# Characterisation of cell-wall-derived polypeptide antigens from different species of *Mycobacterium*

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**Summary.** Cell walls from different species of *Mycobacterium* were purified on a sucrose step gradient. The components derived from these preparations were characterised by sodium dodecyl sulphate—polyacrylamide gel electrophoresis, followed by staining or by Western blotting. Surface-exposed polypeptide molecules were also identified by biotinylation. Many protein and glycoprotein molecules were identified in the cell walls. Some of these molecules were immunogenic in man and experimental animals and showed wide variability from species to species. The data suggest that these molecules could be of significance in the diagnosis and pathophysiology of mycobacterial diseases.

# Introduction

In addition to *Mycobacterium tuberculosis*, other species of *Mycobacterium* cause tuberculosis-like disease in man. This has led to intensive studies of methods to identify and define mycobacterial species in order to develop more effective tools for the diagnosis and prevention of tuberculosis and other mycobacterial diseases.

At present, identification of non-tuberculous mycobacteria is based on colony morphology, pigment production, growth rate, temperature preference, biochemical tests and serotyping.<sup>1</sup> These tests may give ambiguous results because of phenotypic variations among different strains of the same species.<sup>2, 3</sup> Taxonomic studies in mycobacteria have also been performed with the help of serological techniques, including immunodiffusion and immuno-electrophoresis,<sup>4, 5</sup> but cross-reactive antigens present in different mycobacterial species make analysis difficult.

Among the species-specific antigens that have been identified are: the phenolic glycolipid of M. *leprae*,<sup>6</sup> antigen 60 of  $BCG^7$  and antigen 5 of M. *tuberculosis*.<sup>8</sup> There is still considerable need to identify and characterise more such antigens. Cellwall antigens, especially polypeptide antigens, have not been characterised in mycobacteria. Since the cell wall is involved in interaction with the host, these molecules probably have a role in pathogenesis. In this report, we describe the preliminary characterisation of antigens derived from cell walls of a few, selected species of *Mycobacterium*.

# Materials and methods

# Mycobacteria

Species used in this study were: *M. asiaticum* TMC 803, *M. smegmatis* TMC 1548, *M. gordonae* TMC 1324, *M. bovis* BCG US 1037, *M. fortuitum* ND-125 and *M. tuberculosis* H 37 Ra L-3-TMC-201.

# Sera

Sera from patients were obtained from the out-door department of the New Delhi Tuberculosis Centre, New Delhi, India. Rabbit anti-*M. tuberculosis* (H 37 Ra strain) serum was a kind gift from Mr U. Kumar and Dr Saxena, School of Life Sciences, Jawaharlal Nehru University, New Delhi.

# Preparation of mycobacterial antigens

Mycobacteria were grown in Middlebrook 7H9 medium and lysed as described before.<sup>9</sup> Briefly, the cells were harvested by centrifugation at 7000 g for 20 min at  $4^{\circ}$ C and were washed with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.14M NaCl, pH 7.2). The washed cells were resuspended to a concentration of

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250 mg (wet weight)/ml in Tris-buffered saline (110 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 2.0 mM phenylmethylsulphonylfluoride (Sigma). The cell suspension was frozen, and then thawed at  $37^{\circ}$ C. After 10 cycles of freezing and thawing, the broken cells were further disintegrated by ultrasonication for 10 min at 4°C. The cell lysate was stored frozen in separate small volumes at  $-70^{\circ}$ C.

#### Preparation of cell walls

Cell lysate was layered on to a discontinuous sucrose gradient (25%, 33%, 45% w/v; 2·0 ml each) and then centrifuged at 1230 g for 10 min at 4°C. The fraction containing the cell wall was collected from the interphase of the 33 and 45% sucrose layers. This fraction was examined microscopically and was found to be essentially free of unbroken whole cells. Plasma-membrane components were removed by washing with the non-ionic detergent, Triton X-114 (Sigma) 1·0% w/v at 4°C. Cellwall components were dissolved in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 100°C for 5 min.

# **Biotinylation of cells**

Biotinylation was performed as described by Heitzmann and Richards.<sup>10</sup> Briefly, mycobacterial cells were harvested by centrifugation and then washed twice with 0·1 M sodium bicarbonate; 20 mg (wet weight) of cells were suspended in 1·0 ml of 0·1 M sodium bicarbonate. To 500  $\mu$ l of suspension, 0·1 ml of biotinyl-N-hydroxysuccinimide ester (20 mg/ml in dimethylformamide) was added and incubated at room temperature with shaking. After incubation the cells were washed twice with 0·1 M sodium bicarbonate and then suspended in SDS-PAGE buffer.

# SDS-PAGE

Unless otherwise mentioned, antigens were separated by electrophoresis on discontinuous 12% polyacrylamide gels<sup>11</sup> and stained with Coomassie Blue R 250. Molecular weights were determined by comparison with standard mol. wt markers (Sigma).

#### Solubility test

The solubility of cell-wall-derived components was determined by incubation with SDS-PAGE sample buffer with and without 2-mercaptoethanol at different temperatures. After 10 min, the suspension was centrifuged and the supernate was used for electrophoresis.

#### Enzyme treatment

Extracted cell-wall pellets (500  $\mu$ g of protein) were resuspended in 200  $\mu$ l of 10 mM Tris-HCl, pH 8·0, containing proteinase K 125  $\mu$ g/ml. Digestion was allowed to proceed for 1 h at 37°C. The cell-wall pellet was collected by centrifugation and resuspended in SDS-PAGE sample buffer.

The cell wall was also digested with lysozyme (Worthington, USA) 2 mg/ml for 1 h at  $37^{\circ}$ C and processed as for digestion with proteinase K.

# Western blot analysis

After electrophoresis, the separated proteins were transferred electrophoretically to a nitrocellulose membrane in pH 8.3 buffer containing 25 mM Tris-HCl, 192 mM glycine, SDS 0.1% w/v, methanol 20% v/v.<sup>12</sup> Antigens were identified with the help of appropriate antibodies and protein A-peroxidase conjugate (Bio-Rad, USA).

#### Lectin blot

A concanavalin A (Con A) blot was performed as described by Kigimoto-Ochiai *et al.*<sup>13</sup> After transfer, the nitrocellulose membrane was incubated with Con A in 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, and Tween 20 0.05% w/v, containing 1.0 mM Ca<sup>++</sup> and 1.0 mM Mn<sup>++</sup> for 1 h. The amount of Con A bound was determined by incubation with horseradish peroxidase (Sigma).

#### Protein assay

Protein concentrations were determined with bicinchoninic acid reagent (Pierce, USA). Assays were done in 96-well microtitration plates; 50  $\mu$ l of the reagent was added and colour was estimated at 540 nm.

# Results

#### Preparation of mycobacterial cell wall

During cell lysis, some cells were observed to maintain their integrity. A sucrose step gradient was used to fractionate cell lysates and to allow separation of cell envelope from whole cells. The fraction that was insoluble in non-ionic detergent was considered to be derived from the cell wall. The SDS-PAGE pattern of a few species is presented in fig. 1A, which shows a number of bands that could be either polypeptides or glycopolypeptides.

# Characterisation of polypeptides derived from cell wall

To determine which of these polypeptides were glycoconjugates, lectin blots were allowed to react with Con A and the amount of lectin bound was identified by their ability to bind horseradish peroxidase (fig. 1B). Several bands of 150, 80, 67, 57 and 39.5 K da were observed. There was a strong





Fig. 1. Characterisation of cell-wall components by SDS-PAGE. Purified cell wall components were analysed on a discontinuous polyacrylamide 12% gel and stained with Coomassie Blue (A). After separation, the components were transferred electrophoretically to nitrocellulose membranes and probed with lectin Con A (B); the cell surface molecules were also labelled by biotinylation (C). Lanes 1, 4, 7, 8-*M. gordonae*; lanes 2, 5, 9, 10-*M. fortuitum*; lanes 3, 6, 11, 12-*M. asiaticum*; lanes 8, 10, 12-non-biotinylated controls.

straining material at very high mol. wt in some species.

Periodic acid schiff's (PAS) staining failed to detect any glycoconjugates which did not bind Con A.

To identify which of the cell-wall components were exposed at the cell surface, intact mycobacterial cells were biotinylated and the results were compared with the convention gel pattern purified on the sucrose gradient. Fig. 1C shows that several bands were common to the two preparations, but some were different, suggesting that there may not be exposed reactive groups on these molecules.

# Changes in the molecular profile during growth

Cell-wall profiles of mycobacteria harvested in early log phase, middle log phase and late log phase were identical.

#### Solubility of cell wall components

The nature of the interaction between cell-wall components and the insoluble matrix was investigated by checking solubility under different conditions (fig. 2). SDS and high temperature were required to solubilise cell wall components; at low temperature there was less soluble material. 2-Mercaptoethanol was not essential for solubilising cell wall components.

#### Enzyme treatment

The electrophoretic pattern observed after proteolytic digestion showed that most bands were digested by the enzyme except for two bands of 31 and 28 Kda.

There was no difference in electrophoretic pattern of undigested samples and those treated with lysozyme.

# Antigenicity of cell-wall-derived components

The immune human sera recognised two polypeptides of 28 and 31 Kda from BCG-derived cellwall components (fig. 3C, lane 9). The polyclonal rabbit antiserum recognised these two antigens as well as other antigens of 56 and 18 Kda (fig. 3A, lane 3). However, only one antigen (28 Kda) could be demonstrated in *M. asiaticum* preparations with immune human serum. Unlike the result with BCG, polyclonal antibody recognised a different antigen of 24 Kda in *M. asiaticum* (fig. 3C, lane 7).

# Comparison of different mycobacterial species

The feasibility of using the SDS-PAGE pattern of cell-wall polypeptides as markers of different species was investigated (fig. 4). Most bands were found to be different among different species. However, one common band of 47 Kda was observed in all mycobacteria tested.

# Discussion

Suitable methods are needed for the diagnosis of mycobacterial disease and for the development of newer immuno-prophylactic methods as existing methods are inadequate. Many approaches have been used to develop markers for the differentiation of mycobacterial species. However, approaches based on the detection of polypeptide antigens in



Fig. 2. Solubility of cell-wall fraction. Purified cell-wall fraction was incubated in SDS-PAGE sample buffer with (lanes 1, 2 and 4) and without (lane 3) 2-mercaptoethanol at:  $25^{\circ}$ C (lane 1);  $60^{\circ}$ C (lane 2);  $100^{\circ}$ C (lanes 3 and 4).



Fig. 3. Western blot analysis of cell-wall-derived components. The separated proteins were probed with (A) rabbit, anti M. tuberculosis—H 37 Ra; (B) pre-immune rabbit serum; (C) pooled immune serum from tuberculosis patients; (D) normal human serum. Lanes 1, 4, 7, 10—M. asiaticum; lanes 2, 5, 8, 11—M. fortuitum; lanes 3, 6, 9, 12—M. bovis BCG.

the soluble fraction of the cell lysates<sup>8, 14</sup> or identification of non-protein antigens in the cell wall components<sup>15</sup> have been unsuccessful.

No systematic search has been made for characteristic polypeptides, including glycoproteins derived from cell wall. These molecules may be significant in pathogenesis because of their ability to interact with the host cells. In this study, we have attempted to purify and partially characterise these molecules. Since whole cells and membrane components could interfere with our analysis, purification was devised in such a way as to eliminate these fractions. The fraction that was insoluble in nonionic detergent was found to be soluble in SDS-PAGE sample buffer only at high temperature, and 2-mercaptoethanol was not needed, suggesting that these molecules are not anchored to the cell-wall peptidoglycan layer through disulphide linkage. The molecules were soluble only in SDS; in the absence of SDS they tended to precipitate out. Moreover, in the absence of SDS, no blotting was observed during electrophoretic transfer from gel to nitrocellulose (data not shown).

Biotinylation of whole cells mainly modifies cellsurface molecules. Differences between the biotinylated and Coomassie Blue patterns suggest that not all cell-wall-derived polypeptides are exposed on the surface. It is possible that the number of biotinyl residues attached to a polypeptide is not directly proportional to the concentration in the cell wall because some molecules may be labelled to high specific activity despite low concentration in the cell wall.

The surface molecules of bacteria are mostly glycoproteins, but in the case of mycobacteria these molecules are either glycolipids or complex carbo-



**Fig. 4.** SDS-PAGE profile of cell-wall-derived components of different species of *Mycobacterium*. Lane 1 *M. gordonae*; lane 2 *M. fortuitum*; lane 3 *M. bovis* BCG; lane 4 *M. asiaticum*; lane 5 *M. tuberculosis*  $H_{37}$  Ra; lane 6 *M. smegmatis*.

hydrates.<sup>16, 17</sup> Most molecules observed by SDS-PAGE appeared to be either polypeptides or glycopolypeptides for the following reasons: (a) normally, complex sugars or glycolipids show diffuse and broad bands in SDS-PAGE and we observed sharp bands; (b) most were susceptible to proteolytic digestion; (c) many bands also reacted with Con A; and (d) these molecules could be labelled with the biotin reagent.

Not all glycoprotein bands bound Con A, indicating that they do not all contain mannose or glucose. Failure to stain the glycoproteins with PAS may indicate that the amount of components in individual bands may be insufficient. Dense staining of diffuse material in *M. asiaticum* may be due to complex carbohydrates or glycolipids as these molecules are neither susceptible to biotinylation nor stain with Coomassie Blue.

We observed two bands of 31 and 28 Kda which were resistant to proteinase K. These molecules may be buried in the cell wall or may be degraded molecules partially exposed to the enzyme. The lack of any effect of lysozyme on solubility indicates that these molecules are not attached to the cell wall through the peptidoglycan layer.

Some of these molecules stimulate an immune response in patients and in animals. The 28-Kda band reacted only with patient serum, and not with polyclonal antibodies raised in rabbits. This difference suggests that different epitopes may be presented in different hosts. Further work is needed to investigate possible correlations between antibody response to cell-wall components and pathophysiology of mycobacterial disease.

Comparison of a few selected species of *Mycobacterium* by means of SDS-PAGE profiles revealed major differences, although a 47-Kda band was found in all mycobacteria. To investigate the feasibility of using cell-wall-derived SDS-PAGE profiles for taxonomic and diagnostic purposes, other species of *Mycobacterium* and different strains of the same species must be studied.

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