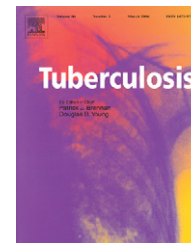




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Monoclonal antibodies to *Mycobacterium tuberculosis* CDC 1551 reveal subcellular localization of MPT51

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Received 6 February 2007; received in revised form 12 July 2007; accepted 19 July 2007

KEYWORDS

Mycobacterium tuberculosis;
Monoclonal antibodies;
MPT51

Summary

Mycobacterium tuberculosis CDC 1551, a highly immunogenic outbreak strain, previously reported to have unique surface distribution of capsular polysaccharide, was used to generate novel monoclonal antibodies (mAbs) to surface mycobacterial targets. Two immunoglobulin G1 (IgG1) mAbs, 16a1 and 16a6 were generated. The mAbs originated from the same B cell, bound strongly to whole cell *M. tuberculosis* CDC1551 and to its cell wall, membrane and cytosol fractions recognizing a 90 kDa protein. Immunoprecipitation using mAb 16a1 isolated a protein with amino acid peptide sequences matching MPT51 from the cytosol. This immunogenic protein of unknown function was previously reported only in culture filtrates of *M. tuberculosis*. Our findings suggest for the first time that this protein is found within the *M. tuberculosis* cell.

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Introduction

The role of antibodies in protection against tuberculosis (TB) was historically discounted. However, recent studies demon-

strated that certain antibodies have beneficial effects on the course of mycobacterial infection.^{1–8} Monoclonal antibodies (mAbs) directed to arabinomannan (AM) and lipoarabinomannan (LAM) were described in several of these studies.^{1,3,5}

The surface of *Mycobacterium tuberculosis* is rich in lipids and carbohydrates with an outermost capsular layer composed primarily of the polysaccharides AM and glucan.⁹ The capsular material was previously shown to be easily removed or shed from the mycobacterial surface.^{10,11} Using

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mAbs to AM, we previously demonstrated that all *M. tuberculosis* strains tested shed AM into the culture media.¹² However, while most of these strains also retained capsular AM on the cell surface, some shed their entire capsular AM.¹² Shedding of capsular material may have implications on the interactions of *M. tuberculosis* with antibodies and host cells. In this study we used *M. tuberculosis* CDC 1551, an outbreak strain from which the capsular AM is largely shed and possibly entirely removed from the surface, to generate novel mAbs to immunogenic non-capsular surface epitopes. These mAbs were important for understanding their target antigen localization.

Materials and methods

Mycobacterial strains

M. tuberculosis CDC 1551 originated from an Albert Einstein College of Medicine collection. Irradiated *M. tuberculosis* CDC 1551 was supplied by J.T. Belisle (Colorado State University). Nine drug-susceptible *M. tuberculosis* strains from sputa of TB patients originated from the Microbiology Laboratory, Montefiore Medical Center, Bronx, NY. *M. tuberculosis* Erdman (TMC 107) originated from Trudeau Institute Mycobacterium Culture Collection, Saranac Lake, NY. *M. tuberculosis* Erdman mutant strain with double deletion $\Delta RD1\Delta panCD$ was previously described.¹³

Culture of mycobacterial strains

Virulent *M. tuberculosis* strains were grown in biosafety level 3 (BSL3). For the generation of mAbs, *M. tuberculosis* CDC 1551 was grown in 7H9 medium (Difco, Detroit, MI) containing 1% glycerol (Sigma, St. Louis, MO) and OADC (Becton-Dickinson, MD) without Tween 80 for 14 days at 37 °C, heat killed at 80 °C for 2 h, washed twice in PBS, sonicated (Branson Ultrasonics, Danbury, CT) for 5 s and frozen at -80 °C. *M. tuberculosis* clinical strains and Erdman were grown and lyophilized as previously described.¹² *M. tuberculosis* $\Delta RD1\Delta panCD$ was grown in 7H9 medium containing 0.05% Tween 80 and pantothenate (24 µg/ml).¹³

Antibodies

mAbs 9d8 (IgG3) to AM, 24c5 (IgG1) to glucan, 5c11 (IgM) to LAM and AM, and 4f11 to a polysaccharide epitope of mycolyl-arabinogalactan-peptidoglycan (mAGP) complex were previously described.^{14,15}

Antigens

M. tuberculosis CDC 1551 whole cell lysate, cytosol, cell wall, membrane and culture filtrate protein as well as *M. tuberculosis* H37Rv LAM, mAGP complex, total lipid fractions, and mycolic acids were supplied by J.T. Belisle. MPT51 was generated as previously described.¹⁶

Generation of mAbs to *M. tuberculosis* CDC 1551

Six to eight week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were each immunized intraperitoneally with 1×10^7 heat killed *M. tuberculosis* CDC 1551 bacilli suspended in 200 µl of PBS and mixed 1:1 with Incomplete Freund's Adjuvant. They were boosted, with 1×10^7 bacilli on days 14, 28, and 42. The mouse with the highest serum antibody titers to *M. tuberculosis* CDC 1551 was boosted again 4 days prior to fusion and its spleen cells were fused with NSO cells as previously described.¹⁵ Hybridomas producing antibodies were screened and cloned as previously described.¹⁴

Whole cell ELISA for *M. tuberculosis*

ELISA was adapted from a previously described protocol¹⁴ using *M. tuberculosis* CDC1551 (heat killed or irradiated) placed in microtiter plate wells and blocked with 5% milk (dry, non-fat). Hybridoma supernatants, were added, serially diluted if necessary and incubated for 1 h at 37 °C. Alkaline phosphatase (AP) goat anti-mouse isotype-specific Ig (1 µg/ml) (Southern Biotechnology Associates, INC) was added and color was developed using *p*-nitrophenylphosphate (PNPP) (Southern Biotechnology Associates, INC). Negative control wells had no *M. tuberculosis*. Whole cell ELISA with *M. tuberculosis* clinical strains and Erdman was performed as previously described.¹²

Immunoelectron microscopy

M. tuberculosis CDC 1551 grown for 9 days in 7H9 media without Tween 80, was fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M sodium cacodylate buffer, dehydrated through a graded series of ethanol, embedded in LR White resin and cut into ultra thin sections. Grids were blocked and then incubated with mAbs (10 and 100 µg/ml) in BSA-gel PB for 2 h at room temperature. No mAb was added to negative control grids. Grids were then incubated with goat anti-mouse Ig conjugated to 5 nm gold spheres (Aurion) in BSA-gel PB, fixed in PB containing 2% glutaraldehyde, washed with PB and distilled water followed by the addition of 4% phosphotungstic (Fisher) and incubation with lead citrate. Grids were viewed with a 1200EX transmission electron microscope (JOEL, Peabody, MA).

Chemical analysis of mAbs epitope

Proteinase K and sodium meta-periodate were used in ELISA as previously described¹⁴ to investigate whether mAbs recognized epitopes containing protein or carbohydrate, respectively.

Binding of mAbs to cellular fractions of *M. tuberculosis* by ELISA

Cytosol, membrane, cell wall and whole cell lysate preparations of *M. tuberculosis* CDC1551 dissolved in PBS at 10 µg/ml, were placed in polystyrene microtiter ELISA plate wells (50 µl/well) and incubated for 1 h at 37 °C. The

plates were blocked with 5% milk for 1 h at room temperature. MAbs at 10 µg/ml in 1% BSA were added and serially diluted. The rest followed as previously described.¹² Binding of mAbs to surface carbohydrate and lipid fractions was determined by ELISA, as previously described.¹⁴ LAM (10 µg/ml) and mAGP complex (1 mg/ml) in carbonate buffer (pH 9.6) were each incubated in microtiter ELISA plates at 37 °C for 1 h. TLF and mycolic acids suspended in 100% ethanol, were added to microtiter plates, serially diluted starting at 1 mg/ml, and air dried overnight prior to proceed with the ELISA protocol.

Western blot analysis

Western blot analysis was performed as previously described.^{17,18} Cytosol, membrane, cell wall, and samples of purified protein preparation suspended in PBS were separated by 10% and 12% SDS-PAGE gel (maximum voltage and current of 250 mA) for 3 h. Gels were trans-blotted onto nitrocellulose membranes (Protran), blocked with BLOTTO (Tris buffered saline with 0.2% Tween, 2% dry non-fat milk, and azide), incubated with 10 µg/ml of mAb diluted in BLOTTO overnight at 4 °C, and then probed with goat anti-mouse-AP labeled IgG1 (Southern Biotechnology Associates, INC) at 1 µg/ml in Tris buffered saline with 0.2% Tween (TBST). Blots were washed with TBST between steps and color development reagents (Bio-Rad Laboratories) were applied. Negative controls consisted of mAb 2H1 to *Cryptococcus neoformans* or omission of primary mAb.¹⁹

Antigen identification

For antigen identification, immunoprecipitation of cytosol was performed with purified mAb 16a1 using Seize[®] Protein G Immunoprecipitation Kit (Pierce, Rockford, IL). The immunoprecipitation product was separated on a 12% SDS-PAGE gel and silver stained. A separate gel was trans-blotted onto a nitrocellulose membrane and incubated with mAb 16a1. The band identified by the immunoblot and silver stain was cut, processed and digested into peptides as previously described.²⁰ Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry was performed using Voyager-DE[™] Biosystems STR Biospectrometry[™] workstation (Applied Biosystems, Foster city, CA). Data base search using MS-FIT was performed at the Mass Spectroscopy Facility of the University of California, San Francisco.²¹ Tandem Mass Spectrometry was performed on an ABI 4700 TOF/TOF (Applied Biosystems) and data search was done using Mascot search engine (Matrix Science).²²

MAB fine specificity studies

To examine the antibody specificity, mAb 16a6 was purified (immunopure L or G Immunoglobulin purification kit, Pierce, Rockford, IL), biotinylated (EZ-Link Sulfo-NHS-LC-Biotinylation kit, Pierce, Rockford, IL) and used in competition ELISA with mAb 16a1 for binding to *M. tuberculosis* CDC 1551 whole cell. Competition ELISA was performed twice; once adding constant concentration of biotinylated mAb 16a6 and

varying concentrations of mAb 16a1, and once adding constant concentration of mAb 16a1 and varying concentrations of biotinylated mAb 16a6. After incubation and washing, alkaline phosphatase conjugated streptavidine was added followed by color development with PNPP in substrate buffer.

MAB sequencing

RNA was prepared from hybridoma cell lines 16a1 and 16a6 as previously described.²³ RNA was used for the synthesis of cDNA from mRNA, using oligo(dT) primer and superscript II reverse transcriptase (GIBCO). The cDNA encoding the immunoglobulin variable domains was then amplified by PCR as previously described,²³ using the following universal 5' (sense) variable-region and specific 3' (antisense) constant region primers: 3' msCgamma, AGA CCG ATG GGG CTG TTG TTT TGG C; 3' msCkappa, TGG ATA CAG TTG GTG CAG CAT CAG C; 5'vh Uni, TGA GGT GCA GCT GGA GGA GTC; 5'Vk Uni GAC ATT CTG ATG ACC CAG TCT.

PCR products (Qiagen) were ligated into pCR2.1 (Invitrogen) according to manufacturer instructions. Constructs containing inserts were detected by restriction digestion with EcoR1 (Roche) and were visualized by 2% agarose gel electrophoresis. Alternatively a one-step PCR Kit (Qiagen) was used according to the manufacturer instruction, under the following conditions: 50 °C for 30 min, 95 °C for 15 min, followed by 94 °C for 1 min, followed by 54 °C for 1 min, and 72 °C for 1 min, followed by a final 10-min extension at 72 °C. Amplified variable domains cDNA samples were sequenced bidirectionally by automated gel sequencing using the appropriate 5' (sense) and 3' (antisense) primers for each variable domain.

Agglutination assay

To evaluate for mAb-mediated agglutination, $\Delta RD1\Delta panCD$ double mutant of *M. tuberculosis* Erdman was thawed from frozen stocks and 100 µl aliquots were added to 10 ml liquid medium and incubated at 37 °C. The aggregation status of mycobacteria was determined visually using Olympus BX61 microscope with Cooke SensiCam^{QE} High Performance camera. For this, 10 µl of culture were placed on glass microscope slide and covered with a coverslip. The slides were sealed with nail polish. To obtain a solution containing only single bacilli, vortexing, precipitation of clumps by gravity or centrifugation for 1 min using VMSC-13 minicentrifuge at 6.5 RPM to precipitate clumped bacilli were employed as necessary. The bacilli status was determined after screening multiple fields. Sets of 3–5 pictures were taken from each preparation for re-evaluation. Aliquots of 100 µl media containing single bacilli were then added to media containing purified mAb at concentrations of 10 or 100 µg/ml. The mycobacterial inocula CFU were 4.88×10^5 and 3.47×10^5 , respectively. At designated times, aliquots were removed to determine the bacilli status by microscopy. Plating on 7H10 agar and absorbance measurements were performed to confirm viability.

Results

Isolation of hybridomas producing mAbs to *M. tuberculosis* CDC 1551

Thirty-six antibody producing hybridoma cell lines were identified by whole cell ELISA. After two cloning procedures in soft agar, 6 stable clones were isolated. We continued our work with two hybridoma cell lines, 16a1 and 16a6, both producing IgG1 with kappa light chains, because they bound both heat-killed and irradiated *M. tuberculosis* (see below) suggesting a heat-stable target epitope.

Binding of mAbs to whole cell *M. tuberculosis* ELISA

To confirm the surface properties of *M. tuberculosis* CDC 1551, mAbs 5c11 (IgM), 9d8 (IgG3) 24c5 (IgG1) and 4f11 (IgM) were reacted with heat killed whole-cell *M. tuberculosis* CDC 1551 by ELISA. Strong reactivity of mAb 5c11 and no reactivity of mAbs 9d8, 24c5 and 4f11 were demonstrated (Figure 1A).

MAbs 16a1 and 16a6 bound similarly heat-killed (Figure 1B), and irradiated *M. tuberculosis* CDC 1551 as well as *M. tuberculosis* Erdman and all clinical strains (TB1 through TB9) (Table 1). No reactivity was detected in negative control wells.

Chemical analysis of epitopes

Binding of mAbs 16a1 and 16a6 to proteinase K treated *M. tuberculosis* CDC 1551 was significantly reduced relative to non-treated mycobacterial cells (Figures 1C and 1D). Binding of mAbs was not affected by sodium *meta*-periodate treatment (which oxidizes carbohydrate sugar rings and can affect the binding of antibodies to polysaccharide epitopes). Neither mAb bound to LAM, mAGP, total lipid fraction or mycolic acids.

Localization of mAb binding

mAbs 16a1 and 16a6 bound strongly to cytosol, membrane, cell wall and cell lysate tested by ELISA. Western blot

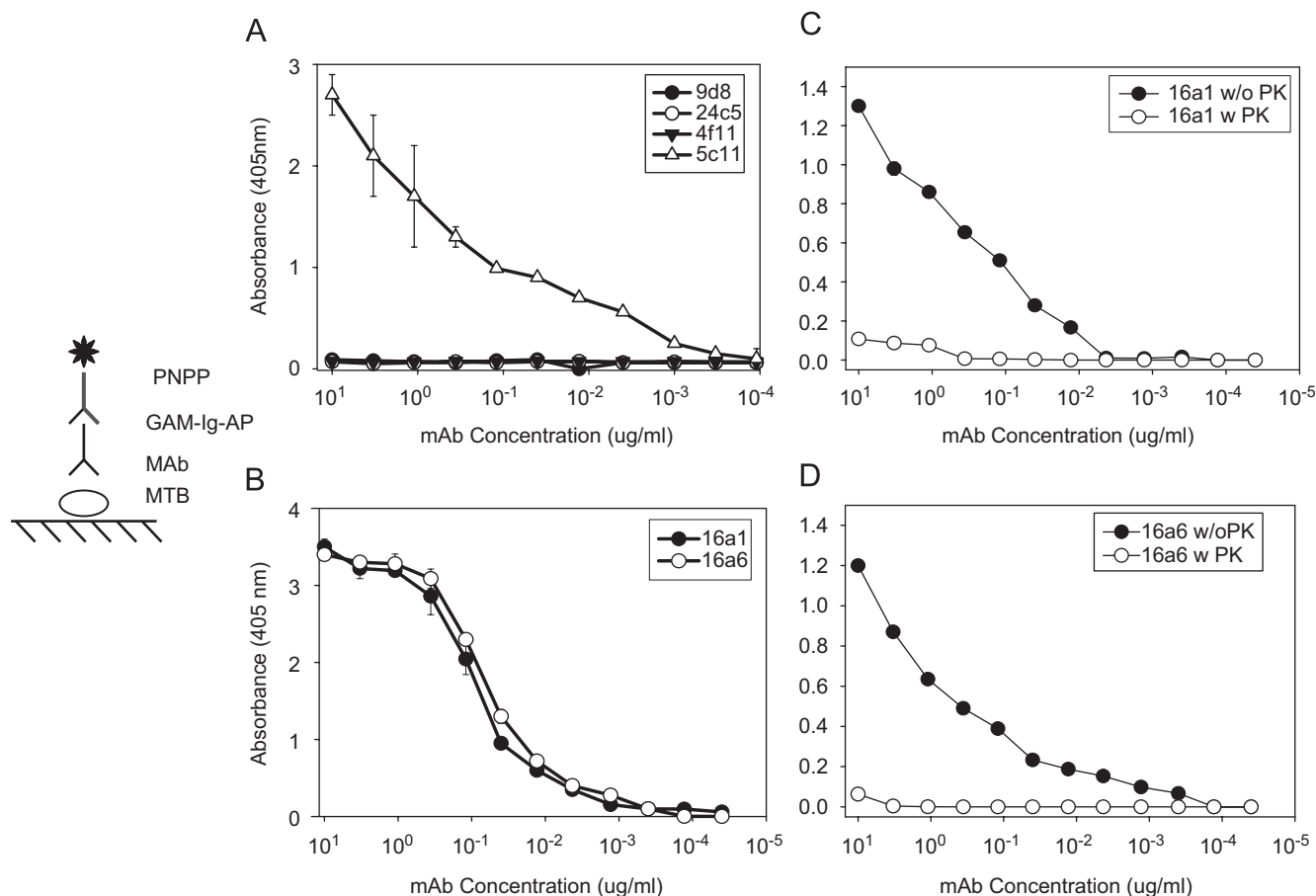


Figure 1 Binding of mAbs to *M. tuberculosis* CDC 1551 by whole-cell ELISA. Binding at different dilutions of (A) mAbs 5c11, 24c5, 9d8 (B) mAbs 16a1, and 16a6. Similar binding of mAbs 16a1 and 16a6 were obtained using irradiated killed *M. tuberculosis* CDC 1551. (C) and (D) represent binding of mAbs 16a1 and 16a6 to *M. tuberculosis* in the presence (open symbols) and absence (filled symbols) of proteinase K, by whole-cell ELISA. The diagrams represent the ELISA configurations. Values represent the average of three measurements. Error bars indicate standard deviation from the mean. Experiment was repeated three times with similar results.; MTB, *M. tuberculosis*; AP, alkaline phosphatase.

Table 1 Analysis of murine mAbs 16a1 and 16a6.

MAB	Isotype	Binding to <i>M. tuberculosis</i> CDC 1551 (Heat-killed)	Binding to <i>M. tuberculosis</i> CDC 1551 (irradiated)	Binding to <i>M. tuberculosis</i> strains Erdman & TB1–TB9	V _H	J _H	V _K	J _K	Peptide sequences of MPT51 identified immunoprecipitation with mAb
16a1	IgG1	+	+	+	7183	4	4 (subgroup 50)	2	APYENLMVPSMGR GISVVAPAPAGGAYSMYTN- WEQDGSK GLAPGGHAAVGAAQGGY- GAMALAAFHPDR WHPWPVHASLLAQNNTR VWVWSPNPGASDPAAMIG- QAAEAMGNSR MFYNQYRS
16a6	IgG1				7183	4	4 (subgroup 50)	2	

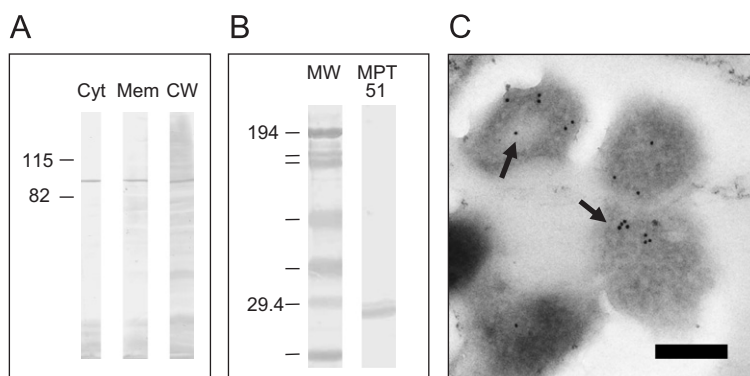


Figure 2 (A) Western blot analysis using mAb 16a6 with *M. tuberculosis* CDC1551 cellular fractions. Assay using mAb 16a1 was identical. (B) Western Blot analysis with MPT 51. Results were identical for mAb 16a1 and 16a6. (C) Immunoelectron microscopy demonstrating the binding of mAb 16a1 to *M. tuberculosis*. Gold particles denote secondary antibody binding to primary mAb. Similar binding patterns were obtained with mAb 16a6. Control experiments with irrelevant isotype-matched mAbs revealed no binding of gold particles to mycobacteria (data not shown). Bars = 200 nm.

analysis with *M. tuberculosis* cell fractions confirmed the ELISA results and demonstrated that both mAbs bound to a 90 kDa protein antigen (Figure 2A) in all cell fractions tested.

Immunoelectron microscopy

Gold labeling localized primarily within the mycobacterium when mAbs 16a1 and 16a6 were used (Figure 2C). No gold labeling was detected in control samples, including those utilizing mAbs 9d8 and 24c5.

Antigen identification

The immunoprecipitation product obtained using mAb 16a1 migrated to the 90 kDa range on SDS-PAGE gel. Mass spectroscopy of the protein recovered, obtained unique monoisotopic ions used for peptide mass fingerprinting of

the Mycobacterium NCBI database. The top 2 hits, with MOWSE scores of 2.049e+06 and 4.603e+04, identified 5 peptides that matched with peptides of fbpC1 also known as MPT 51. Tandem Mass Spectrometry data search using Mascot²² identified 2 peptide sequences that belong to MPT51, with a search score of 70 and 39. Altogether 6 peptides of MPT51 were identified covering 124 of the 299 (41%) amino acids of the protein (Table 1). The binding of mAbs 16a1 and 16a6 to MPT 51 was confirmed by running purified recombinant MPT51 on SDS-PAGE gel and performing a western blot as described above (Figure 2B).

MAB fine specificity

Due to the similarities in binding characteristics of mAbs 16a1 and 16a6, competition ELISA was done to assess the mAb fine specificity. Inhibition of the binding of biotinylated mAb 16a6 to *M. tuberculosis* CDC 1551 was observed in the

presence mAb 16a1 both when mAb 16a1 concentration was kept constant and mAbs 16a6 concentration was variable and vice versa (not shown).

mAb variable domain sequencing

Due to the similarity in the specificity, localization and isotype of mAbs 16a1 and 16a6, we sequenced their VH and VK domains. A 100% sequence homology for the heavy and light chain variable domains was observed for the 2mAbs. Based on 317 nucleotides, the sequence of the variable heavy chain (V_H) was demonstrated to use the germ line family V_H 7183, and J_H family J_H4 . The sequence of the variable light (V_L) chain (based on 325 nucleotides) used the germ line family V_K 4 subgroup 50 and J_K family J_K2 (Table 1). The consensus sequences were deposited in GenBank (accession numbers EF 409992 and EF 409993).

Determination of agglutination by mAbs

M. tuberculosis has a natural tendency to clump.²⁴ Thus establishing the role of antibody in the agglutination of mycobacteria can be difficult. To overcome this problem, we devised a method for identifying single bacilli visually prior to the addition of antibodies and observing their aggregation status thereafter. When mAbs 16a1 and 16a6 were used at a concentration of 10 $\mu\text{g}/\text{ml}$, no clumping was observed 3 days after the addition of mAbs. At a mAb concentration of 100 $\mu\text{g}/\text{ml}$, widespread clumping was observed (Figure 3B), while control samples containing mAb 18B7 or no mAb had predominance of single bacilli (Figure 3A). Microscopic observation 20 days later, demonstrated predominance of single bacilli (with only few clumps) in samples to which mAbs 16a1 and 16a6 were added, regardless of antibody purification method (by protein A or L). Viability of bacilli was confirmed by growth of bacilli on 7H10 agar plates and increasing absorbance values.

Discussion

The beneficial effect of mAbs to AM on the outcome of *M. tuberculosis* infection^{1,3,5} combined with the reports that AM conjugate vaccine candidates elicited antibody responses,¹² lowered CFU early after mycobacterial infection¹² and prolonged survival,²⁵ suggest that AM is a useful antigen to be considered in vaccine development against TB. However, the shedding of capsular AM from the mycobacterial surface which in few *M. tuberculosis* strains appears to be complete,^{12,26} raises concerns regarding the ability of antibodies directed towards certain capsular epitopes to protect effectively against those strains. Hence, we directed our efforts toward identifying additional surface antigens that could potentially be useful for eliciting protective antibody responses. We hypothesized that using a *M. tuberculosis* strain that sheds the capsular polysaccharide completely will allow an enhanced exposure of other surface antigens to the immune system. We used *M. tuberculosis* CDC 1551, which was shown in our previous studies¹² to lack the capsular AM on its surface after washing, to generate mAbs. This strain of *M. tuberculosis* was previously found to induce a more vigorous host response *in vivo* and *in vitro* as compared to other strains.²⁷

Our results of whole-cell ELISA and immunoelectron microscopy showed that mAb 9d8 and mAb 24c5 (directed to the capsular polysaccharides AM and glucan, respectively) did not bind the surface of *M. tuberculosis* CDC 1551, confirming that their respective epitopes were absent from the cell surface after separation from the growth media and washing. The lack of binding of mAb 4f11 which recognizes a polysaccharide epitope of mycolyl-arabinomannan-peptidoglycan (mAGP),¹⁴ could suggest either the absence of this epitope from the surface of *M. tuberculosis* CDC 1551, or a different distribution of this epitope with respect to the cell surface of this strain.

The two new IgG1 mAbs generated, mAbs 16a1 and 16a6, bound to *M. tuberculosis* CDC 1551, by whole-cell ELISA, immunoelectron microscopy and western blot. Each mAb recognized protein epitopes not affected by irradiation or

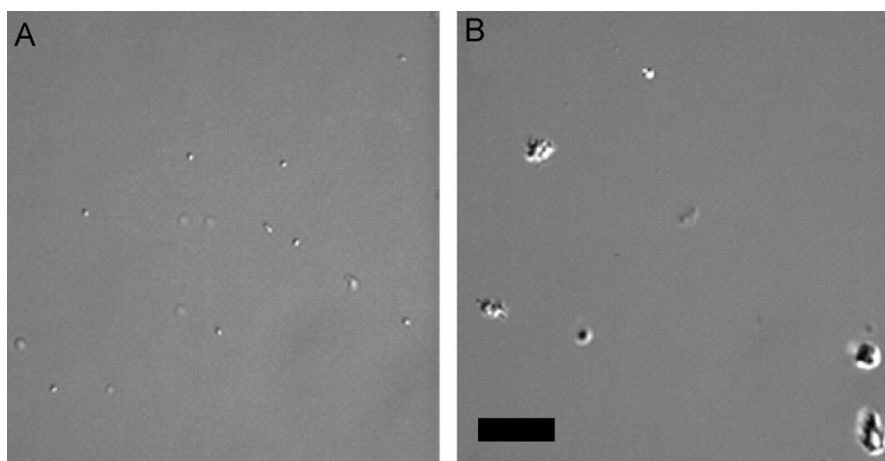


Figure 3 Figures of *M. tuberculosis* $\Delta RD1\Delta panCD$ under DIC microscope. (A) Control sample 3 days after inoculation with *M. tuberculosis* $\Delta RD1\Delta panCD$ without addition of mAb, demonstrating single bacilli. (B) Antibody (mAb 16a1)-treated sample demonstrating clumps; similar results were obtained with mAb 16a6 (Bar 20 μm).

heat killing of *M. tuberculosis* CDC 1551, suggesting a heat-stable target. The binding of these mAbs to cytosol, membrane and cell wall fractions suggests that their epitopes were found on the cell surface as well as within the mycobacterial cell. The reactivity of both mAbs with all clinical strains tested suggests a target epitope that is ubiquitous among *M. tuberculosis* isolates.

Although the cell lines producing the 2 mAbs differed in the amount of antibody produced (as manifested by significantly different mAb concentrations in equal amounts of culture supernatants and ascites), the similarities between the mAbs in binding characteristic and isotype, led us to hypothesize that they may be structurally very similar or perhaps identical. Inhibition ELISA further indicated that the 2 mAbs have similar specificities. The 100% homology of the variable light (V_L) and heavy chains (V_H) sequences, suggest that these mAbs originated from the same ancestral B cell that underwent clonal expansion. Thus, the experimental results obtained with mAbs 16a1 and 16a6 provide a valuable internal confirmation for our findings.

Immunoprecipitation of the cytosol fraction with mAb 16a1 identified a protein with peptides mass and sequences that matched MPT 51. MPT 51 is a 27–30 kDa protein, the exact function of which is unknown. It has about 40% sequence homology with members of the antigen 85 complex (A, B, and C) of *M. tuberculosis*. These are secreted proteins with molecular weight of 27–31 kDa^{16,28,29} that catalyze mycolate transfer resulting in the formation of α , α' -trehalose monomycolate (TMM) and α , α' -trehalose dimycolate (TDM), the later also known as cord factor.³⁰ Ag 85C has an additional role in the covalent attachment of mycolic acids to arabinogalactan.³¹ Several conserved residues of these proteins represent potential sites for interaction with human fibronectin,^{32,33} and they are also known as fibronectin-binding proteins (Fbp) A, B and C. The degree of sequence homology between MPT 51 and the members of the Ag 85 complex and the previous demonstration that it can bind fibronectin,³⁴ led to speculations that this protein might constitute a forth member of this complex. However, MPT51 does not have structural elements that are required for mycolyltransferase activity,¹⁶ suggesting that it may have function/s that is/are different than that/those of the members of Antigen 85 complex.

MPT 51 was identified as a B-cell antigen with specific antibodies detected in patients with TB, especially HIV-infected.^{34,35} Detection of antibodies prior to the development of clinical TB,³⁶ suggested that this antigen is expressed during active replication of *M. tuberculosis*. Protective cell-mediated immunity induced in mice with recombinant attenuated strains of *Listeria monocytogenes* carrying an expression plasmid for MPT51, suggest a role for MPT 51 as a T-cell antigen as well.

Previous studies demonstrated the presence of MPT51 in culture filtrate, but not in the cell wall or cytosol.³⁶ Our results indicate for the first time the presence of this protein in subcellular fractions including cytosol and cell surface. The identification of 6 peptides with amino acid sequences that are unique to MPT51 and do not match with any of the Ag 85 complex members, provide a strong evidence for the existence of this protein in the cytosol. The migration of this protein in our SDS-page gels as a 90 kD protein suggests that

it may be present in a trimeric form in these subcellular fractions.

The localization of proteins is of paramount importance to the definition of their function.³⁷ Furthermore, the localization of MPT 51 to cell wall and cytosol, suggests that it may play more than one role.³⁰

Antibodies can cause agglutination of the particles bearing their target antigen.³⁸ Given the natural tendency of *M. tuberculosis* to clump it may be difficult to study antibody-mediated agglutination. Although sonication is often used to break clumps, our experience demonstrated that clumps could be present in significant amounts following the procedure. $\Delta RD1\Delta panCD$ is an attenuated double mutant strain of *M. tuberculosis* Erdman¹³ which has been approved for use under Biosafety Level 2 (BSL2) conditions at the Albert Einstein College of Medicine and was suitable for DIC microscopy. Clumping of mycobacteria resulting from the addition of mAbs 16a1 and 16a6 at a concentration of 100 μ g/ml, but not at 10 μ g/ml, confirm the presence of these mAbs epitope on the surface of *M. tuberculosis* and provide useful information regarding the antibody concentration that can be used without causing agglutination. Agglutination is a potential mechanism of antibody function since clumping helps localize the microbe to the site of infection and agglutinated mycobacteria could potentially be handled differently by the immune system as compared to single bacteria. Additional studies are required to determine the role of antibody-mediated agglutination on the course of mycobacterial infections.

In conclusion, the use of a *M. tuberculosis* strain that sheds easily capsular epitopes resulted in the generation of MPT51-binding mAbs, which enabled the identification and extraction of the molecule from subcellular fraction. We also developed a method to evaluate for antibody-mediated agglutination and demonstrated a dose-dependent agglutination of mycobacteria by mAbs 16a1 and 16a6. The clumping provides further confirmation to the existence of their epitope on the mycobacterial surface. Further studies are needed to establish the function of MPT51, to define its exact role in the pathogenesis of Tb and the potential of MPT51-binding antibodies to modify the course of *M. tuberculosis* infection.

Acknowledgments

A.G-F., A.C. and W.R.J. Jr. are Center for AIDS Research investigators at the Albert Einstein College of Medicine. Special gratitude to Louis Weiss for critically reviewing the manuscript and helpful suggestions, John Belisle for providing research materials as part of contract N01 AI-75320 (Tuberculosis Materials and Vaccine Testing), Samir Hasan for technical support and advise, the Clinical Microbiology Laboratory at the Montefiore Medical Center, the Analytical Imaging facility, Fang Wang and Eddie Nieves from the laboratory of Macromolecular analysis and Proteomics at the Albert Einstein College of Medicine.

Funding: This work was supported by a grant from the center for AIDS research at the Albert Einstein College of Medicine to A.G-F and in part by NIH grants AI001691 and AI053192 to A.G-F.

Competing Interests: None declared

Ethical Approval: Not required

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