

Full Length Research Paper

Potential of *Mycobacterium tuberculosis* Cord Factor Antigen for Laboratory Diagnosis of Tuberculous Meningitis

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In this retrospective study, an indirect enzyme- linked - immunosorbent assay (ELISA) was standardized to measure the IgG antibody titers in cerebrospinal fluid (CSF) specimens against cord factor antigen of *Mycobacterium tuberculosis* and evaluated its application for the laboratory diagnosis of tuberculous meningitis (TBM). ELISA with cord factor antigen was specific and did not yield false positive result in any one of the 80 CSF specimens of patients with non-tuberculous neurological diseases. The assay showed an overall sensitivity of 86.66 % in 50 patients with a clinical diagnosis of TBM. Based on the results of this study, it is being recommended that cord factor antigen of *M tuberculosis* has potential application for the diagnosis particularly in patients in whom the bacteriological methods in CSFs did not confirm the diagnosis of TBM.

Key words: Tuberculous meningitis (TBM); *Mycobacterium tuberculosis*; Cord factor-Trehalose 6- 6' dimycolate (TDM); Cerebrospinal fluid (CSF); Enzyme-Linked- Immunosorbent Assay (ELISA).

INTRODUCTION

Tuberculous meningitis (TBM) is one of the common clinical and neuropathological manifestations of extra-pulmonary tuberculosis. The incidence and prevalence of TBM have shown an upward trend especially after the outbreak of HIV infection (Chandramuki et.al 2002). A confirmatory ('gold standard') diagnosis of TBM depends upon the demonstration of the causative agent of the disease *Mycobacterium tuberculosis* in cerebrospinal fluid (CSF) specimens by the bacteriological methods such as Ziehl - Neelsen staining or cultures. The conventional culture for *M tuberculosis* by Lowenstein-Jenson medium as well as by radiometric short- term culture methods lack sensitivity and often yield false

negative results in CSF specimens of patients with TBM (Caws et.al. 2000). In an earlier publication, we reported that the lumbar CSF specimen contain fewer *M tuberculosis* bacilli than does cisternal or ventricular CSFs in patients with TBM (Radhakrishnan et.al. 1991). However for the routine bacteriological investigations, cisternal or ventricular CSF specimens in patients with TBM cannot be obtained since these procedures at bedside, can lead to fatal consequences. Because of the catastrophic nature of the disease and because effective as well as specific chemotherapy is available for this potentially curable infectious disease, clinicians cannot wait for a confirmative bacteriological diagnosis and antituberculosis chemotherapy (ATT) are often administered to these patients on an empirical basis because a delay in diagnosis and treatment would invariably culminate into irreversible neurological sequelae (Chandramuki et.al. 2002).

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During the past two decades, several indirect methods have been employed as an adjunct for the laboratory diagnosis of TBM. These include immunological (Chandramuki et.al 2002; Thomas et.al 2008; Murakami et.al 2008; Kim et.al 2010) and molecular biological (Sumi et.al 2002; Baker et.al 2002; Quan et.al 2006) methods. All these studies are aimed at the analysis of CSF and measure either the host immune response to infection or estimate the DNA of *M tuberculosis* bacilli in the CSF specimens from patients with TBM.

Cord factor (Trehalose 6-6' dimycolate-TDM) is one of the major lipid antigens of *M tuberculosis* and it plays an important role in the immunopathogenesis of tuberculosis. Cord factor is known to induce macrophage proliferation as well as promotes the granuloma formation in tuberculous lesions (Yamagami et.al 2001 Hunter et.al 2006). Besides these, several earlier published studies estimated serum antibody concentration to cord factor antigen and emphasized its diagnostic significance in sputum smear- negative and culture - negative patients with pulmonary tuberculosis (Maekura et.al 1993; Laszlo et.al 1992; Julian et.al 2001). However the estimation of antibody to cord factor in CSF specimens and its role for the laboratory diagnosis of TBM have not been previously reported.

In the present study an indirect ELISA was standardized to measure the IgG antibody concentration to cord factor antigen of *M tuberculosis* in CSF specimens for the laboratory diagnosis of TBM. The sensitivity of the assay was evaluated in CSF specimens from culture proven patients with TBM and specificity was critically assessed in CSF specimens from patients with non-tuberculous neurological diseases. Technical protocols used for the isolation of cord factor antigen from the cultures of *M tuberculosis* in the study as well as potential application of this immunoassay for the diagnosis of TBM are highlighted in this study.

MATERIAL AND METHODS

In this retrospective study, serum and CSF specimens were obtained from 50 patients with a clinical diagnosis of TBM. These patients were admitted to the Neurology unit of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala State; India - a tertiary care referral center for neurological diseases. At the time of admission, none of the patients had associated diseases such as diabetes, acquired immunodeficiency syndrome. With the exception of two, none of the 50 patients had earlier clinical manifestations of pulmonary tuberculosis, neurotuberculosis or had previously received chemotherapy for tuberculosis. The diagnosis of TBM in all the 50 patients were based on relevant clinical features such as neck rigidity, positive Kernig's sign and compatible CSF biochemical and cytological features

such as elevated protein levels (60-400 mg%; mean 98 mg%), low glucose concentrations (15-30 mg%; mean 21 mg%) and pleocytosis (30-700 cells/ cm³.

During the hospital stay, lumbar CSF specimens (3-4 ml) from the 50 patients with a clinical diagnosis of TBM were collected and were centrifuged at 5000 X g for 30 min. The deposits were directly inoculated into Lowenstein - Jensen medium for culturing for *M tuberculosis*. The supernatants from the centrifuged CSF specimens were dispensed into aliquots and stored at - 70°C until used for ELISA. After 8 weeks, the culture results showed the presence of *M tuberculosis* bacilli in CSF specimens in five patients and these five patients were regarded as having 'confirmed' TBM. For the remaining 45 patients, repeated bacteriological investigations were negative for *M tuberculosis*, fungi (aspergillus), bacteria (*pneumococci*, meningococci, *haemophilus influenzae*). India- ink preparation was negative for *C neoformans*. IS- 6110-targeted PCR for tuberculosis was reported to be positive for tuberculous etiology in 28/50 CSF specimens. Magnetic Resonance Imaging (MRI) scans in patients with TBM showed varying degree of exudates in the base of the brain as well as mild to moderate degree of hydrocephalus. Since the clinical and neuroimaging features were suggestive of TBM, the culture negative patients(n=45) were categorized as 'probable' cases of TBM. Patients with 'confirmed' and 'probable' TBM after their CSF studies were given anti- tuberculosis treatment (ATT-rifampicin 450 mg, isoniazid 300 mg, streptomycin 500 mg and ethambutol 80 mg) daily during their hospital stay. These patients were advised to continue ATT for 3 months. Serial CSF analyses in these patients were not indicated and hence data on IgG antibody titer to cord factor antigen during the ATT chemotherapy could not be assessed. All these patients were monitored in the infectious disease clinic for the assessment of their clinical response to ATT. CSF specimens from 80 patients with non-tuberculous neurological diseases were collected and used as 'disease' control for this study. (Bacterial meningitis- due to *Haemophilus influenzae* n= 5, *Neisseria meningitides* n=4, partially treated pyogenic meningitis n= 34, Cryptococcal meningitis n= 5, Japanese B viral encephalitis n= 8, CNS tumors n= 26). 10 ml of cisternal CSF was collected at autopsy from a culture proven patient with TBM and this was used as the 'positive' control in this study. All the CSF specimens from tuberculous, 'disease' control as well as 'positive' control were dispensed in sterile aliquots (1ml) and stored at - 70.C⁰ until used.

Isolation of cord factor antigen from *M tuberculosis*

M tuberculosis H₃₇R_v strain was grown in Sauton's medium for 6-8 weeks. At optimum growth, the cultures



Figure.1: Thin layer chromatogram showing cord factor antigen. Lane 1: Cord factor isolated from *M tuberculosis* H37 Rv strain in the laboratory. Lane 2: Reference cord factor antigen supplied by the Colorado State University, USA

were autoclaved and bacillary sediments in pellet form were recovered by centrifugation. The autoclaved pellets were weighed and sonicated in chloroform/ methanol (4:1, vol /vol) for 15 min at +4°C. Deionized distilled water was added (1:20 total volume) and the aqueous phase was sequentially extracted with chloroform/ methanol (3:1 and 2:1 vol/vol), centrifuged at 5000 G for 30 min and the deposit was allowed to dry at room temperature and was treated with acetone. The insoluble phase containing – Trehalose—6' dimycolate (TDM) was separated by repeated centrifugation. The TDM fraction was precipitated by 'drop- wise' addition of methanol at 4°C and then dissolved in tetrahydrofuran and reprecipitated by 'drop-wise' addition of methanol at 4°C to a final ratio of 1: 2 tetrahydrofuran / methanol (vol/vol). The precipitated TDM fraction was then dissolved in chloroform/acetone (8:2 vol/vol) and then loaded onto a column of silica gel and TDM was eluted with chloroform/ methanol (9:1, vol/vol). The elute containing TDM was weighed and the purity was assessed by thin layer chromatography (TLC) using 10 X 10 cm silica plates in comparison with reference cord factor obtained from Colorado state university, USA (Fig.1). TDM at concentrations of 1000 ng/ ml were dispensed in *n*-

hexane and stored in sterile aliquots at + 4°C

ELISA for the IgG antibody to Cord factor in CSF and serum

An indirect ELISA was standardized in polyvinyl chloride microtiter plates (Nunc Laboratories; Roskilde, Denmark). The assay volume and reaction temperatures were 100 ul and 21°C respectively. Each well in the microtiter plate was coated with cord factor antigen (100 ng / per well, dissolved in *n*-hexane) for 2 h and subsequently quenched with 1 % bovine serum albumin (BSA) for 1 h. Serially diluted CSF specimens (1: 10-1:100 dilution) from 'confirmed', 'probable' TBM and 'disease' control (in duplicate) were added at their respective wells in the microtiter plates and incubated for 12 h. The microtiter plates were then washed thoroughly with 0.05% Tween – 20 in 0.15 M phosphate- buffered- saline (PBS-T) and incubated with anti- human IgG- alkaline phosphatase conjugate (1: 1000 dilution) for 2 h. Following several washing in PBS-T, the colour reaction was developed with a substrate containing *p*- nitrophenyl phosphate (1 mg in 1 ml of 10 % diethanolamine buffer pH 9.6). Controls used in the microtiter plate consisted of (a) positive control well, contained -cisternal CSF from culture proven patient with TBM (b) negative control well, contained *n*-hexane (c) blank well contained CSF, enzyme conjugate and substrate. At the end of 30 min, a color reaction developed in the positive control well and 25 ul of 3N NaOH was added to each well to stop the reaction. The microtiter plates were then read at 405 nm using an automated ELISA reader (BioRad; Hercules, California, USA). The absorbances in CSF specimens in tuberculous and control groups were separately recorded.

Statistical analysis

For the calculation of geometric antibody titer in CSF, the serial dilution data was normalized by the logarithmic transformation, using the formula $X = \log_2(\text{titer}) / 10$ where the titer is taken as the reciprocal of end- point dilution. Student's 't' test and standard error for the unpaired data was used to compare the IgG concentration in CSF and sera in tuberculous and disease control (non-tuberculous) groups.

Serum IgG antibody to cord factor

The IgG antibody to cord factor antigen in sera in tuberculous and disease control groups was measured using the above technical protocol except that the sera

Table-1: IgG antibody to cord factor in CSF by indirect ELISA

Estimations used	'Confirmed' TBM (N= 5)	'Probable' TBM (N=45)	'Disease' control (N= 80)
End-point titer in CSF			
< 10	5	45	64
25	5	45	16
50	5	39	0
100	5	39	0
Absorbance at 1: 50			
Range	0.8 - 1.34	0.72-1.31	0.24- 0.48
Mean	1.02±0.028	0.98 0± 026	0. 38+0.019
Sensitivity at 1: 50(%)	100	86.66	0.0
Specificity at 1: 50(%)	---	---	100
Mean CSF- IgG index	0.78	0.74	0.46
Mean serum antibody titer	0.63	0. 56	0.53
Mean CSF- antibody index	0.52	0.48	0.17

specimens were used at 1: 2000 dilution, The absorbances in the sera in tuberculous and controls groups were also tabulated separately.

CSF- IgG index

Then CSF IgG index was determined in all the specimens using the formula- CSF IgG index= CSF IgG: Serum IgG / CSF albumin: Serum albumin (normal range is 0.1 –0.5). Albumin and IgG in CSF specimens were quantitated by an in - house developed low concentration partigen immunodiffusion plates.

CSF/ serum antibody index

The CSF antibody index was determined in all the CSF specimens using the formula: CSF antibody titer: serum antibody titer. The data in tuberculous and non-tuberculous groups of patients were individually recorded.

The IgG antibody to cord factor antigen estimation in CSF and sera were performed in batch of 10 specimens at a time. In order to eliminate an observer bias, the assay was performed on coded samples of CSF and serum, without the clinical details of the patient. In order to evaluate the reproducibility as well as to assess inter-assay variation, the ELISA was repeated on the same CSF/ sera on two different occasions by two technical staff under supervision (VVR). A ratio between serum and CSF IgG antibody to cord factor antigen in patients in tuberculous control groups were calculated. The mean antibody index in tuberculous and control groups were evaluated for assessing the statistical significance.

RESULTS

The IgG antibody titers in CSFs from 45 'probable' (culture negative) and 5 'confirmed' (culture positive) patients with TBM and 80 patients with 'disease' (non-tuberculous) patients are shown in Table-1. It is apparent that none of the 80 CSFs in non- tuberculous patients showed positive reaction in the ELISA at 1:50 dilution. Hence 1:50 end-point titer in the ELISA was a chosen as a 'cut-off' point to score a CSF specimen positive for tuberculous etiology. At this end- point dilution, the mean absorbance in the non-tuberculous patients was 0.38 ± 0.019 (2 SD). The ELISA gave positive result in all the five culture proven patients with TBM and the absorbance at 1:50 dilution ranged between 0.8-1.34(mean 1.02 ± 0.028). In 39 out of 45 culture negative (probable) patients with TBM the absorbance at 1: 50 dilution ranged between 0.72-1.3(mean 0.98 ± 0.026) and were regarded as positive for tuberculous etiology. In the remaining 6 patients the absorbance ranged between 0.24-0.49(mean 0.37 ± 0.12) and did not support for tuberculous etiology. The absorbances in positive and negative controls in ELISA were 1.09 and 0.21 respectively.

The data on CSF antibody to cord factor antigen was correlated with other clinical and laboratory parameters in patients with TBM. The geometric mean antibody titers in CSFs were correlated with the tuberculin reactor status. An intradermal tuberculin test was considered positive if the reaction diameter is more than 10 mm in diameter. 36 out of 45 'probable' patients with TBM had shown a positive intradermal tuberculin test while in 9 patients the intradermal tuberculin test was recorded to be negative. The geometric mean antibody titers in tuberculin positive and negative patients were 164 and 154 respectively. Thus no correlation was observed between intradermal

tuberculin reactor statuses Vs the CSF antibody titer to cord factor antigen in patients with TBM. The IgG antibody to cord factor antigen in patients with TBM were not altered by the total lymphocytes count in CSF. The antibody titers were also correlated with the IgG concentration in CSF. The results indicated that the antibody to cord factor antigen was present in significant titers in all CSF specimens with IgG concentrations >2.5 mg%. In 4 patients with TBM, the CSF IgG < 2.5 mg% had lesser antibody concentration than patients with greater than IgG 2.5 mg % in their CSF specimens.

To determine whether the IgG antibodies are synthesized within the CNS in patients with TBM, levels of IgG and albumin in both serum and CSF were measured and the ratio in both TBM and 'disease' control groups were compared. Similarly the ratio between CSF and serum antibody to cord factor antigen was compared in tuberculous and 'disease' control groups. The mean CSF-IgG antibody index in TBM patients were statistically higher than in 'disease' control group ($p < 0.05$). This observation would suggest that the IgG antibodies to cord factor antigen of *M tuberculosis* are produced by the lymphocytes and plasma cells within the CNS during the active stages of the disease. This observation would also eliminate the possibility that the IgG antibody in CSF occurs as a result of passive transfer across the blood brain barrier in patients with TBM. The mean absorbance in ELISA regarding the end-point serum antibody titers (at 1:2000) between probable TBM and disease control was 0.56 and 0.53 respectively and was not significant ($p > 0.05$).

IS6110 targeted PCR in CSF specimens

This was performed in all CSF specimens using the technical protocol as described previously (Sumi et.al 2002). The PCR gave positive results in three out of five CSFs from 'confirmed' patients with TBM. In 'probable' patients with TBM, the PCR gave positive results in 28 out of 45 CSF specimens. False positive results were also encountered in 12 CSFs from patients in the 'disease control' group. Thus the results would indicate that PCR less sensitive and less specific than the indirect ELISA in our study.

DISCUSSION

The most characteristic and one of the hallmark neuropathological features in patients with TBM is the occurrence of dense fibrinous exudates that encases the leptomeninges as well as cisterns in the base of the brain. At the time of autopsy, acid-fast bacilli (AFB) are often demonstrated in Ziehl- Neelsen stained preparation of smears from the basal fibrinous exudates. It needs

to be emphasized here that the fibrinous exudates pose as a mechanical 'barrier' and prevent the circulation of AFB in the spinal CSF. This could be one of the reasons for the low isolation rate of *M tuberculosis* in lumbar CSF by cultures. Secondly and perhaps more importantly most patients with TBM received a course of ATT before they are referred to tertiary referral center for further management. CSF specimens in partially treated patients with TBM seldom contain optimum numbers of viable tubercle bacilli and hence *M tuberculosis* bacilli are seldom isolated in CSF specimens at cultures. Thus direct microscopic and cultural techniques often inadequate for diagnosing TBM. In these instances immunodiagnostic assays would appear to have a major role to play. However for these assays to be successful, immunodominant mycobacterial antigens need to be identified and protocols to produce them in highly pure and large quantities are essential for the development of appropriate immunodiagnostic assays. The choice of antigens for TB immunodiagnosis is also complicated due to heterogeneity in antibody response of different individuals to the same antigen. Thus antigens that give more uniform and sustained antibody response need to be identified and evaluated in different clinical settings.

In earlier published reports, attempts have been made to demonstrate either mycobacterial antigens or antibodies directed against protein antigens of *M tuberculosis* in CSF specimens for the diagnosis of TBM (Chandramuki et.al. 1989; Chandramuki et.al 2002). However there are few reports in which the lipid antigen of *M tuberculosis* was used for the diagnosis of TBM. Chandramuki et.al (1989) applied an indirect ELISA to antibody response against lipoarabinomannan in cerebrospinal fluid specimens from 74 patients with TBM and compared the results with three protein antigens of *M.tuberculosis* –i.e. 14,19 and 38kDa. They observed, antibody responses to lipoarabinomannan and 14 kDa antigens were immunodominant and showed higher sensitivity than the protein antigens in the assay. However in their study, false negative results were encountered in patients with non-tuberculous subjects. A detailed literature survey till 2010 revealed no published reports regarding the application of cord factor of *M tuberculosis* for the immunodiagnosis of TBM. Hence the results of this study assume significance. A comparison of the cord factor with other mycobacterial antigens for the immunodiagnosis of TBM indicated that (a) cross-reacting antibodies that are often present in CSF specimens, but they did not react with cord factor in ELISA when compared with protein antigens of *M tuberculosis* (*unpublished personal observation*). (b) In ELISA with cord factor antigen did not yield false- positive results in any one of the 80 CSF specimens from patients with non-tuberculous neurological diseases. (c) Cord factor antigen is stable and can be stored in n- hexane for longer period and unlike the protein antigens, the cord

factor does not undergo any degradation or loses its antigenic potency. (Personal observation)

A localized immunological response within the CNS is well known to occur with several inflammatory diseases of CNS including TBM. As a consequence, the proportion of intrathecally-synthesized immunoglobulin (antibody) to *M tuberculosis* in the CSF is likely to be higher in CSF than in serum antibody titer. This phenomenon was clearly demonstrated in this study. The ratio between CSF and Serum antibody titers calculated in tuberculous and 'disease' control groups. The mean anti- cord factor antibody index in patients with TBM was statistically higher than in patients in the 'disease' control. Elevated anti- cord factor antibody index would also indicate that the antibodies to cord factor CSF are synthesized within the CNS during active stages of the disease and it is not as a result of passive transfer from serum across the blood-brain- barrier.

In conclusion, TBM is a devastating neurological disease in which a delayed diagnosis and institution of ATT would worsen the already appalling prognosis. Since the existing bacteriological methods seldom establish the diagnosis, an alternative diagnostic assay that would support the bedside management in patients with TBM would be immensely helpful. In order to meet this challenge, we advocate the application of ELISA with cord factor antigen to detect the specific antibodies in CSF for the following reasons (a) It carries a very high degree of specificity and without any danger of potential false positive results in non- tuberculous subjects (b) the assay is highly reproducible (c) A patient with positive for tuberculous etiology in ELISA may be given the benefit ATT and subsequently can be evaluated for optimum clinical response and neurological recovery. However prospective studies in CSF specimens in a larger population of patients with a clinical diagnosis of TBM is essential for a proper validation and clinical application of this assay. Currently efforts are being made in this direction.

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