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On the linkage between mycolic acid and arabinogalactan in phenol-treated mycobacterial cell walls

It has been reported[1] that phenol isolates a complex of mycolic acid, arabinogalactan and mucopeptide (murein or peptidoglycan) in mycobacterial cell walls. Likewise, the ester linkage between mycolic acid and arabinogalactan has been demonstrated[1]. That the linkage between mycolic acid and arabinose residue might be the main linkage between mycolic acid and arabinogalactan is proposed herein.

Isolation and chemical analyses of the mycolic acid–arabinogalactan–mucopeptide complex in both *Mycobacterium tuberculosis* BCG and *Mycobacterium smegmatis* ATCC 14468 were performed as previously described[1].

Isolation and column chromatography of the mycolic acid–sugar complex were carried out as follows: 5 g of the whole complex were suspended in 100 ml of 0.1 M HCl, and then incubated at 37°. In order to suspend the complex homogeneously, sonic treatment was occasionally applied. Lipids from the reaction mixtures were extracted with ether daily for 1 month. Approx. 90% of the mycolic acid was recovered in the ether extracts. The extracted lipids were purified as described before[1]. Yield: *M. tuberculosis* BCG, 2.1 g; *M. smegmatis*, 1.7 g. Silicic acid column chromatography of the lipids was performed according to the method of AZUMA AND YAMAMURA[2]. The mixture of silicic acid and celite (Sigma Chemical Co., St. Louis, Mo.) (2:1, by wt.) was dried for 24 h at 110° before use. The lipids (1.4 g of *M. tuberculosis* BCG or 1.2 g of *M. smegmatis*) were dissolved in 50 ml of chloroform, placed on a column of 25 g of silicic acid–celite in chloroform and eluted with 300 ml (or 400 ml) of chloroform, chloroform–methanol (95:5, by vol.), chloroform–methanol (90:10, by vol.) and chloroform–methanol (80:20, by vol.), successively. Fraction b (650 mg), eluted with chloroform–methanol (95:5, by vol.), was rechromatographed on a 20-g column of the same adsorbent, using as eluent chloroform–methanol (98:2, by vol.) and chloroform–methanol (95:5, by vol.), successively.

Mild acid hydrolysis of the arabinogalactan–mucopeptide complex was performed as follows: 30 mg of the complex, which was isolated by saponification from the mycolic acid–arabinogalactan–mucopeptide complex[1], were incubated in 2 ml of 0.005 M H_2SO_4 at 85° for 1.5 and 3 h. The reaction mixtures were neutralized with Dowex 1 (OH^- form) and filtered. Sugars liberated in the filtrates were concentrated by lyophilization and then examined by means of paper chromatography[1].

To calculate the molar ratios of the mycolic acid–sugar complexes, 1260 and 1195 were used as molecular weights for the mycolic acids of *M. tuberculosis* BCG and *M. smegmatis*, respectively[1].

The patterns of the first silicic acid column chromatography of the mycolic acid–sugar complexes were similar in both mycobacteria (Table I). In Fraction b approx. 70% of the amount applied was eluted with chloroform–methanol (95:5, by vol.) and then rechromatographed (Table II). In both mycobacteria, Fraction I was mainly composed of mycolic acid and arabinose in an approximate molar ratio of 1:1 and gave a positive reaction with aniline hydrogen phthalate. After acid hydrolysis, arabinose was observed on paper chromatograms, and after saponification, more than 90% of the lipid moiety was recovered in the mycolic acid fraction (*M. tuberculosis* BCG, m.p. 53–55°; *M. smegmatis*, m.p. 63–65°). The elemental analyses and the

TABLE I

FIRST SILICIC ACID–CELITE COLUMN CHROMATOGRAPHY OF MYCOLIC ACID–SUGAR COMPLEXES OF *M. tuberculosis* BCG AND *M. smegmatis*

Figures in parentheses are the molar ratio of sugar to mycolic acid. The percentage of mycolic acid was calculated subtracting the percentage of sugars from 100 %.

Fraction	*Eluted with 100 ml of*	*M. tuberculosis BCG*		
		Yield (mg)	*Arabinose (%)*	*Galactose (%)*
a	Chloroform	132	1.1 (0.09)	1.4 (0.10)
	Chloroform	36		
	Chloroform	8		
b	Chloroform–methanol (95:5)	796	15.5 (1.66)	6.4 (0.57)
	Chloroform–methanol (95:5)	147		
	Chloroform–methanol (95:5)	35		
	Chloroform–methanol (95:5)	8		
c	Chloroform–methanol (90:10)	60	21.4 (2.84)	15.3 (1.70)
	Chloroform–methanol (90:10)	20		
	Chloroform–methanol (90:10)	10		
d	Chloroform–methanol (80:20)	68	24.5 (3.71)	20.0 (2.52)
	Chloroform–methanol (80:20)	12		
	Chloroform–methanol (80:20)	2		
Recovery		1334	95.4 %	
		M. smegmatis		
		Yield (mg)	*Arabinose (%)*	*Galactose (%)*
a	Chloroform	108	2.2 (0.18)	1.6 (0.11)
	Chloroform	28		
	Chloroform	13		
b	Chloroform–methanol (95:5)	743	12.0 (1.18)	7.2 (0.59)
	Chloroform–methanol (95:5)	45		
	Chloroform–methanol (95:5)	22		
	Chloroform–methanol (95:5)	8		
c	Chloroform–methanol (90:10)	101	20.0 (2.38)	13.3 (1.32)
	Chloroform–methanol (90:10)	30		
	Chloroform–methanol (90:10)	10		
d	Chloroform–methanol (80:20)	57	25.8 (3.50)	15.5 (1.75)
	Chloroform–methanol (80:20)	10		
	Chloroform–methanol (80:20)	2		
Recovery		1177	98.0 %	

melting point of Fraction I were as follows: *M. tuberculosis* BCG, C 76.55 %, H 12.38 %, m.p. 43–45°; *M. smegmatis*, C 76.08 %, H 12.81 %, m.p. 42–45°. Infrared spectra of these fractions were almost identical to those of the arabinose mycolate[2] and to the mycolic acid–sugar complex reported previously[1]: absorption maxima, 3450, 2920, 2850, 1735, 1460, 1400, 890 and 720 cm^{-1} and broad absorption between 1200 and 920 cm^{-1}. The amount of mycolic acid in Fraction I accounted for 44.4 % (*M. tuberculosis* BCG) and 38.0 % (*M. smegmatis*) of the total mycolic acid of the isolated mycolic acid–sugar complexes.

TABLE II

SECOND SILICIC ACID–CELITE COLUMN CHROMATOGRAPHY OF FRACTION b OF *M. tuberculosis* BCG AND *M. smegmatis*

Figures in parentheses are the molar ratio of sugar to mycolic acid.

Fraction	*Eluted with 100 ml of*	*M. tuberculosis BCG*		
		Yield (mg)	*Arabinose (%)*	*Galactose (%)*
I	Chloroform–methanol (98:2)	284	11.0 (1.05)	1.2 (0.10)
	Chloroform–methanol (98:2)	94		
	Chloroform–methanol (98:2)	17		
II	Chloroform–methanol (95:5)	134	16.5 (1.88)	9.7 (0.92)
	Chloroform–methanol (95:5)	20		
	Chloroform–methanol (95:5)	9		
Recovery		558	85.8%	
		M. smegmatis		
		Yield (mg)	*Arabinose (%)*	*Galactose (%)*
I	Chloroform–methanol (98:2)	268	11.6 (1.06)	1.3 (0.10)
	Chloroform–methanol (98:2)	88		
	Chloroform–methanol (98:2)	19		
II	Chloroform–methanol (95:5)	181	15.4 (1.64)	9.8 (0.87)
	Chloroform–methanol (95:5)	25		
	Chloroform–methanol (95:5)	8		
Recovery		589	90.6%	

To examine esterified hydroxyl groups of arabinose, Fraction I (100 mg) of both mycobacteria was subjected to methylation following the method of AZUMA AND YAMAMURA[2]. However, methylated arabinose could not be identified on paper chromatograms. The reducing group of arabinose moiety may become oxidized during the methylation procedure in which methyl iodide and silver oxide were used.

Only arabinose was liberated after 1.5 h of mild acid hydrolysis of the arabinogalactan–mucopeptide complexes. Arabinose, galactose and various oligosaccharides were liberated after 3 h. These results indicate the existence of arabofuranoside at a nonreducing arabinogalactan terminal[3].

It can be concluded that at least 40% of the mycolic acid in the mycolic acid–arabinogalactan–mucopeptide complex is linked to the arabinose residue. Fractions c, d and II, have a significant amount of galactose in addition to arabinose. Although this suggests that there might be a linkage between mycolic acid and galactose, it seems reasonable to assume that galactose links to the arabinose moiety of the arabinose mycolate, since there is no fraction which has only galactose as sugar moiety. A review of the literature shows reports on the isolation of arabinose mycolate from bound lipids[2,4] and wax D[5,6] of various strains of mycobacteria, but none on the presence of galactose mycolate. In addition, VILKAS, DELAUMENY AND NACASCH[6], and I. AZUMA (personal communication) suggested that almost all mycolic acid may link to the hydroxyl group of C-5 of the arabinose residue at a nonreducing terminal of

mycobacterial polysaccharides. Although this report does not describe in detail the structure of arabinogalactan because of the difficulty in preparing pure arabinogalactan from the phenol-treated cell walls, its structure may be similar to that of highly branched arabinogalactan, having arabinose at a nonreducing terminal, isolated from cell wall[3], wax D[6] and whole cell[7] by other investigators. The existence of arabinose at a nonreducing terminal of polysaccharides also supports the possible linkage between arabinose and mycolic acid.

It is concluded that mycolic acid links to arabofuranoside of arabinogalactan in the mycolic acid–arabinogalactan–mucopeptide complex of mycobacterial cell wall.

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Secretory response to the stimulation of amphibian skin glands

There is a diverse series of reports in the literature on the time course of secretion from the amphibian skin glands in response to stimulation. SHARE AND USSING[1] referred to unpublished observations which demonstrated that the secretion from the skin glands of *Rana temporaria* had a transient character with a duration of about 20 min. SELDIN AND HOSHIKO[2] reported that, when adrenaline was applied to the inner (corium) surface of isolated skin of *Rana pipiens*, small volumes of fluid were extruded from the glandular pores within 30 sec and this secretory process was apparently completed within 2 min; LINDLEY[3] found a similar time course of secretion during cutaneous nerve stimulation.

There have been no published accounts of attempts to record simultaneously the time course of glandular secretion and the electrical response[4–6] of the isolated amphibian skin under these conditions. I have obtained volumetric records of the secretion from the glands of the isolated skin of the toad, *Xenopus laevis*, and the contemporaneous changes in the potential difference and resistance of the skin.