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Immunoreactive antigens of a candidate leprosy vaccine: *Mycobacterium habana*

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Summary *Mycobacterium habana* (*M. simiae* serovar-1) is a candidate vaccine for mycobacterial infections on the basis of the protection shown by this strain. We prepared 3 fractions of *M. habana*, i.e. the cell wall (CW), the cell membrane (CM) and the cytosol (CS). Protein antigens of these fractions were resolved by SDS-PAGE and subsequently probed with the sera of leprosy and tuberculosis patients and also antiBCG antibodies.

We saw 3 major protein bands at ≃33 kD in the CW, ≃38 kD in the CM and ≃22 kD in the cytosol (CS) after coomassie blue staining of the gels. Pool leprosy patients' serum had identified proteins of ≃26 kD in CW, ≃35 and ≃18 kD in CM and ≃24 kD in the CS which have not been seen by the TB patient's serum pool. Pool serum of tuberculosis patients has identified 1 protein at ≃10 kD in the CW and a broad band between 20 and 24 kD and 1 at ≃4 kD in the CM which have not been visualized in the pool leprosy patient's serum lane. The proteins of *M. habana* which are recognized only by leprosy antisera or only by tuberculosis antisera could be exploited for developing diagnostic agents against these infections.

Introduction

Integral mycobacterial cells contain a pool of potentially pathogenic/redundant constituents which compromise the protective efficacy of such cells as vaccine. These vaccines may lead to the development of adverse immunological phenomenon on immune suppression[1,2] autoimmunity[3] and tissue necrosis.[4] It is imperative to identify the immunologically active molecules in such cells to further our understanding about the antigenic molecules in order to develop a subunit vaccine or diagnostic agents for mycobacterial infections.

Mycobacterium habana, TMC 5135 (now known as *M. simiae* serovar-1; the derivation is given elsewhere) is a cultivable[5] nonpathogenic mycobacterium which has shown promising protective efficacy against experimental leprosy[6] and tuberculosis[7] in a series of studies. It is an interesting organism, and its antigens deserve further exploration.

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In this study we have identified the immunoreactive protein antigens in *M. habana* by probing them with the sera of leprosy and tuberculosis patients and also with antiBCG antibodies with a view to recognize immunoligically important antigens of this organism.

Materials and methods

GROWTH OF *MYCOBACTERIUM HABANA*

Mycobacterium habana was grown in liquid Sauton's medium containing glycerol and L-asparagine in a shaking incubator at 37°C. Defined mid-log phase (of 10 days) growth of the mycobacterium was harvested at 4000 × ***g*** at 4°C and washed 3 times with normal saline.

FRACTIONATION OF *M. HABANA*

The mycobacterial harvest was suspended in Tris-buffered saline (TBS; 0.01 M Tris, pH 7·4) containing $MgCl_2$ (10 mM) and protease inhibitors (1 mM) at a concentration of 200 mg ml^{-1}. The suspension was sonicated for 10 min at >20k cycles s^{-1} in phasic manner (50% s^{-1}) in Ultrasonic Processor XL Heat systems, sonicator. The sonicate was subjected to differential centrifugation to isolate the subcellular fractions of cell wall, cell membrane and the cytosol according to the procedure described by Brodie with slight modifications.[8] Briefly, the sonicate was first centrifuged at 15,000 × ***g*** for 30 min at 4°C to isolate the cell wall (pellet). The pellet was washed 3 times with TBS. The supernatant was again centrifuged at 150 000 × ***g*** for 60 min at 4°C, to obtain membrane (pellet) and cytosol (supernatant), fractions, respectively. Pure membrane was obtained by repeated centrifugation at 150 000 ×***g***.

ANTIBODIES

*Anti-*M. leprae *and anti-*M. tuberculosis *antibodies*

Sera from 12 lepromatous leprosy (LL) and 8 tuberculosis (TB) patients were collected separately from the Mission Leprosy Hospital, Barabanki and Tuberculosis Hospital, Rajendar Nagar, Lucknow. Separate pools of the sera of LL and TB patients were prepared. The serum pools, respectively, served as sources of polyclonal anti-*M. leprae* and anti-*M. tuberculosis* antibodies. Peroxidase conjugated antibodies against rabbit and human immunoglobulins (Igs) were procured from Sigma Chemicals (USA).

Anti-BCG Antibodies

Commercially available anti-BCG antibodies (rabbit raised; Dakoppats, Denmark) were used.

SDS-PAGE and Immunoblotting

Proteins of cell wall, cell membrane and cytosol fractions were separated on discontinuous 12·5% SDS-polyacrylamide gels (SDS-PAGE).[9] After electrophoresis, the resolved proteins were either stained or transferred to nitrocellulose paper (NCP, S&S,

0·45 μm).[10] After transfer the paper was cut into 4 mm wide strips and blocked with 3% skimmed milk powder (Anik-Spray, Lipton India Ltd) in TBS (20 mM Tris-HCl, pH 7·2, 500 mM NaCl) containing 0·05% Tween-20 (TTBS; dilution buffer) for 2 hr at room temperature (RT). Blocked strips were probed with heterologous antibodies diluted in 1% milk-TTBS (antiBCG antibodies 1 : 250 and anti-*M.leprae* and anti-*M. tuberculosis* antibodies 1 : 25) for 2 hr at room temperature. After washing (5 × 5 min each) with TTBS the strips were treated for 2 hr at room temperature (30°C) with peroxidase conjugated antirabbit and antihuman immunoglobulins (Igs) (1 : 500 in 1% milk TTBS). Strips were washed (5 × 5 min) with TTBS and finally once with TBS. Strips were developed with 4-chloronaphthol and reaction was stopped by extensive washing with distilled water.

Results

Analysis of the subcellular fractions of whole cell sonicate of *M. habana* revealed that the cell wall (CW) constituted 32%, the cell membrane (CM) only 2% and the cytosol (CS) about 46% of the cellular mass (in terms of proteins).

SDS-PAGE pattern of the proteins of CW, CM and CS after coomassie blue staining are shown in Figure 1. Cytosol fraction showed the greatest number of individual bands. It is apparent that the major protein bands are at ≃33 kD in CW, ≃38 kD in CM and ≃22 kD in CS. The consistency in protein migration pattern of fractions prepared in 2 different batches is also apparent in Figure 1.

Subcellular location of immunologically active protein antigens has been determined by probing the antigen loaded strips of the 3 fractions with heterologous polyclonal antibodies.

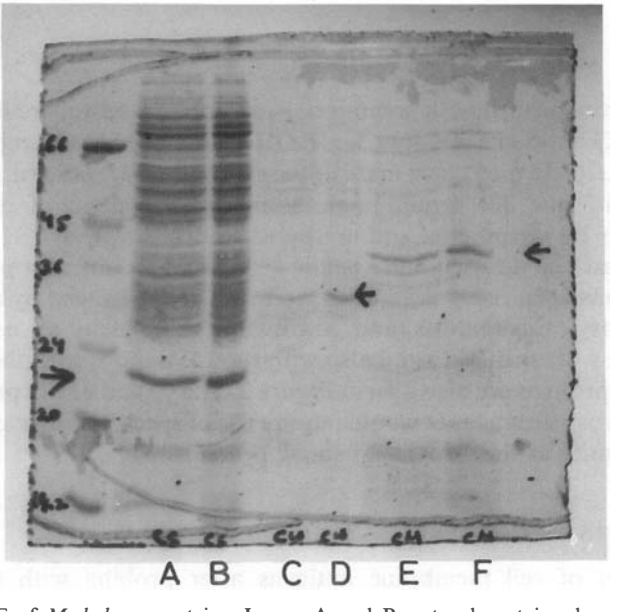

Figure 1. SDS-PAGE of *M. habana* proteins. Lanes: A and B, cytosol proteins; lanes C and D, cell wall proteins: E and F, cell membrane proteins. Molecular weight markers (MWM) are shown on the left.

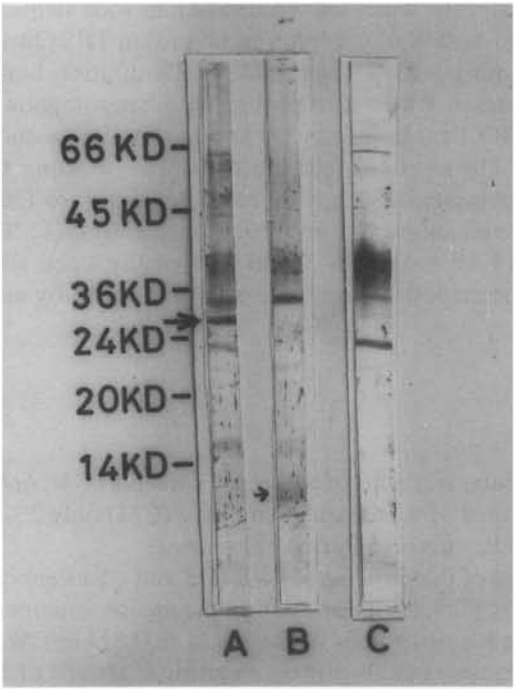

Figure 2. Probing of *M. habana* cell wall antigens with heterologous antibodies. Lane A: anti-*M. leprae* abs; lane B: anti-*M. tuberculosis* abs; and lane C: antiBCG abs. Molecular weight markers are shown on the left.

THE CELL WALL ANTIGENS

These antigens were probed with serum pools of leprosy and tuberculosis patients and also with antiBCG antibodies (Figure 2). The LL serum pool has recognized a dominant protein band at ≃26 kD molecular mass in the cell wall of *M. habana*, which is not seen after probing with the TB serum pool. Similarly, a protein of ≃10 kD has been recognized by the TB serum pool and not by LL sera.

A strong broad and diffused band below 40 kD and 1 dominant protein at ≃30 kD molecular mass has been recognized in the cell wall of *M. habana* by all the polyclonal antibodies (leprosy, tuberculosis and antiBCG). A protein at ≃23 kD has been prominantly seen with anti-BCG and also with the LL but not with the TB serum pool.

Several other proteins are also seen in Figure 2. The 60 and 65 kD proteins seen in the antiBCG and leprosy serum lanes would require use of specific monoclonal antibodies to confirm their identity as the Groel heat shock proteins.

THE CELL MEMBRANE ANTIGENS

The western blot of cell membrane antigens after probing with these 3 types of polyclonal antibodies (*vide supra*) is shown in Figure 3.

Proteins of ≃35 kD and ≃18 kD have been recognized only by the LL serum pool

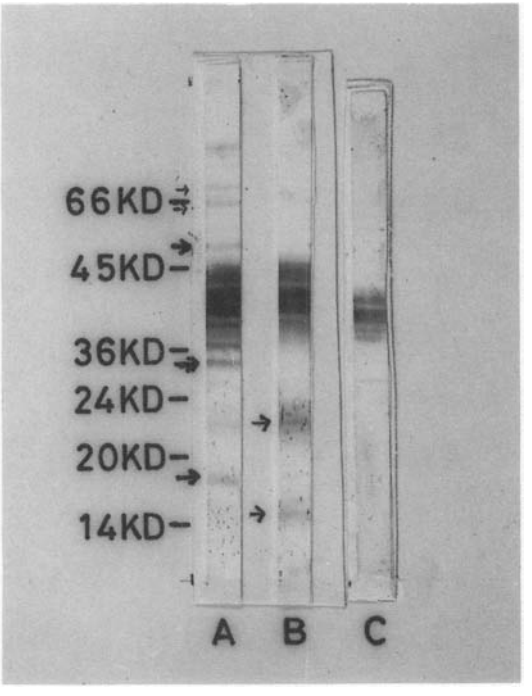

Figure 3. Probing of *M. habana* cell membrane antigens with heterologous antibodies (see Figure 2).

and not by the TB sera. Several other protein bands, i.e. at ≃70, ≃65, and ≃50 kD molecular mass are evident in the LL serum pool but is not so clearly seen in other lanes.

A diffuse broad band at 20–24 and 1 at ≃14 kD are seen in the TB serum lane which are not so well marked in the LL serum lane.

AntiBCG serum has recognized a broad and diffuse band of proteins at 36–40 kD which are also recognized by LL and TB serum.

CYTOSOLIC ANTIGENS

Cytosolic antigens of *M. habana* after probing with these 3 types of polyclonal antibodies have been shown in Figure 4.

A broad band at ≃24 kD and several other bands have been seen in the LL serum lane but not in TB serum lane.

AntiBCG serum has recognized several protein bands between 40–65 kD molecular mass which are also seen faintly/prominantly in the LL sera and feebly in the TB sera lane.

Discussion

Integral mycobacterial vaccines have generally provided less than desirable protection.[11] This may possibly be due to an adverse effect of certain mycobacterial constituents.

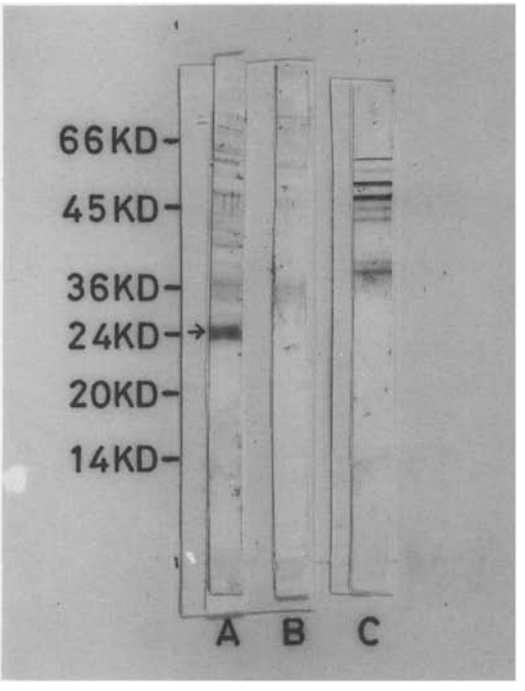

Figure 4. Probing of *M. habana* cytosol antigens with heterologous antibodies (see Figure 2).

Protection against intracellular mycobacterial infection is regulated through cell-mediated immune (CMI) response.[12] CMI response, in turn, is induced by the activation of relevant T cells (helper, $CD4^+$) by appropriate protein/peptides.[13] Mycobacterial proteins are known to bear both B and T cell epitopes and induce, through them, humoral and cell-mediated responses.[14–16] Research on mycobacterial proteins is still in a descriptive phase. Nevertheless, searching the antigens that might be involved in eliciting a protective CMI response by probing the epitopes that are recognized by B cell[17] is considered a justified approach. Therefore, immunological characterization of these proteins is done by studying their B cell reactivity through corresponding antibodies. Moreover, according to a prevalent view, protective, antigens of mycobacteria are those which are shared by other mycobacterial species rather than species specific.[6,18] These facts put together inspired us to undertake a systematic study to identify the myriad of protein antigens present in *Mycobacterium habana*, an atypical mycobacterium. The mycobacterium offers protection against experimental tuberculosis[7], leprosy[6], buruli ulcer[19] and shows antigenic cross-reactivity with *M. tuberculosis*, *M. leprae* and BCG.[20]

In this study we have identified some protein antigens in *M. habana* which are identified only by pooled LL serum and not by pooled TB serum or vice versa. These may be important for immunodiagnosis and/or immunoprophylaxis in these mycobacterial infections. In order to analyze the antigenic mosaic, mid-log cultures of *M. habana*

have been used to avoid the known contamination of autolytic cell products and irrelevant polysaccharides in old cultures.[21] Subcellular location of immunologically important proteins within the cell have been determined by preparing 3 broadly defined fractions, namely, the cell wall, the cell membrane and the cytosol. Protein profile of these subcellular fractions have been analysed by polyacrylamide gel electrophoresis under reducing conditions (SDS-PAGE) and 3 major proteins, ≃22 kD in cytosol, ≃38kD in membrane and 33 kD in wall fraction have been recognized by direct protein staining.

We identified 4 antigens of *M. habana*, i.e. ≃26 kD of CW, ≃35 kD and ≃18 kD of CM and ≃24 kD of CS, only by pooled LL serum. Similarly, 3 antigens ≃10 kD of CW, 20–24 kD and ≃14 kD of CM have been identified only by pooled TB serum. These findings indicate that these antigens may bear epitopes which are common between *M. habana*, and *M. leprae* only or *M. habana* and *M. tuberculosis* only. These may have diagnostic and/or protective potential in leprosy and tuberculosis.

A ≃40 kD antigen of cytosol fraction has been strongly recognized by anti-BCG antibodies. Similarly, ≃23 kD cell wall protein has been identified by anti-*M. leprae* and antiBCG antibodies. A ≃30 kD antigen of CW has been recognized by all the 3 antibodies. These observations suggest antigenic homology between *M. habana* and *M. leprae*, *M. tuberculosis* and BCG.

A 35 kD antigen of *M. leprae*[15,16] and *M. tuberculosis*[14] and a 14 kD antigen of *M. tuberculosis*[22] have already been reported to be of high protective and/or diagnostic value. Interestingly the proteins of corresponding molecular weights have been identified in *M. habana* also and more importantly by patient sera. Therefore, findings of this study will provide more appropriate information about the particular antigen of interest. The present effort to find subcellular location for some of the important predominant proteins may help in assigning a functional role of them.

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