

Demonstration of Components of Antigen 85 Complex in Cerebrospinal Fluid of Tuberculous Meningitis Patients

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Tuberculous meningitis (TBM) is the most common form of chronic infection of the central nervous system. Despite the magnitude of the problem, the general diagnostic outlook is discouraging. Specifically, there is no generally accepted early confirmative diagnosis protocol available for TBM. Various *Mycobacterium tuberculosis* antigens are now recognized as potential markers for diagnosis of TBM. However, their presence remains questionable, and many of these antigens are reported in the blood but not in the cerebrospinal fluid (CSF). This study identifies a specific protein marker in CSF which will be useful in early diagnosis of TBM. We have demonstrated the presence of a 30-kDa protein band in CSF of 100% ($n = 5$) of confirmed and 90% ($n = 138$) of suspected TBM patients out of 153 TBM patients. The 30-kDa band was excised from the gel, destained extensively, and digested with trypsin. The resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Partially purified proteins from CSF samples of TBM were analyzed by two-dimensional polyacrylamide gel electrophoresis and Western blotting. Immunoblotting and enzyme-linked immunosorbent assay (ELISA) were performed to confirm the presence of proteins in the 30-kDa protein band. The antigen 85 (Ag 85) complex was detected in CSF of TBM patients by indirect ELISA using antibodies against Ag 85 complex. The results of this study showed the 30-kDa protein band contained MTB proteins Rv3804c (Ag85A) and Rv1886c (Ag 85B), both members of the Ag85 complex. This was also confirmed by using immunotechniques such as indirect ELISA and the dot immunobinding assay. Detection of Ag85 complex was observed in CSF of 89% (71 out of 80) of suspected TBM patients that were 30-kDa protein positive. The observed 30-kDa protein in the CSF is comprised of the MTB Ag85 complex. This protein was earlier reported to be present in the blood of patients with extra-central nervous system tuberculosis. Therefore, this finding suggests that this protein can be used as a molecular marker for any type of tuberculous infection. It also provides a more sensitive immunoassay option for the early and confirmatory diagnosis of TBM.

Tuberculosis is the leading cause of death among communicable diseases, killing nearly 2 million people each year (2, 13). Among tubercular infections, tuberculous meningitis (TBM) leads to multiple central nervous system (CNS) complications and remains a major health problem in underdeveloped and developing countries (1, 9, 16). Even in developed countries where a decade ago it was rare, it has reappeared following human immunodeficiency virus infection (6, 14, 29). Early and rapid confirmatory diagnosis is still difficult due to the clinical picture of TBM and similarities of TBM cerebrospinal fluid (CSF) to that of partially treated pyogenic meningitis, which results in frequent diagnostic confusion (4, 21, 28, 33). Early and reliable diagnosis of TBM still poses a great challenge. In most of the cases, diagnosis relies on clinical observations, imaging of the infected area, and detection of *Mycobacterium tuberculosis* in CSF by acid-fast bacillus (AFB) staining and culturing. A number of immunological and molecular methods have been reported, but these cannot be em-

ployed in standard pathology laboratories and are often not useful for early confirmatory diagnosis of TBM (3, 7, 11, 15, 22, 23, 25, 26, 31, 35).

To overcome the deficiencies in TBM diagnosis, we set forth to identify specific protein markers unique to CSF samples collected from suspected patients with TBM. By analyzing the total sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile of such CSF, we observed a 30-kDa protein band specific to these patients (18). Further, all 30-kDa protein-positive CSF samples tested with polyvalent antibodies against culture filtrate proteins (CFP) of *M. tuberculosis* strain H₃₇Rv by the single radial immunodiffusion (SRID) method indicated that the CSF of TBM patients possesses *M. tuberculosis* antigens (17).

These early investigations led us to hypothesize that molecular identification of the TBM-specific 30-kDa CSF protein would allow for development of specific reagents and protocols for the rapid and accurate diagnosis of TBM. In this report, the application of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) identified two mycobacterial proteins of the antigen 85 (Ag85) complex (Rv3804c and Rv1886c) and one host-derived protein (immunoglobulin [Ig] kappa light

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chain VLJ region; accession no. BAC01690.1) as the components of the TBM-specific 30-kDa band. Simultaneously, antibodies generated against the 30-kDa protein were found to react with purified Ag85 complex. These antibodies as well as those previously generated against the Ag85 complex were found to react with the majority of CSF samples of TBM patients.

MATERIALS AND METHODS

Patients and samples. A total of 240 patients, including 80 TBM patients (clinically suspected = 75, confirmed cases = 5) admitted to the Neurology Department, Central India Institute of Medical Sciences (CIIMS), Nagpur, between June 2000 and January 2004, on whom diagnostic lumbar puncture was performed, were selected for Ag85 complex detection in CSF. The ages of the patients ranged between 5 and 65 years, and there were 44 males and 36 females. CSF examination included total and differential cell count, biochemistry, and microscopic evaluation after Gram, India ink, and AFB staining. Patients were divided into the following groups.

(i) **Tuberculous meningitis.** Diagnosis of TBM was based on the clinical features, which included subacute or chronic fever and signs of meningeal irritation with or without other features of CNS abnormality. CSF findings in these patients could be described as increased protein levels, decreased glucose (CSF/blood glucose ratio, ≤ 0.5), and pleocytosis with lymphocyte predominance. Patients in whom AFB was demonstrated by smear and/or cultures were considered as "confirmed" cases of TBM. In the remaining cases, response to antituberculous drugs was considered as a criterion for the diagnosis and these patients were considered as "suspected/probable" cases of TBM.

(ii) **Nontuberculous infectious meningitis.** This group included the patients having pyogenic, viral, or fungal meningitis. Pyogenic meningitis was suspected in patients with acute high-grade fever with features of meningitis and often with associated altered sensorium, as well as CSF findings of increased proteins, very low sugar (CSF/blood glucose ratio, < 0.2), and pleocytosis with polymorphonuclear predominance. However, CSF findings in partially treated cases of pyogenic meningitis can be very similar to TBM. Response to appropriate antibiotics was also considered as one of the diagnostic criteria. Viral meningitis was suspected in patients who presented with acute onset of fever and symptoms and signs of meningeal irritation. In such patients, CSF showed mild increase in protein, glucose was often normal (CSF/blood glucose ratio, ≥ 0.5), and pleocytosis was predominantly lymphocytic. None of them had clinical evidence of extra-CNS tuberculosis. Those with clinical suspicion of herpes encephalitis were treated with acyclovir, and other viral meningitis was treated symptomatically. Fungal meningitis demonstrates the same CSF profile as TBM; however, the India ink stain shows the presence of etiological agents such as *Cryptococcus*. This was further confirmed by culturing on selective media.

(iii) **Noninfectious neurological disorders.** All other patients who had no clinical features of meningitis and had no evidence of CNS or extra-CNS tuberculosis were grouped into the noninfectious/control group.

Characterization of the 30-kDa protein. (i) **CSF protein profile.** SDS-PAGE was initially used to show the presence of the 30-kDa protein associated with TBM patients as reported earlier (18). All CSF samples showing the presence of 30-kDa protein were further analyzed by SRID (17). In this method, the polyvalent antibodies of CFP were allowed to react in an agarose matrix with CSF samples and interaction was demonstrated using Coomassie brilliant blue R-250 as reported earlier (17).

Electroelution. Following separation of protein from the CSF of patients with TBM by SDS-PAGE, the 30-kDa protein bands were sliced from the gels and pre-equilibrated in the elution buffer (0.15 M phosphate-buffered saline [PBS], pH 7.4). The gel slices were electroeluted in a whole-gel elution system (BIOTECH, INDIA) for 90 min at 30 V (20), harvested from the unit, and dialyzed against PBS, and the protein content was measured by Bio Lab kit. The purity of protein was checked by running native PAGE.

Production of 30-kDa polyclonal antibody. Female rabbits were injected subcutaneously at multiple sites along the back with eluted 30-kDa protein antigen (200 $\mu\text{g}/\text{ml}$ in PBS) from TBM patients' CSF emulsified in an equal volume of complete Freund's adjuvant and boosted at 4-week intervals with the same dose. Blood was collected from the marginal ear vein 2 weeks after each booster dose. Serum was obtained by allowing the blood to stand for 3 to 5 h at room temperature followed by overnight cold storage at 4°C to form the clot. The clot was gently removed with the aid of a wooden stick, and the serum was centri-

fuged for 10 min at 250 rpm at 4°C. The supernatant was removed and stored at -20°C in 1-ml aliquots.

One- and two-dimensional polyacrylamide gel electrophoresis. Partially purified proteins from six TBM CSF samples were precipitated with ammonium sulfate (40%), and the precipitate was washed with saturated ammonium sulfate. The pellets were hydrated with water and dialyzed against 10 mM ammonium bicarbonate. The protein concentration of each sample was measured by bicinchoninic acid assay (Pierce Chemical Co.). An aliquot (10 μg) of each sample was resolved by SDS-PAGE using a 15% Tris-glycine gel, transferred to nitrocellulose, and probed with the monoclonal antibody against the Ag85 complex (CS-90). Color development was achieved using anti-mouse antibody conjugated to alkaline phosphatase followed by development with 4-bromo, 3-chloro, 2-indoyl, 1-phosphate and nitroblue tetrazolium.

Two samples containing sufficient protein were additionally analyzed by 2D-PAGE. Samples were applied to isoelectric focusing strips with linear pH 4 to 7 gradients and focusing conducted using a ZOOM IPGRunner (Invitrogen, Carlsbad, CA). Strips were resolved by SDS-PAGE using 4 to 12% Bis-Tris gels (Invitrogen) followed by either staining with Coomassie or transfer to nitrocellulose. Proteins of the Ag85 complex were identified by Western blot analysis.

Liquid chromatography-tandem mass spectrometry. To partially purify the 30-kDa protein, the CSF was resolved by SDS-PAGE and the 30-kDa protein was electroeluted as reported earlier (20). This partially purified material was again resolved by SDS-PAGE, the gel was stained with Coomassie blue, and the 30-kDa band was excised from the gel. The protein was destained, digested in the gel with trypsin, and the peptides were extracted by standard methods (10). Recovered peptides were hydrated with 5% acetonitrile in 0.1% glacial acetic acid and applied to a 0.2- by 50-mm C₁₈ reversed-phase capillary high-performance liquid chromatography column (Michrom Bio Resources, Inc., Auburn, CA). The peptides were eluted with an increasing gradient of acetonitrile at a flow rate of 5 μl per min using an Eldex MicroPro capillary solvent delivery system (Napa, CA). The effluent was introduced directly into a Thermo-Finnigan LCQ electro spray ion trap mass spectrometer (San Jose, CA) where data-dependent MS/MS was employed. Specifically, the parent ion yielding the greatest relative abundance from the full MS scan was selected for MS/MS fragmentation a maximum of two times before being placed on the dynamic exclusion list for 1 min. Ion fragmentation was achieved using 40% normalized collision energy. MS/MS data were interrogated against the *M. tuberculosis* database (12) and nonredundant database housed at the National Center for Bioinformatics using the BioWorks 3.1 software suite (ThermoFinnigan) and SEQUEST (8).

M. tuberculosis antigens. Ag85 complex and its three components, Ag85A, Ag85B, and Ag85C from *M. tuberculosis* strain H₃₇Rv, were obtained from mid-log-phase cultures as previously described (5). Briefly, sterile culture filtrate proteins were precipitated with 40% saturated ammonium sulfate. Proteins were equilibrated into phosphate buffer (pH 6.8) and loaded onto a phenyl Sepharose column. Proteins were eluted via a pH gradient (pH 6.8 to 8.0) followed by an increasing concentration of ethylene glycol. Ag85 complex and individual components A, B, and C were cleaned up using either a second phenyl Sepharose column or size exclusion chromatography with a G-75 Sephadex column or both.

Antibodies. The monoclonal antibody to the purified Ag85 complex (CS-90) and polyclonal sera to Ag85 complex (04.Ag85.1.11.32.rp) and culture filtrate proteins of *M. tuberculosis* strain H₃₇Rv (C193) were obtained from Colorado State University through the TB Research Materials and Vaccine Testing Contract (NO1-AI-75320).

Western blotting. The CSF of 30-kDa protein-positive CSF patients was subjected to SDS-PAGE and electroblotted to nitrocellulose membrane as per standard protocols (22). The membrane was probed with a 1:1,000 dilution of polyvalent antibodies (04.Ag85.1.11.32.rp) to Ag85 complex for 2 h at room temperature, followed by the secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP). Antibody reactivity was visualized by detection of HRP activity with tetramethylbenzidine-hydrogen peroxide (TMB/H₂O₂) as substrate. The Ag85 complex was used as a positive control in the Western blot analysis. A total of 31 CSF samples, including those from 25 TBM patients (clinically suspected = 20, confirmed cases = 5), were randomly selected for the Ag 85 complex detection.

Demonstration of Ag85 complex components using 30-kDa protein antibodies. Wells of flat-bottom microtiter plates were coated with 100 μl (10 $\mu\text{g}/\text{ml}$) of Ag85 components (Ag85A, Ag85B, Ag85C) in a separate wells and incubated overnight at 4°C. The wells were then washed with PBS (pH 7.4) and blocked by the addition of 100 μl of 2.5% bovine serum albumin (BSA) in PBS at 37°C for 60 min. After removal of blocking solution, antibodies generated against 30-kDa protein were added to all the wells (1:5,000) and incubated at 37°C for 60 min. The wells were again washed with the PBS, 100 μl of affinity purified HRP-conjugated anti-rabbit IgG (Bangalore Genei, India) (1:10,000) was added to the

wells, and this mixture was incubated at 37°C for 60 min. The wells were washed extensively with PBS; TMB/H₂O₂ substrate solution (100 µl) was added into the wells and incubated at room temperature for about 20 min for development of the color. The reaction was stopped with addition of 100 µl of 2.5 N H₂SO₄. The absorbance of each well was read at 450 nm (20).

Demonstration of the 30-kDa protein using polyclonal antibody (04.Ag85.1.11.32.rp) to Ag85 complex. The same procedure was used for 30-kDa protein demonstration with the Ag 85 complex antibodies as described above, with little modification. Briefly wells of microtiter plates were coated with 100 µl (5 µg/ml) of eluted 30-kDa protein antigen and incubated overnight at 4°C. The antigen was then blocked by addition of 100 µl of 2.5% BSA in PBS and incubated at 37°C for 60 min. After washing with PBS, polyclonal antibody (04.Ag85.1.11.32.rp) to Ag85 complex was added and incubated at 37°C for 60 min. The wells were again washed with the PBS, and 100 µl of affinity purified HRP-conjugated anti-rabbit IgG (Bangalore Genei, India) (1:10,000) was added to the wells and incubated at 37°C for 60 min. The wells were washed extensively with PBS. TMB/H₂O₂ substrate solution (100 µl) was added to the wells and incubated at room temperature for about 20 min for development of the color. The reaction was stopped with addition of 100 µl of 2.5 N H₂SO₄. The absorbance of each well was read at 450 nm (20).

Dot immunobinding assay. A total of 240 CSF samples, including 80 from TBM patients (clinically suspected = 75, confirmed cases = 5), were selected for detection of Ag 85 complex. Strips of nitrocellulose were cut and placed on glass slides. Three circles were marked on the each strip and numbered 1, 2, and 3, respectively. Two microliters of Ag85 complex (10 µg/ml) (positive control), 2 µl of CSF (10 mg/ml) from patients with noninfectious neurological disorders (negative control), and 2 µl of CSF from TBM patients were deposited into circles 1, 2, and 3, respectively. The nitrocellulose strips were blotted dry and blocked with 2.5% BSA in PBS for 60 min at 37°C. Polyclonal antibody (04.Ag85.1.11.32.rp) to Ag85 was added at a concentration of 1:2,000, and the nitrocellulose strips were processed as described earlier (19).

Indirect ELISA. CSF samples (100 µl of a 1:10 dilution) from TBM patients and non-TBM patients were added to the microtiter wells and blocked with 2.5% BSA in PBS. After washing with PBS, polyvalent antibody (04.Ag85.1.11.32.rp) against Ag85 complex was added and plates were incubated at 37°C for 60 min. The wells were washed, followed by addition of the secondary antibody (goat anti-rabbit IgG-HRP) and incubation for 60 min at 37°C. The antibody reactivity was detected by addition of the TMB/H₂O₂ substrate as described for antibody detection. A total of 240 CSF samples, including 80 from TBM patients, were selected for this study.

Detection of antibodies to Ag85A, Ag85B, and Ag85C in CSF samples of TBM patients. Indirect ELISA as described earlier by Kashyap et al. (20) was employed with minor modification. The flat-bottom microtiter wells were coated with 100 µl (10 µg/ml) of Ag85 components (Ag85A, Ag85B, Ag85C) in separate wells and incubated overnight at 4°C. The wells were then washed with PBS solution, pH 7.4, and then nonspecific sites of coated antigen were blocked by the addition of 100 µl of 0.5% BSA-PBS at 37°C for 60 min. One hundred microliters of CSF from TBM patients was diluted in PBS (1:10), added to the wells, and incubated at 37°C for 60 min. The wells were again washed with the PBS. One hundred microliters of affinity-purified HRP-conjugated anti-human IgG (Bangalore Genei, India) diluted 1:10,000 in PBS was added to the wells and incubated at 37°C for 60 min. After another washing with PBS, 100 µl of TMB/H₂O₂ substrate solution was added to the wells and incubated at room temperature for about 20 min. The reaction was then stopped with 100 µl of 2.5 N H₂SO₄. The absorbance of each well was read at 450 nm (20).

RESULTS

The CSF samples of patients admitted to the Neurology Department of CIIMS hospital were grouped into three different categories on the basis of clinical observations and biochemical and pathological analyses of CSF samples. These CSF samples were then subjected to SDS-PAGE analysis to observe total protein profile and to demonstrate and assign the specificity of the band(s) to TBM.

In our earlier publications using SDS-PAGE, we demonstrated the presence of a 30-kDa protein band in the CSF of 92% ($n = 37$) of suspected TBM patients out of 40 cases and when the sample set of non-TBM patients ($n = 105$) was analyzed, it only gave 6% ($n = 6$) of nonspecificity in terms of

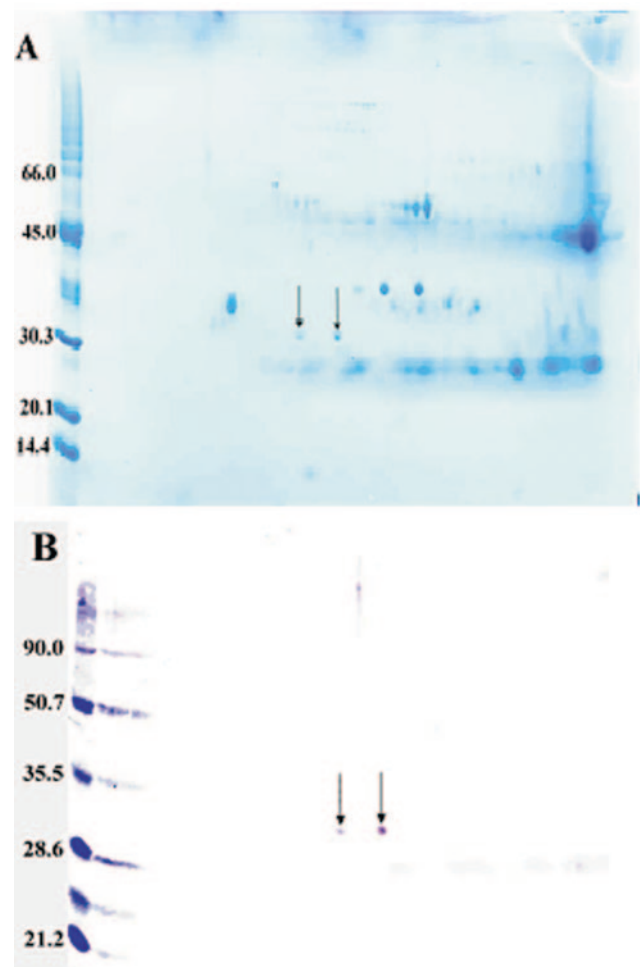


FIG. 1. Characterization of 30-kDa protein by 2D-PAGE. (A) Coomassie-stained 2D-PAGE profile of proteins resolved from partial purification of the 30-kDa CSF protein. (B) Western blot analysis of partially purified proteins demonstrating reactivity to the monoclonal antibody for Ag85, CS-90. Arrows indicate proteins from the CSF reactive to CS-90 (B) and stained with Coomassie blue (A).

presence of the 30-kDa protein (18). In the last 6 years, we have analyzed CSF samples of 153 TBM patients (showing 90% [$n = 138$] positivity for 30-kDa protein) and 531 non-TBM patients (showing only 2.3% [$n = 12$] false-positive results for 30-kDa protein antigen) (data not shown). We have also demonstrated the presence of *M. tuberculosis* antigens in the CSF of 30-kDa protein-positive TBM patients using polyvalent antibodies to CFP. This has been demonstrated using the SRID assay, where 94% ($n = 17$) of TBM patients out of 18 patients gave positive results (17).

Since these earlier reports strongly suggested the 30-kDa protein as a marker in the diagnosis of TBM, a full characterization of the 30-kDa protein was initiated. The 30-kDa protein band, along with other CSF proteins, was harvested from the CSF as described above, and the protein extract was analyzed by 2D-PAGE (Fig. 1A). Probing of the 2D-PAGE protein profile with the monoclonal antibody for Ag85 (CS-90)

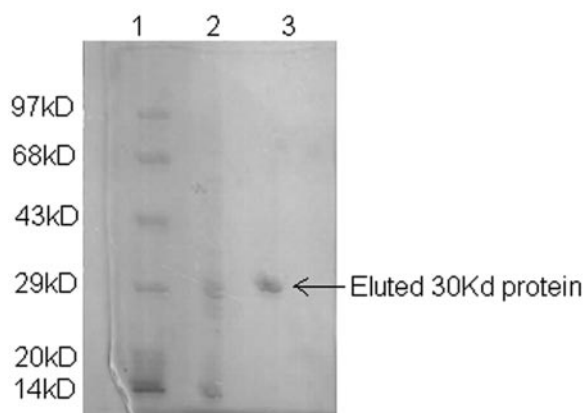


FIG. 2. SDS-PAGE with protein staining to illustrate the purification of 30-kDa protein antigen from CSF of confirmed TBM patients. Lane 1, molecular mass marker; lanes 2 and 3, fraction (30-kDa protein) obtained after the purification step.

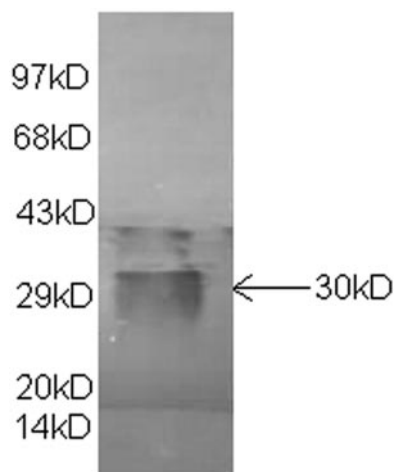


FIG. 3. Western blot analysis of CSF of confirmed case of TBM patient. Reactivity of 30-kDa protein antigen with the polyvalent antibodies of Ag85 complex indicated by arrowheads along with medium-range molecular mass marker.

demonstrated reactivity against two protein spots in the size and pI range of the Ag85 proteins (Fig. 1B).

A more highly purified 30-kDa protein band of the CSF of TBM patients was recovered by electroelution (Fig. 2). Analysis by LC-MS/MS of peptides derived through a trypsin digestion of the 30-kDa band revealed the presence of Ag85A (Rv3804c) and Ag85B (Rv1886c) (Table 1). A single peptide derived from the human immunoglobulin kappa light chain VLJ region (accession no. BAC01690.1) was also identified.

The polyvalent antibody against Ag85 complex of MTB was tested against 30-kDa protein-positive TBM CSF samples by Western blot analysis (Fig. 3). This finding was also confirmed by dot immunobinding assay and reverse dot immunobinding assay. Further, the reactivity of antisera generated against the 30-kDa CSF protein was demonstrated by indirect ELISA for the individual components of the Ag85 complex, Ag85A, -B, and -C, purified from *M. tuberculosis* (Fig. 4, top), while no reactivity was observed with LAM or native GroES antigen (not shown). The bottom panel of Fig. 4 demonstrates the

reactivity of eluted 30-kDa protein (isolated from CSF of TBM patients) with polyclonal antibody (04.Ag85.1.11.32.rp) to Ag85 by indirect ELISA. The highest activity was observed in a 1:40 dilution.

The detection of Ag85 complex in the CSF of TBM patients ($n = 80$) containing the 30-kDa protein versus non-TBM patients ($n = 160$) was confirmed by dot immunobinding assay and indirect ELISA using anti-Ag85 complex polyclonal sera. Seventy-four (93%) of the TBM patients tested positive for the Ag85 complex versus 4 (2.5%) non-TBM patients. Figure 5 shows the diagnostic tree for Ag 85 complex detection generated from STARD (Standards for Reporting of Diagnostic Accuracy [<http://www.consort-statement.org/stardstatement.htm>]) analysis (Fig. 5). The specific detection of antibodies against Ag85A, Ag85B, or Ag85C in the CSF samples of 10 of these positive TBM patients was determined by indirect ELISA. A high concentration of antibodies against Ag85A was

TABLE 1. MS identification of the 30-kDa CSF band in TBM patients

Protein	Peptide sequence	Amino acid position ^a	Mass ^b	% Total ^c
Rv1886c	PGLPVEYLQVPSPSMGR	4-20	1,828.1	5.29
	VQFQSGGNNSPAVYLLDGLR	24-43	2,136.4	6.18
	WETFLTSELPOWLSANR	97-113	2,079.3	6.01
	FQDAYNAAGGHNAVFNFPNGTHSWEYWGAQLNAMK	240-275	4,013.3	11.61
	FQDAYNAAGGHNAVFNFPNGTHSWEYWGAQLNAMKGDLOSSLGAG	240-285	4,899.2	14.17
% Coverage				31.65
Rv3804c	PGLPVEYLQVPSPSMGR	4-20	1,828.1	5.03
	VQFQSGGANSPALYLLDGLR	24-43	2,107.4	5.92
% Coverage				10.95

^a Amino acid position of the peptide in the mature protein.
^b Monoisotopic mass calculated for each peptide.
^c Percentage the peptide contributes to the total mass of the mature protein.

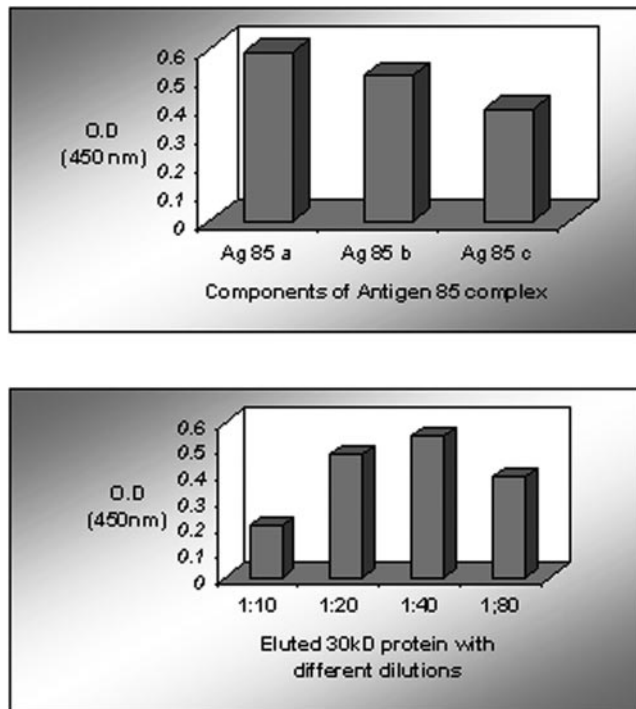


FIG. 4. (Top) Reactivity of 30-kDa protein antibodies generated using the 30-kDa protein eluted from CSF of TBM patients with components of antigen 85 complex (Ag85A, Ag85B, Ag85C). All the components reacted with 30-kDa antibodies (1:100). (Bottom) Reactivity of eluted 30-kDa protein (isolated from CSF of TBM patients) with polyclonal antibody (04.Ag85.1.11.32.rp) to Ag85 by indirect ELISA. Highest activity was observed in a 1:40 dilution.

observed in all the samples (Fig. 6). Non-TBM patients were not specifically reactive toward these individual antigens.

DISCUSSION

TBM remains one of the most serious chronic medical conditions resulting from infection with *M. tuberculosis*, if not treated promptly. Clinical observation coupled with CSF analysis of TBM patients can be similar to other infectious sequelae, such as partially treated pyogenic meningitis, fungal meningitis, and viral meningitis (21). Further, diagnostic confirmation of TBM via observation of tubercle bacilli in the CSF by AFB staining is rare and does not exceed 50% even when adequate CSF samples are obtained (32).

In an attempt to develop a rapid test for the diagnosis of TBM, we analyzed CSF samples from confirmed and suspected TBM patients along with control CSF samples from patients with non-TBM and noninfectious CNS disorders. The SDS-PAGE protein profile of TBM patient CSF samples demonstrated the presence of the 30-kDa protein band (100% of confirmed and 92% of suspected patients). Analysis of 30-kDa protein-positive CSF samples by SRID analysis with polyclonal antisera generated against Ag85 complex demonstrated reactivity against the 30-kDa protein, indicating that this protein may be comprised of the Ag85 complex from *M. tuberculosis*.

Further, visualization of this protein by SDS-PAGE analysis of CSF in TBM patients treated with anti-TB medication (such as isoniazid, pyrazinamide, rifampin, and ethambutol) is reduced (18). This strengthens our argument that the 30-kDa protein band observed in the CSF of TBM patients not only is an important marker for early diagnosis of TBM but also can be useful in assessing the response to therapy.

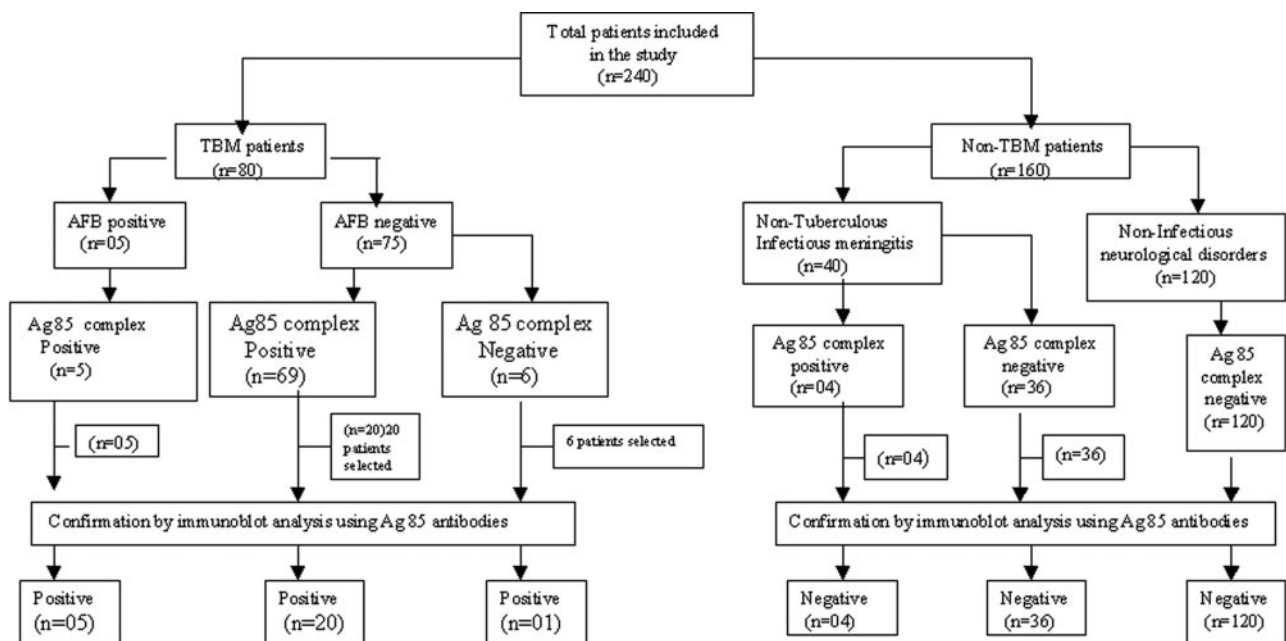


FIG. 5. Diagnostic classification tree showing Ag85 complex detection in CSF of TBM and non-TBM patients.

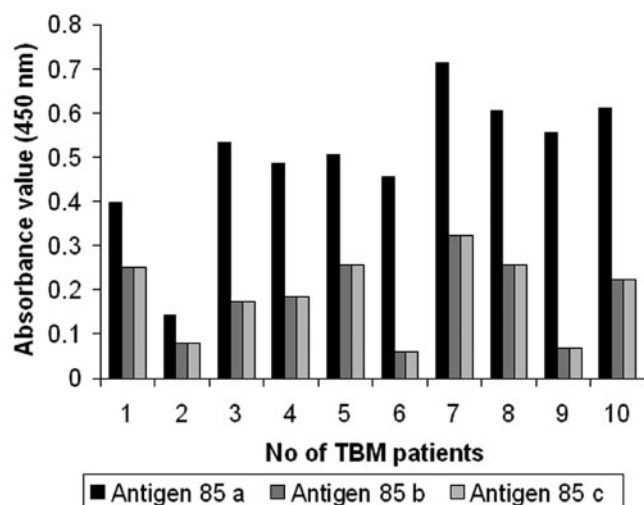


FIG. 6. Demonstration of antibodies (IgG) to Ag85A, Ag85B, and Ag85C in 10 confirmed CSF samples from TBM patients using their respective antigens by ELISA.

We have shown the presence of the 30-kDa protein band in the CSF of TBM patients (18); a similar report by Katti also demonstrated 30- to 32-kDa antigen in CSF of TBM patients with 94% sensitivity and 99% specificity (22). At the time, it was hypothesized that this protein represented an *M. tuberculosis* cell wall antigen, though its discrete identity was not confirmed.

In this report, the 30-kDa protein band from the CSF of patients with confirmed and suspected cases of TBM was demonstrated to react with antibodies directed against Ag85 complex. Additionally, antibodies produced against the 30-kDa CSF band reacted with the Ag85 complex, and the individual components of the Ag85 complex, Ag85A, -B, and -C. Thus the 30-kDa band was partially purified from the CSF and the excised band was digested with trypsin and analyzed by LC-MS/MS and 2D-PAGE. These studies cumulatively identified two mycobacterial antigens, Rv1886c and Rv3804c (Ag85B and -A, respectively) in the CSF of TBM patients.

The 30- to 31-kDa Ag85 complex has been the focus of intensive research for several years and comprises three related proteins, Ag85A (31 kDa), Ag85B (30 kDa), and Ag85C (31.5 kDa). Interestingly, these have been found in the blood of non-CNS tuberculous patients (24, 27, 30, 35). More recently, these antigens have been demonstrated in the sputum of pulmonary tuberculosis patients (34). We have also observed these antigens in the serum of patients with sputum AFB-positive pulmonary tuberculosis (our unpublished observations).

After confirming the presence of proteins from the Ag85 complex in the CSF, 80 CSF samples from 30-kDa protein-positive TBM patients and 160 CSF samples from 30-kDa protein-negative non-TBM cases were selected for detection of the Ag85 complex. The Ag85 complex was detected in 93% of the CSF samples of TBM patients. This was also confirmed by the immunoblot analysis.

The literature suggests that this is the first report of demonstration of the Ag85 complex in the CSF of TBM patients.

This coincides with other studies demonstrating the presence of the Ag85 complex in the serum of patients with pulmonary and other non-CNS tuberculosis. Ag85 complex expression in the CSF of TBM patients might provide prospective insights in the area of infection by *M. tuberculosis*.

In conclusion, the 30-kDa protein band noted in the CSF of TBM patients is comprised of mycobacterial products, including components of the Ag85 complex, and can be used to develop an immunodiagnostic assay with increased sensitivity and specificity. Such a test would be rapid, sensitive, and cost-effective and could be easily performed in any standard pathology laboratory.

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Central India Institute of Medical Sciences has filed a complete specification of the patent for the development of diagnostic kit for diagnosis of tuberculous meningitis in CSF (Vide No-91/mum/2004) at Mumbai, India.

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