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Tuberculous Meningitis in Patients Without Systemic Focus of Miliary Tuberculosis

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Abstract: Antigen 85 (Ag 85) complex, a component of *M.tuberculosis* bacilli (MTB) have been the focus of extensive research for diagnosis of tuberculosis for last several years. The aim of the present study to evaluate the status of primary tuberculosis infection in confirmed tuberculous meningitis (TBM) patients by estimating Ag 85 complex in Cerebrospinal Fluid (CSF) and serum specimen. A prospective study was also conducted to evaluate response of B cells derived from the CSF and serum of TBM patients after challenging with the Ag 85 complex. CSF and blood samples with and without heparin were collected from all the study subjects. An indirect ELISA was performed using monoclonal antibody (mAb) against Ag 85 complex for the demonstration of Ag 85 complex antigen in CSF and serum specimen of TBM patients. Cell ELISA was also done to evaluate the response of B cells derived from CSF and serum of TBM patients against Ag 85 complex. The CSF positivity for Ag 85 complex in TBM patients was 100% (35/35) however serum of same patients was positive for only 9% (3/35) cases. B cell response against Ag 85 complex was observed only in cells isolated from CSF rather than blood cells. Ag 85 complex activity and B cell response was found to be negative for control group (within 72 h). The absence of Ag 85 complex activity and B cell response in blood sample suggest a systemic focus of tuberculosis is possibly absent in blood and B-cell response lacking systemic feedback control. Ag 85 complex turn out to be an important molecular marker to understand the immune response in TBM patients associated with localized humoral response in central nervous system.

Key words: Tuberculous meningitis · tuberculosis · antigen 85 complex

INTRODUCTION

Tuberculous meningitis (TBM) remains a major health problem in underdeveloped and developing countries [1, 2]. It has been recognized long before modern understanding of it evolved: in Vedic hymn dated approximately, 2000 B.C. invokes a treatment ritual for a "consumption seated in the head" [3]. It accounts for 70 to 80 percent cases of neurological tuberculosis. Incidence of TBM in patients with tuberculosis has been reported to vary from 7.4 to 11.8 percent from various centers in India. Even though, the recent figures of neurotuberculosis in India are unavailable. In earlier Indian studies upto 42%cases of systemic tuberculosis have been reported to involve meninges in clinical studies and this figure rose to 65.5% when autopsies were performed on such patients. It has been reported that 4.1% of all pediatric admissions in a hospital, Bombay were victims of TBM. More or less identical figures have been reported from other centers in India [4, 5].

TBM is secondary to tuberculous infection elsewhere in the body from where *M.tuberculosis* bacilli reach the CNS by hematogenous route. It has been suggested that tuberculous lesion (Rich Foci) develop in the CNS after primary tuberculous infection [6].

Antigen 85 (Ag 85) complex are major secretary proteins of *M.tuberculosis* which have been the focus of extensive research for several years and comprises three related proteins i.e. Ag85A (31 kD), Ag85B (30 kD) and Ag85C (31.5 kD) which have also been demonstrated in the sputum of pulmonary TB patients [7]. Various forms of Ag 85 complex have been previously evaluated for antibody detection in extra central nervous system TB [8]. This antigen has been variously called in literature as, alpha Antigen fibronectin binding protein Antigen 6 and AG 85 Complex [9]. 30kD is one of the early antigen to be

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secreted [10] and highly immunogenic to the host, it is a suitable candidate for the early diagnosis. In our earlier study, we have demonstrated the presence of 30 kD protein antigen in Cerebro Spinal Fluid (CSF) of confirmed and suspected patients of tuberculous meningitis (TBM) by one-dimensional electrophoresis [11]. This 30 kD protein was analyzed by liquid chromatography tandem mass spectrometry. These studies cumulatively identified two mycobacterial antigens Rv 3804c and Rv1886c (Ag 85 A and B respectively, both members of Ag 85 complex) and one host derived protein (immunoglobulin [Ig] Kappa light chain VLJ region; accession no. BAC01690.1) as the components of TBM specific 30 kD protein antigen [12].

A number of studies have been carried out to understand the immune response against *M. tuberculosis* antigens either in blood or CSF specimen however no attempt appear to have been made in both specimen of same patient [13-16]

The present study was aimed to evaluate the status of primary tuberculosis infection in confirmed TBM patient by estimating Ag 85 complex in CSF and the serum specimen. A prospective study was also conducted to evaluate the response of B cells derived from CSF and serum of TBM patients after challenging with the Ag 85 complex.

MATERIALS AND METHODS

CSF and serum samples of different group of patients, which included confirmed culture positive TBM (n=35) and non-TBM infectious meningitis (n=20) which included pyogenic and viral meningitis patients and non-infectious neurological disorders (n=15) which included stroke, epilepsy; headache, etc were analyzed in the present study. Serum and plasma samples (heparinsed blood) were also obtained from same group of patients as well as from healthy subjects (n=15). Patients included in the study are those admitted to the Neurology Department of Central India Institute of Medical sciences (CIIMS). Ag 85 complex antigen estimation was done in the CSF and serum samples obtained before starting any treatment. All subjects were negative for HIV infection. All TBM and controls groups included in the study were vaccinated with BCG. The institutial Ethics Committee of Central India Institute of Medical Sciences, Nagpur, India approved this study. All patients were grouped as follows:

Confirmed TBM: This group included culture positive cases with one or all of the following observations. Sub-acute or chronic fever with features of meningeal irritation such as headache, neck stiffness and vomiting with or without other features of CNS involvement. CSF findings showing increased proteins, decreased glucose (CSF: blood glucose ratio<0.5) and/or pleocytosis with lymphocytic predominance.

Non-TBM group:

Non-TBM infectious meningitis: This group included patients of pyogenic and viral meningitis. a) Pyogenic meningitis: Presence of pathogenic bacteria such as *Staphylococcus* sps., *Streptococcus* sps, *Haemophilus* sps in CSF by staining and/or culture with CSF findings showing increased proteins, decreased glucose (CSF: blood glucose ratio <0.2) and/or pleocytosis with a predominance of polymorphonuclear cells and good clinical response to broad-spectrum antibiotics b) Viral meningitis: Mainly caused by Enterovirus and Herpesvirus. This group included suspected patients with following observations: Acute onset of fever and symptoms and signs of meningeal irritation. CSF findings showing mild increase in protein, glucose often normal and pleocytosis, predominantly lymphocytic. No clinical evidences of extra cranial tuberculosis.

Non-infectious neurological disorders: All other patients who had no evidence of CNS or extra CNS bacterial or viral infections were grouped in the non-infectious/control group. Patients included in this group are of chronic intractable headache, status epileptics, stroke etc.

Specimen: CSF sample was collected by standard lumbar puncture. Approximately 3ml of CSF was obtained. It was used for total and differential cell count, biochemistry and smear for Gram's, India ink and AFB staining and for detection of Ag 85 complex antigen by ELISA. Serum and plasma samples (heparinsed blood) were obtained from same group of patients as well as from healthy subjects (control group). The samples were stored at -20°C until further analysis.

Antigen and antibody: The purified Ag 85 complex and monoclonal antibodies to the purified Ag 85 complex (CS-90) were obtained from Colorado State University through the TB Research Materials and Vaccine Testing Contract (NO1-AI-40091).

Preparation of CSF Cells: One milliliter of CSF collected from TBM patients was centrifuged at 400 rpm for approximately 20 min. The supernatant was then

discarded and the cell pellet was washed two times with PBS and then diluted in RPMI 11640 tissue culture medium containing 10% fetal calf serum.

Preparation of blood cells: Heparinised blood sample were obtain from TBM and Non-TBM patients and healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated using density gradient cell histopaque -077 (SIGMA co, st. Louis MO, USA). The Buffy coat containing PBMC cell were washed thrice in RPMI 1640 containing 2mM HEPES, L glutamine, sodium biarbonate, 1 mg dl^{-1} media of antibiotic antimycotic mixture (HIMEDIA, INDIA) containing 10,000 Unit penicillin10 mg streptomycin 25 g and amphoterian in 0.9% Nacl 10% and New born calf serum (SIGMA CO, st. Louis MO, USA).

ELISA: Microtiter plates were coated with 100 ul samples {CSF (1:5) /Serum 1:200} and incubated overnight at 4°C. The sample was then blocked by addition of 100 µl of 0.5% 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 HRPconjugated 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/H_2O_2 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 H_2SO_4. The absorbance of each well was read at 450 nm.

Cell ELISA method: Flat-bottomed, 96-well ELISA plates were coated with Ag 85 complex (10 µg ml^{-1} diluted in PBS, pH 7.2). Following overnight incubation, the plates were washed with PBS and then coated with 5% BSA-PBS for 4 h. The plates were then washed five times with PBS. Two-hundred µl of the cell preparation derived from CSF and blood of patients with TBM were then separately added to the wells and coated. Each sample was prepared in duplicate. Plates were maintained overnight at 37°C in 5% CO_2 in a carbon dioxide incubator. The following day, the plates were washed with PBS and horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (1:10,000) was then added to the plates. After 2 h incubation at 37°C, the plates were washed again with PBS and 100 µl TMB/ H_2O_2 substarte solution was added. The TMB/ H_2O_2 served as a substrate for HRPO. After 15-min incubation, 100 µl stop solution (2.5 N sulphuric acid) was added and the plates were then read with an ELISA reader at 450 nm.

Statistical analysis: Results are expressed as mean±SD. To compare means among the TBM, non-TBM infectious meningitis and non-infectious neurological disorders, we used unpaired t test with Welch correction to compare the CSF/Serum Ag 85 complex activity of TBM patients with that of control group. Similarly simple unpaired t test was used to compare the serum Ag complex activity with control group serum P value less than 0.05 was considered significant.

RESULTS

The clinical data of the TBM patients with clinical history and CSF picture suggestive of TBM are given in Table 1. Thirty-five cultures positive confirmed TBM patients were included in the study. All the TBM patients were HIV negative and responded well to anti-TB medication. However, all of them had normal chestX ray and ESR and AFB negative for sputum. None of the patients showed sign and symptoms of primary tuberculosis infection elsewhere. CSF findings showed increased proteins (118 mg dl^{-1} [64-1002]), decreased glucose (27 mg dl^{-1} [19-44]) and CSF: blood glucose ratio (0.29 [0.11-0.49]) and/or pleocytosis with lymphocytic predominance (83% [40-100]).

Figure 1 shows a box plot of the Ag 85 complex activity obtained with indirect ELISA in CSF/serum of TBM and non-TBM group. Results obtained with indirect ELISA for Ag 85 complex activity in CSF and serum of TBM patients are shown in Fig. 1. CSF specimens showing absorbance > 0.283 absorbance (mean+ SD of controls) were taken as positive for the assay however in serum specimen showing absorbance >0.477 absorbance (mean+ SD of controls) were taken as positive for the assay. Using these cut off values all the 35 TBM patients were positive for Ag 85 complex, however Ag 85 complex activity was found to be positive in only three serum samples of the TBM patients. Ag 85 complex activity was absent in all the control CSF/serum group. Sensitivity and specificity of 100% was noted when CSF samples of TBM patients when compared with the CSF samples of control group. Similarly sensitivity and specificity of 17 and 70% respectively was observed when serum sample of TBM was compared with the control serum. Figure 2 shows the ELISA absorbance of IgG to the Ag 85 complex antigen in cells isolated from

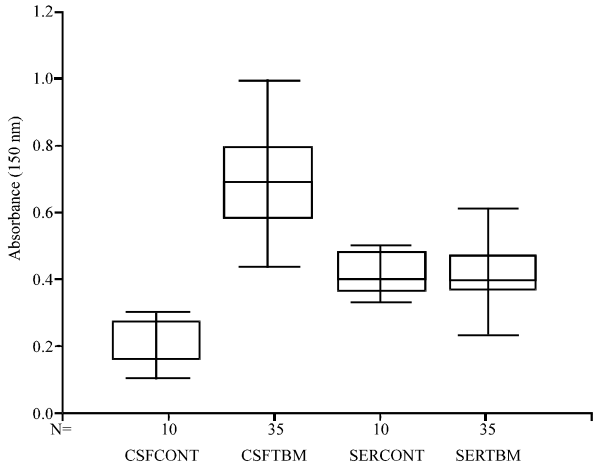

Fig. 1: Shows a box plots of CSF Ag 85 complex activity in CSF/serum of control and TBM group and The box plot show the outliers (O), 90th percentiles (bars), 75th and 25th percentiles (boxes) and median (bars in boxes). N-numbers of individual in each group. (CSFCONT: Control CSF samples; CSFTBM: CSF of TBM patients; SERCONT: serum of control group; SERTBM: serum of TBM patients)

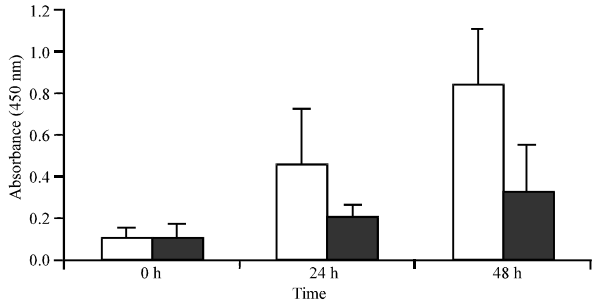

Fig. 2: Shows the response of cells derived from CSF and blood specimen of TBM patients after induction of antigen 85 complex in 0, 24 and 48 hrs by cell ELISA

CSF and blood of TBM patients. High-titer values for IgG antibody production against Ag 85 complex was observed in cells isolated from CSF of TBM patients at different time intervals. However, the titer in blood cell of same patients was much lower than that observed in cells isolated from CSF of TBM patients. No response was noted in the CSF cells isolated from pyogenic and viral meningitis patients, similarly blood cells isolated from healthy volunteer showed no IgG antibody production against Ag 85 complex.

Table 1: TBM Patient's (n=35) clinical and CSF finding. (*except one case, TLC-total leukocyte count, DLC-differential leukocyte count, P-polymorphs, L-lymphocytes, M-monocytes)

	Number (%) or median (90%range)
Male	16 (46%)
Age (years)	30 (18-59)
Duration of disease (days)	10 (6-37)
Neck stiffness	Present
CSF findings	
Color	Colorless
Appearanc	turbid
Coagulum	absent*
Xanthochromia	absent
TLC cu.mm	198 (85-790)
DLC	
P%	12 (2-29)
L%	83(40-100)
M%	01 (1-6)
Sugar (mg dl^{-1})	27 (19-44)
Protein (mg dl^{-1})	118 (64-1002)
CSF: Blood glucose ratio	0.29(0.11-0.49)
Chest x ray	Normal
ESR	5 (10-25)
Sputum AFB	negative
Past history of TB	none

DISCUSSION

TBM develops most often when a caseating meningeal or sub-cortical focus, the Rich focus, discharges its contents into the subarachnoid space. It is well established that both humoral and cell mediated immunity play an important role in the TBM infection [17-19]. It is also recognized that TBM is frequently accompanied by milliary tuberculosis, but the relationship between the development of the Rich focus and milliary TB remain controversial [12].

In the present study, we have evaluated the status of primary tuberculosis infection in patients with TBM by estimating Ag 85 complex activity in CSF and the serum specimen and evaluated the B cells response of cells derived from CSF and serum of TBM patients after challenging with the Ag 85 complex. The CSF of all the TBM patients were positive to Ag 85 complex however except three cases Ag 85 complex activity was absent in the serum specimen of the same group of patient. PBMC isolated from the blood of TBM patients did not proliferate after induction of Ag 85 complex however, the cells isolated from CSF recognized the same antigen and proliferated. The cells obtained from

CSF of TBM patients gave an early response, presumably because they were already sensitized against the TBM antigen. However, when challenged with the Ag 85 complex, the cells obtained from blood of healthy volunteers and CSF of control group not give a response within 72 h suggesting they are not sensitized against this antigen. This observation confirms our earlier findings which show that B-cell response (production of antibodies) against *M. tuberculosis antigen* was significantly higher in CSF derived mononuclear cell than those from peripheral blood [11].

There are several studies which supports finding of our results, Kinnman *et al*. [16] and Baig [11] have showed that CSF derived lymphocytes had significantly higher proliferative response to PPD than to peripheral lymphocytes in patient suggesting an intrathecal response [20, 21]. In another study Malashkhia and Geladze [18] with the help of autoradiographic studies showed that the CSF lymphocytes isolated from TBM patients showed high response of the cells to purified protein derivative than the PBMC [22]. Similarly Schneider *et al.* [21] demonstrated that T & B-cell specific immune response to purified protein derivative in CSF maintained and regulated independently of systematic immune control. The selective enrichment of antibodies in the CSF but not in the patient's serum further indicated T and B cell responses lacking systemic feedback control [23]. In another study it was also shown that TBM is associated with localized humoral response in CNS as it was proved by the detection of Anti BCG IgG secreting cell, which was isolated from the CSF of TBM patient [24].

The absence of Ag 85 complex activity and B cell response in blood sample suggest a systemic focus of tuberculosis is possibly absent in blood and B-cell response lacking systemic feedback control. However it is unclear how *M.tuberculosis* organism directly affects the meninges of the brain without any involvement of blood. In conclusion Ag 85 complex turn out to be an important molecular marker to understand the immune response in TBM patients associated with localized humoral response in central nervous system.

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