glycolipid, D-arabinose-5-mycolate, from wax D fractions of strain Aoyama B after acid treatment, and we also suggested that arabinose mycolate seemed to represent a combining site for mycolic acid in the wax D molecule. This paper consists of a description of the chemical and immunological properties of the polysaccharide portion of wax D fractions extracted from human tubercle bacilli of strain Aoyama B.

Wax D fraction was extracted from acetone-dried cells of human tubercle bacilli strain Aoyama B by the method of J. Asselineau (D.Sc. Thesis, Univ. of Paris, 1951) and was acetylated by the following procedure. To 10 g of wax D fraction dissolved in 100 ml of pyridine, 80 ml of acetic anhydride was added. The reaction mixture was warmed at 30 C for 12 hr, and five volumes of ethyl alcohol were added. The resulting precipitate was collected by centrifugation (3,000 rev/min for 20 min) and was washed repeatedly with ethyl alcohol. From the results of infrared spectrum analysis of the acetylated wax D fraction, it was shown that the wax D fraction is almost completely acetylated in this way. The ability of the acetylated wax D fraction to induce adjuvant arthritis was examined by S. Kishimoto (Proc. Gen. Assembly Japan Med. Congr. 17th 2:394, 1967), and it was shown that adjuvant arthritis could not be induced by the injection of acetylated wax D fraction prepared in the present experiments.

Acetylated wax D fraction (10 g) was chromatographed on a column of silicic acid-Celite (1:1) and was eluted with benzene-chloroform-methanol (95:5), chloroform-methanol (9:1), and chloroform-methanol (8:2). The fractions were designated as D1, D2, D3, and D4, respectively. The results of chemical analysis of each acetylated wax D subfraction showed that peptide was present in fractions D3 and D4 and in trace amounts in fraction D1, but not in fraction D2. Alanine, glutamic acid, and α,ϵ-diaminopimelic acid were detected as major amino acid components of the peptide portion by paper chromatography after acid hydrolysis. Adjuvant activity of each acetylated wax D subfraction was examined by S. Yamazaki et al. (Ann. Rept. Japan-U.S.A. Med. Coop. Tuberc. Sect., p. 305, 1966) and adjuvant activity was observed in acetylated D3 and D4 fractions. These results suggest that the adjuvant activity of wax D is not affected by acetylation and that the peptide portion is essential for the development of adjuvant activity in wax D fractions.

In the present experiments, chemical and immunological properties of the polysaccharide moiety of the original wax D and acetylated D2 fractions were investigated. The polysaccharide portions of wax D and acetylated D2 fractions were obtained by alkaline hydrolysis. Pooled acetylated D2 fraction (5 g) was dissolved in a mixture of 60 ml of benzene, 30 ml of chloroform, and 10 ml of 5% methanolic potassium hydroxide solution. The mixture was boiled in the water bath for 5 min and was evaporated to dryness under reduced pressure. The residue was extracted with water repeatedly. The water-soluble fraction, which was dialyzed against running water for 72 hr, was concentrated to one-half the original volume under reduced pressure, and crude poly-

TABLE 1. *Chemical and immunological properties of polysaccharides obtained from wax D and acetylated wax D subfractions of human tubercle bacillus strain Aoyama B*

Polysaccharide obtained from	$[\alpha]_D^{25}$ (in water)	Elementary analysis	Sugar composition (ratio)[a]	Precipitation test (Ag titer)[b]	Complement fixation reaction[c]	Passive hemagglutination reaction[d]
		%				
Wax D.....	Not examined	C, 41.03 H, 6.17 N, 1.73	Arabinose, 2.8 Galactose, 1.0 Mannose, trace	1:512,000	Ag, 0.024 μg (Ab, 1:640)	1:20,480
Acetylated D2 fraction.....	+ 22.2 (c = 1.011)	C, 41.93 H, 6.28 N, trace	Arabinose, 2.8 Galactose, 1.0	1:512,000	Ag, 0.024 μg (Ab, 1:640)	1:20,480

[a] Amino sugar was not determined.
[b] Antigen (Ag) titer was determined in a "ring test," using rabbit antiserum.
[c] Results are expressed as micrograms of antigen necessary for 50% lysis in the system; Ab, antibody.
[d] Passive hemagglutination tests were carried out by the method described previously by Azuma et al. (2). Titer is expressed as the highest dilution of rabbit antiserum which gave a 2+ positive reaction.

saccharides (0.8 g) were obtained by the addition of five volumes of ethyl alcohol. Crude polysaccharide was dissolved in a small amount of water and was purified by the column chromatography on diethylaminoethyl cellulose. Purified polysaccharide, 0.55 g, was obtained by elution with water. The chemical and serological properties of polysaccharides obtained from original wax D and acetylated D2 fractions are given in Table 1. Sugar composition of polysaccharides was determined by gas-liquid chromatography according to the method described by Azuma et al. (2). The polysaccharide obtained from the acetylated D2 fraction was composed of arabinose and galactose in a molar ratio of 2.8:1.0. The results of elementary analysis indicate that only a trace of nitrogen was contained in this polysaccharide, whereas 1.73% nitrogen was detected in the polysaccharide obtained from the original wax D fraction. The optical rotation of the arabinogalactan polysaccharide obtained from the acetylated D2 fraction was almost identical with that of arabinogalactan purified from culture filtrates or defatted cells of human tubercle bacilli strain Aoyama B (2, 3). In the polysaccharide obtained from the original wax D, arabinose, galactose, and a trace of mannose were found.

Arabinogalactan obtained both from the acetylated D2 and the original wax D fractions showed high titers in precipitation test, complement fixation, and passive hemagglutination reactions with rabbit antiserum obtained by the intramuscular injection of heat-killed tubercle bacilli strain Aoyama B. By immunodiffusion analysis by Ouchterlony's method (5), the precipitin line of arabinogalactan obtained from acetylated D2 fraction fused with those of arabinogalactan purified from the culture filtrate and defatted cells of human tubercle bacilli strain Aoyama B. It was also shown by immunodiffusion analysis that the polysaccharide fraction obtained from the original wax D contained two kinds of polysaccharides, arabinogalactan and arabinomannan.

From the above results, it was concluded that arabinogalactan was the main serologically active component of the wax D fraction of human tubercle bacilli strain Aoyama B, and the chemical and immunological properties of arabinogalactan obtained from acetylated wax D subfraction (D2 fraction) were almost identical with those of arabinogalactan purified from the culture filtrate and defatted cells of human tubercle bacilli strain Aoyama B. It seems that in wax D, mycolic acid is combined with the arabinose residue of arabinogalactan of the wax D molecule. The details of chemical structure and immunological activity of wax D of tubercle bacilli and other mycobacteria are now being investigated in our laboratory.

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