Cell Wall Composition in *Mycobacterium lepraemurium*

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SUMMARY

Cell-wall fractions from *Mycobacterium lepraemurium* were examined chemically and immunologically. The major sugar and amino acid components detected were arabinose and galactose, alanine, glutamic acid, α,ϵ -diaminopimelic acid (DAP) and hexosamine, but substantial amounts of aspartic acid, glycine, valine, serine, threonine, leucine and *iso*-leucine were also present in preparations not extracted with neutral lipid solvents and alkaline ethanol. However, after such extraction and treatment with proteolytic enzymes, alanine, glutamic acid and DAP (1.5:1.0:1.0) were present in much larger amounts than any other amino acids (not more than 0.15), indicating that these three are the mucopeptide constituents. Cell-wall agglutination tests indicated the presence of an antigen shared with other mycobacteria.

INTRODUCTION

Examination of cell wall fractions prepared from a number of strains of mycobacteria have shown a common pattern of components which these organisms share with strains of nocardia and some corynebacteria (Cummins & Harris, 1958; Cummins 1962). This common pattern is characterized by the presence of the sugars arabinose and galactose, and of alanine, glutamic acid and DL-diaminopimelic acid as mucopeptide amino acids. In addition, it has been noted (Cummins, 1962) that organisms whose cell walls show this pattern have a cell-wall antigen in common.

Mycobacterium lepraemurium is the causative organism of a chronic infection in wild rats known as rat leprosy. The organism has not been grown in ordinary bacteriological medium; however, it has been grown in tissue culture (Rees & Garbutt, 1962). *M. lepraemurium* has a generation time of about 10 days in tissue culture and *in vivo*. Therefore it seemed of interest to examine cell-wall fractions from this very slowly growing and obligate intracellular species of mycobacterium to see whether its cell wall was of the same general type as that of all other mycobacterial species examined.

METHODS

Preparation of Mycobacterium lepraemurium suspensions. The Douglas strain of *M. lepraemurium* (Balfour-Jones, 1937) was used; suspensions of the organism were prepared by homogenizing heavily infected liver from mice injected intravenously 4-6 months previously with *M. lepraemurium* (Rees, Valentine & Wong, 1960). The liver homogenates were prepared in 0.01 M-phosphate buffer (pH 7.1) and partially

purified suspensions of organisms obtained by repeated centrifugation and washing in buffer.

Electron microscopy. Samples were suitably diluted with distilled water and thin layers allowed to dry on electron microscope specimen supports covered with carbon films. The preparations were shadowed with platinum before examination in the electron microscope.

Preparation of cell walls. This was done either by the method of Cummins & Harris (1956) (omitting ribonuclease treatment) or by disintegration in a Mickle shaker followed by centrifugation in a sucrose gradient in which the walls formed a layer above 40 % (w/v) sucrose.

Extraction with neutral solvents. This was done at 37°. The material was first extracted twice with ethanol+ether (1+1, v/v), and then twice with chloroform+methanol (1+1, v/v), the total extraction period being several weeks. During this time the extraction mixtures were shaken occasionally by hand.

Extraction with alkaline ethanol. Absolute ethanol containing 0.5 % KOH was used, and the extraction was continued for 2–3 days at 37°, with occasional shaking. The mixture was centrifuged, and the extracted, deposited material washed twice with absolute alcohol and then three times with distilled water.

Chromatography. Material was hydrolysed in 6N-HCl for 18 hr at 105°, the liquid filtered and the filtrate evaporated to dryness at 100° over a water bath. The soluble material was taken up in a small quantity of distilled water. To identify the amino acids two-dimensional ascending chromatograms were run in 10 in. squares of Whatman no. 1 paper. The first solvent was butanol+acetic acid+water (120+30+50, by vol.) and the second phenol+ammonia+water (phenol 80+water 20, v/v; +1 ml. sp.gr. 0.880 ammonia per 200 ml.). The spots were made visible with 0.2 % ninhydrin in 95 % (v/v) acetone in water.

For the separation of diaminopimelic acid (DAP) isomers descending chromatograms were run using the methanol+pyridine+HCl+water solvent (Hoare & Work, 1957).

For sugars the method was that of Cummins & Harris (1956), except that one-dimensional descending chromatograms were used, the solvent being ethyl acetate+pyridine+water (9+2+2 by vol.).

Quantitative estimation of amino acids. This was done by the method of Atfield & Morris (1961) with high-voltage paper electrophoresis. The results were expressed as molar ratios, taking DAP = 1.0.

Cell-wall agglutination tests. The method was that of Cummins & Slade (1961) except that an MSE sonic disintegrator was used to redisperse the cell wall fragments.

RESULTS

Chemical analyses were done on samples of *Mycobacterium lepraemurium* cell walls prepared in four different ways (see Table 1). Quantitative amino acid analyses were made on all four samples, but qualitative examination for amino acids and sugars were also done on sample B, as was the determination of the type of DAP present.

Electron micrographs of the fractions analysed showed them to be very largely composed of cell walls, as can be seen in Pl. 1, fig. 1, which shows a preparation made from sample C. A preparation from sample D was also examined (Pl. 1, fig. 2). The

cell walls of sample C were generally almost intact, while in sample D they were mostly fragmented and showed a greater tendency to aggregate. However, considering that sample D had been prepared from sample C by procedures which resulted in an 85 % loss of weight (see Table 1) there is surprisingly little difference to be noted in the appearance of the cell-wall fragments in the two samples.

Table 1. *Methods of preparation of Mycobacterium lepraemurium cell-wall fractions*

Sample	Method of preparation
A	Formalin-killed suspension disintegrated, deposit treated with trypsin and pepsin, extracted with 0.5% KOH in ethanol for 24 hr at 37°; finally washed with ethanol and distilled water
B	Organisms allowed to autolyse for 1 month at 37°, disintegrated, and cell-wall fraction separated by centrifugation in sucrose gradient
C	Fresh suspension disintegrated, cell walls separated by centrifugation in sucrose gradient: no other treatment except for washing
D	Prepared from part of sample C by thorough extraction at 37° with neutral lipid solvents (ethanol+ether 4 weeks, chloroform+methanol 6 weeks), followed by 0.5% KOH in ethanol (3 days at 37°). Finally the residue was washed with absolute ethanol and then distilled water, and treated with trypsin and pepsin. Final yield = 15%: i.e. 13.4 mg. of sample C yielded 2.0 mg. of sample D.

Table 2. *Qualitative composition of Mycobacterium lepraemurium cell walls; sample B*

Amino acids		Sugars and amino sugars	
Ala Glu DAP (DL and/or DD-)	} + + +	{ Arabinose + + + Galactose + + Mannose trace	
Asp Gly Ser Threonine Valine Leucines			} +
Lysine Arginine			
		Hexosamines +	

Qualitative analyses

The results of chromatography of acid hydrolysates of *Mycobacterium lepraemurium* cell walls is shown in Table 2. The principal amino acids found were alanine, glutamic acid and DAP, the latter the DL-isomer (or DL+DD), and the principal sugars identified were arabinose and galactose, with a trace of mannose. This pattern of sugar and amino acid components is that to be expected in a mycobacterium (Cummins & Harris, 1958; Cummins, 1962). The identity of the hexosamines is not known for certain, since the chromatographic method used did not separate glucosamine and muramic acid, but it may be assumed that both are present.

Quantitative amino acid analyses

The high-voltage electrophoretic method used enabled analyses to be done on samples of 1 mg. or less. The results obtained with samples A-D are recorded in

Table 3; these results are the mean of two estimations in each case. It is obvious from an examination of these results that the method of preparation of cell-wall fractions has a very considerable influence on the total amino acid content and also on the molar ratios of the different amino acids present, especially the latter. The most interesting comparison is between samples C and D, since the latter was prepared from the former by a rather extensive extraction with lipid solvents followed by treatment with proteolytic enzymes. This resulted in a very considerable loss of weight: for example, 13.4 mg. of sample C after treatment gave only 2.0 mg. of sample D as the final product, a yield of about 15 %. It might be expected that such treatment would remove all amino acid-containing material except mucopeptide. The results suggest that this had in fact happened to a large extent, since over 80 % of the total amino acid content of sample D was made up of alanine, glutamic acid and diaminopimelic acid. In view of this it seems reasonable to suppose that these are the mucopeptide amino acids in *Mycobacterium lepraemurium*. In the starting material C, however, which had no treatment except separation in a sucrose gradient followed by washing in distilled water, the picture was very different; appreciable amounts of aspartic acid, glycine, leucines, valine and serine, were present, with rather smaller amounts of 7 other amino acids. The pattern is similar in samples A and B, although the amounts of non-mucopeptide amino acids are lower.

Table 3. Mean molar ratios of amino acids in cell-wall fractions prepared from *Mycobacterium lepraemurium* in different ways

Constituent	A	B	C	D*
Diaminopimelic acid	1.0	1.0	1.0	1.0
Glutamic acid	1.5	2.0	3.0	1.0
Alanine	2.0	3.0	3.5	1.5
Aspartic acid	0.6	0.7	2.0	0.1
Glycine	0.8	0.7	2.0	0.15
Leucine isomers	Leuc + isoleuc 0.65	Leuc + isoleuc 1.0	Leuc 1.3 Isoleuc 0.7 Allo isoleuc 0.7	Leuc + isoleuc 0.15
Valine	0.3	0.4	1.5	0.1
Serine	0.25	0.3	1.4	0.1
Threonine	0.2	0.2	0.95	0.05
Lysine	0.15	0.15	0.5	Trace
Histidine	Not detected	Trace	0.3	Not detected
Phenylalanine	Not detected	0.1	1.0	0.1
Tyrosine	Trace	Trace	0.2	Not detected
Arginine	0.2	0.25	0.8	Trace
Methionine sulphone	Not detected	Not detected	0.2†	Trace
Approx. % amino acids	21	14	25	15

* DAP = 1.0.

† Confirmed as methionine sulphone by nitroprusside test.

Serological tests with Mycobacterium lepraemurium cell-wall preparations

The results of cell-wall agglutination tests are given in Table 4, and show clearly that *Mycobacterium lepraemurium* contains a cell-wall antigen shared with other mycobacteria, since suspensions of cell walls prepared from it agglutinated with anti-smegmatis serum in the same way as cell-wall fractions from the BCG and avian strains of *M. tuberculosis*. In fact, *M. lepraemurium* cell-wall suspensions agglutinated

to the same titre as those of the avian strain. Antiserum 583 G, which had been prepared against *M. lepraemurium* itself, seemed to contain only a little antibody against the common mycobacterial antigen.

Agglutination tests were not possible with many of the samples of cell walls prepared from *Mycobacterium lepraemurium* suspensions because of auto-agglutination in saline; broadly speaking it was only when the bacteria were extracted with neutral solvents and then with alkaline ethanol that the cell-wall fractions made from them were sufficiently stable in saline to be used in agglutination tests, i.e. samples A and D. Table 1. This parallels similar findings for other mycobacteria (see Cummins & Harris, 1958; Cummins, 1962). However, during the present work it was noticed that cell-wall suspensions prepared in a sucrose gradient after disintegration of freshly made suspensions of *M. lepraemurium* were stable in saline and gave a titre against *M. smegmatis* antiserum similar to that recorded in Table 1 for suspension of extracted cell walls.

Table 4. *Agglutination tests with cell-wall suspensions from Mycobacterium lepraemurium and some other mycobacteria*

Serum	Cell-wall suspension† from	20	40	80	160	320	640	SAL
Antiserum SMEG R11/62	<i>M. smegmatis</i> *	++	++	++	++	+	±	—
	<i>M. tuberculosis</i> BCG	++	++	++	++	+	±	—
	<i>M. tuberculosis</i> , avian type	++	++	++	+	—	—	—
	<i>M. lepraemurium</i> (sample D)	++	++	++	+	Tr	—	—
Antiserum <i>M. lepraemurium</i> 583 G	<i>M. tuberculosis</i> BCG	+	±	—	—	—	.	—
	<i>M. lepraemurium</i> (sample D)	±	Tr	—	—	—	.	—
Normal rabbit serum	<i>M. lepraemurium</i> (sample D)	—	—	.	.	.	.	—

* Homologous suspension.

† The cell-wall suspensions used in these tests were prepared from organisms extracted by neutral solvents, followed by 0.5% KOH in ethanol.

. = not done.

Because of the limited amount of material available it was not possible to do full absorption tests. However, in a single test, 1 ml. of a 1/80 dilution of *Mycobacterium smegmatis* serum was absorbed with 2 mg. of *M. lepraemurium* cell walls. On testing the absorbed samples against *M. smegmatis* cell-wall suspension it appeared that all antibodies had been removed.

DISCUSSION

These results provide clear evidence that the cell walls of *Mycobacterium lepraemurium* closely resemble those of other mycobacteria, both chemically and serologically. The presence of arabinose and galactose as principal cell-wall sugars, and of alanine, glutamic acid and DAP as mucopeptide amino acids, coupled with evidence that the walls contain a high proportion of lipid (loss of weight on extraction with neutral lipid solvents) are all typical of this group of organisms. Unfortunately it was not possible to analyse the lipids extracted, as it would have been of interest to know whether or not they contained mycolic acid, and also whether the chloroform-soluble

fractions had any adjuvant activity of the type described by White, Coons & Connolly (1955) in Wax D from other mycobacteria.

As mentioned above, the considerable differences in amino acid patterns between samples C and D make it clear that alanine, glutamic acid and DL-diaminopimelic acid are the mucopeptide amino acids in this organism. However the rather large number of amino acids in sample C indicate the presence of other peptide material in the unextracted wall.

If it is assumed that in samples A, B and C a certain amount of alanine and glutamic acid are also 'non-mucopeptide', it appears possible that there is a peptide in these cell-wall samples, not associated with mucopeptide, which is largely composed of glutamic acid, alanine, aspartic acid, glycine, leucines, valine and serine. It seems that thorough treatment with lipid solvents, followed by proteolytic enzymes, is necessary to remove this peptide, which is perhaps associated with lipid; but these treatments were not fully investigated in all combinations. It is interesting to compare these results with those obtained by Belknap, Camien & Dunn (1961) for other mycobacteria. These workers examined 4 strains, 2 of *Mycobacterium tuberculosis*, one of *M. ranae*, and an unclassified 'Battey' strain, and found the major components in each case were alanine, glutamic acid and diaminopimelic acid. They also found appreciable amounts of aspartic acid, glycine, serine, leucines, threonine and valine, although not all of these were present in every strain. The cell-wall material prepared by Belknap *et al.* was made from organisms which had been extracted rather briefly with acetone and ether and then dried; after disintegration, the cell-wall fractions were treated with trypsin and pepsin.

Aspartic acid and glycine have also been found as the principal 'non-mucopeptide' amino acids in the peptido-glycolipids of Wax D in avian, bovine and saprophytic strains of mycobacteria (Jollès, Samour & Lederer, 1963). Misaki, Yukawa, Tsuchiya & Yamasaki (1966), who have made detailed studies of the composition of the cell wall of *Mycobacterium tuberculosis* BCG, considered that glycine was a mucopeptide constituent in the strain they examined because it was the only amino acid (other than alanine, glutamic acid and diaminopimelic acid) which was not removed by treatment with 'pronase'. In their strain, glycine was present in pronase-treated walls to the extent of 0.33 (diaminopimelic acid = 1.0). Similar studies on cell walls of *M. phlei* by Takeya, Hisatsume & Inoue (1963) showed 'basal layer' (= mucopeptide) amino acids to be alanine, glutamic acid and diaminopimelic acid in ratio 1.9:1.7:1.0, with no other amino acid present in more than 20 % of the amount of diaminopimelic acid. Both these groups of workers found arabinose and galactose to be the principal sugars present in the strains they examined.

The total amino acid content of the different fractions, also shown in Table 2, probably varies with a number of factors, the principal ones being the amounts of lipid and non-mucopeptide protein present. These latter factors might be expected to act in opposite directions, since a large amount of protein would raise the amino acid content and a large amount of lipid would make it relatively smaller; but in the absence of more definite information about the exact amounts of protein and lipid present the figures for total amino acid content are not very informative. However, the decrease from 25 % amino acids in sample C to 15 % in sample D presumably represents a considerable removal of non-mucopeptide protein.

In previous work (Cummins, 1962) all strains of mycobacteria, corynebacteria and

nocardias examined which had arabinose and galactose as characteristic cell-wall sugars showed cross agglutination when suspensions of their cell walls were tested against antisera to *Mycobacterium smegmatis*. From the results obtained with *M. lepraemurium* cell walls (Table 4) there seems to be no doubt that this antigen is present in them also.

It was somewhat unfortunate that the only *Mycobacterium lepraemurium* antiserum available appeared to contain very little antibody to the cell-wall antigens, but the results of cell-wall agglutination tests with *M. smegmatis* antiserum seemed quite conclusive of the presence in *M. lepraemurium* cell walls of the common mycobacterial antigen, since in the tests the titre at which *M. lepraemurium* cell walls reacted was at least as high as that for *M. avium* (Table 4). Moreover, the absorption test, using *M. lepraemurium* cell wall to absorb *M. smegmatis* antiserum, provides confirmatory evidence of the presence of the common antigen in the cell walls of *M. lepraemurium*.

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EXPLANATION OF PLATE

Electron micrographs of *Mycobacterium lepraemurium* cell-wall fractions. (Pt shadowed.)
× 7,300.

Fig. 1. Sample C.

Fig. 2. Sample D, prepared from sample C by thorough extraction with lipid solvents (see text for details).