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Cell Wall of *Mycobacterium lepraemurium* Strain Hawaii

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The chemical properties of the cell wall of *Mycobacterium lepraemurium* strain Hawaii were investigated. Five subunits of the cell wall, arabinose mycolate, mycolic acids, tetrapeptide (Ala-Gln-diaminopimelic acid-Ala), disaccharide (*N*-acetylglucosaminyl-β-1,4-*N*-glycolylmuramic acid), and arabinogalactan, were obtained, and their chemical structures were identified.

It is well known that *Mycobacterium lepraemurium* is the causative microorganism of murine leprosy, a chronic infection in the rat and the mouse. Its taxonomy has not yet been established. Uyeda (18, 19) claimed that, from the morphological features of this bacillus, it should not be classified as a mycobacterium, but rather should be placed in a new genus related to nocardia. However, results of recent works on the chemical analysis of the *M. lepraemurium* cell wall (7-9, 14) suggest that its chemical composition qualitatively resembles that of the cell walls of mycobacteria (15). Also, Fukui and co-workers (12) have reported that the crude extracts of eight strains of *M. lepraemurium* contain a cross-reactive material whose antigenic determinants are partially in common with an alpha antigen of *Mycobacterium avium*. This finding also suggests the propriety of the serological typing of *M. lepraemurium* as a *Mycobacterium*.

In this study, the chemical and immunological properties of the cell wall and polysaccharides of *M. lepraemurium* strain Hawaii have been examined in detail, and, based on these properties, it has been concluded that this microorganism is a *Mycobacterium*.

Collection and fractionation of cells. A 0.2-ml portion of an emulsion of *M. lepraemurium* Hawaii leproma, diluted 1,000 times with Hanks solution, was inoculated subcutaneously into 400 4-week-old C_3H mice. Six months later, the lepromas were excised and homogenized, and the *M. lepraemurium* Hawaii cells were collected by differential centrifugation according to the method of Mori et al. (17). The bacilli (8.7 g, dry weight) were delipidated by repeated extractions with ether-ethanol (1:1), chloroform, and chloroform-methanol (2:1).

Preparation of cell walls. Five grams of delipidated cells were suspended in 200 ml of water and disrupted by sonic oscillation at 20 kc for 30 min in an ice bath. The disrupted cells were centrifuged several times at 600 × *g* for 20 min to remove the unbroken cells and cell debris, after which the walls were collected by centrifugation at 20,000 × *g* for 1 hr. The cytoplasmic residue was removed from the cell wall material by treatment with trypsin, chymotrypsin, and Pronase followed by washing with saline, water, acetone, ether, and chloroform-methanol (2:1), consecutively (4). These purified cell walls (650 mg, dry weight) were used as starting material for chemical and immunological studies. The chemical analyses revealed that they were composed of 34.3% lipid (mycolic acid), 30.4% neutral sugars (arabinose and galactose in a molar ratio of 2.8:1), 13.8% amino acids, 8.1% amino sugars, and 0.27% phosphorus. Amino acid analysis indicated that they contained alanine, glutamic acid, diaminopimelic acid (Dpm), glucosamine, and muramic acid in a concentration of 3.36, 1.65, 1.89, 1.42, and 1.41 μmoles per 10 mg, respectively, as major amino acid and amino sugar constituents.

The cell walls thus obtained from *M. lepraemurium* Hawaii therefore contain the constituents responsible for potent adjuvanticity and "adjuvant-polyarthritogenicity" just as well as do cell walls of other mycobacteria (2, 3).

Chemical structure of the cell wall. The

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cell walls of corynebacteria, nocardia, and mycobacteria have been shown to possess in common a "mycolic acid-arabinogalactan-mucopeptide" complex structure (6, 15). In this study, we have characterized five cell wall subunits, arabinose-mycolate, mycolic acids, arabinogalactan, disaccharide, and tetrapeptide, from the cell wall of *M. lepraemurium* Hawaii.

Arabinose-mycolate and mycolic acids. In previous studies (5, 6), we purified the glycolipid, D-arabinose-5-mycolate, from the cell wall of mycobacteria by treatment with 0.1 N HCl followed by column chromatography and concluded that mycolic acid is combined by the ester linkage with the 5-hydroxy group of the D-arabinose residue of arabinogalactan. Four hundred milligrams of cell walls of *M. lepraemurium* Hawaii were treated with 20 ml of 0.1 N HCl at 60 C for 12 hr and recovered by centrifugation. They were then washed with water and methanol, and the resulting product, which was designated "acid-treated cell walls," was extracted repeatedly with chloroform and ether. The "bound lipid" thus obtained was further purified by column chromatography on Florisil-Celite (2:1) as described previously (5). The glycolipid (67 mg), mp 39 to 41 C, which was composed of arabinose and mycolic acid, was characterized as arabinose-5-mycolate by infrared spectrum, reductive cleavage with $LiAlH_4$, methylation studies, and chemical analyses by the same method described previously (5).

Mycolic acids. Seventeen milligrams of the mycolic acid moiety was obtained from 20 mg of arabinose-mycolate by saponification with 2.5% NaOH in methanol-benzene (1:1) and transformed into the methyl ester by using 2% hydrochloric acid in methanol. It was further purified by preparative thin-layer chromatography on silica gel H (Merck) with petroleum ether-diethyl ether (8:2 v/v) as developing solvent. Two major fractions of mycolic acid methyl esters of *M. lepraemurium* Hawaii, designated "mycolic acid-2 and -3 methyl esters," were obtained in the ratio of 1:2.5, respectively. They were investigated by mass spectrometry using JEOL mass spectrometers, types JMS-06SG and JMS-01SC.

The mass spectrum of mycolic acid-2 methyl ester gave peaks at m/e 1,102, 1,130, 1,146, 1,157, 1,160, 1,172, 1,188, 1,202, and 1,216 at high mass region. On the other hand, the spectrum of mycolic acid-3 methyl ester gave peaks at m/e 896, 910, 922, 936, 938, 950, 964, 978, 992, 1,006, and 1,020 as high mass region. In the mass spectra of both mycolic acid-2 and -3 methyl esters, the peak of greatest intensity was observed at m/e 382 and another of medium intensity at 410. From the above data, it was assumed that mycolic acid-2 and -3 methyl esters derived from the cell wall of *M. lepraemurium* Hawaii are a mixture of high-molecular-weight fatty acids having the main aliphatic branch of $C_{22}H_{45}$ (and another of $C_{24}H_{49}$) at α carbon and a hydroxy group at β carbon. The molecular weight of mycolic acid-2 methyl ester ($C_{77}H_{140}O_3$-$C_{85}H_{164}O_3$) is higher than that of mycolic acid-3 methyl ester ($C_{62}H_{120}O_3$-$C_{71}H_{136}O_3$). Similar findings have been reported for several kinds of mycobacteria, especially in mycolic acids of *M. smegmatis* by A. H. Etemadi (thesis, Univ. of Paris, 1965). Further detailed investigation of the chemical structure of both of the mycolic acids of *M. lepraemurium* Hawaii is being undertaken.

Arabinogalactan. As the polysaccharide moiety of the cell wall of *M. lepraemurium* Hawaii, arabinogalactan (32 mg) $[\alpha]_D = +25.4$ (in water), which is composed of arabinose and galactose in a molar ratio of 2.55:1, was removed from 180 mg of the cell walls by extraction with 0.5 N NaOH solution at 60 C and precipitation with ethanol followed by purification with column chromatography on diethylaminoethyl-cellulose as described in earlier papers (6, 16, 21, 22). Methylation studies were performed by a previously described method (16, 22), and results suggest that this arabinogalactan consists of 1→5-linked arabinofuranosyl, 1→4-linked galactopyranosyl (or 1→5-linked galactofuranosyl) and a small amount of 1→2-linked arabinofuranosyl residues. Arabinofuranosyl residue was detected as a nonreducing terminal. These findings are almost identical with those of similar studies on the arabinogalactans from mycobacterial cell walls (16, 21, 22).

Based on results of quantitative precipitation tests, arabinogalactan isolated from *M. lepraemurium* Hawaii displayed potent antigenicity with anti-*M. lepraemurium* Hawaii as well as with anti-*Mycobacterium bovis* BCG rabbit sera. Arabinogalactan from *M. lepraemurium* Hawaii reacts with anti-*Nocardia rubra* and anti-*Corynebacterium diphtheriae* strain PW 8 rabbit sera, but less antigenicity was displayed with these antisera than with the homologous and heterologous mycobacterial antisera.

From the protoplasmic fraction, arabinomannan, which consists of arabinose and mannose in a molar ratio of 1:0.94, was obtained. The results of chemical and immunological studies on these polysaccharides suggest that the main serological polysaccharides of *M. lepraemurium* Hawaii, as well as of other mycobacteria (21,

22), are arabinogalactan and arabinomannan.

Mucopeptide (tetrapeptide and disaccharide). The "acid-treated cell wall" (150 mg) was digested with *Myxobacterium* AL 1 enzyme (10) and then gel-filtrated on Sephadex G-25 as described previously (4). Three major fractions, designated 1, 2, and 3, were obtained as shown in Fig. 1. Results of amino acid analysis indicated that fraction 3 was composed of alanine, glutamic acid, and Dpm in a molar ratio of 2.1:1.1:1.0. The results of mass spectrometric, thin-layer chromatographic, and high voltage electrophoretic analyses (20) of this fraction were identical with those of tetrapeptide, Ala-Gln-Dpm-Ala obtained from *M. bovis* BCG (20). Fraction 1 was further treated with lysozyme and separated into three fractions (A, B, and C) by gel filtration on Sephadex G-15 according to a previously described procedure (1, 4). Chemical analysis showed that fraction C was composed of equal moles of glucosamine and muramic acid. Paper chromatographic analysis with *N*-butanol-acetic acid-water (5:1:2) by the descending method (1, 4) in-

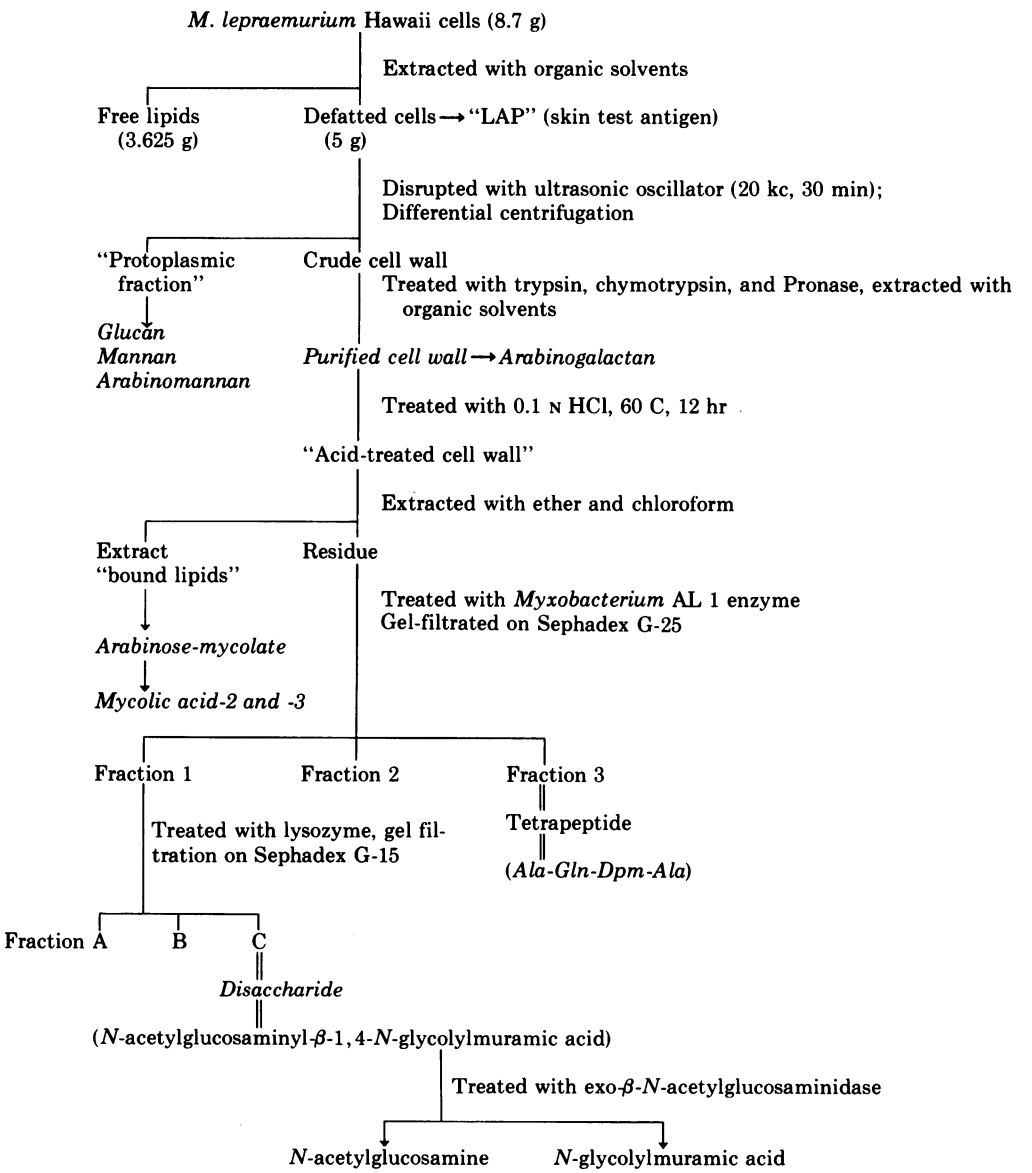

FIG. 1. *Fractionation of Mycobacterium lepraemurium Hawaii cells.*

dicated that it had the same value (R_{GlcNAc} = 0.70) as the disaccharide, *N*-acetylglucosaminyl-β-1,4-*N*-glycolylmuramic acid, which has also been isolated from lysates of the mucopeptide of the cell wall of mycobacteria (1, 4) and nocardia (13). This finding was supported by the result of mass spectrometric analysis of this disaccharide after esterification with diazomethane and peracetylation with acetic anhydride in pyridine. Upon treatment of the disaccharide with exo-β-*N*-acetylglucosaminidase (11), kindly supplied by S. Kotani, the presence of *N*-acetylglucosamine (R_{GlcNAc} = 1.0) and *N*-glycolylmuramic acid (R_{GlcNAc} = 1.35) was detected with the aid of paper chromatography with *N*-butanol-acetic acid-water (5:1:2, v/v) by the descending method (1, 4).

Results of these studies suggest that the structure of the cell wall of *M. lepraemurium* Hawaii is a "mycolic acid-arabinogalactan-mucopeptide" complex and that the detailed chemical structure of its subunits is almost the same as that of mycobacteria.

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