Correlation between culture of *Mycobacterium tuberculosis* and detection of mycobacterial antigens in cerebrospinal fluid of patients with tuberculous meningitis

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Summary. A retrospective study was done to correlate culture of *Mycobacterium tuberculosis* and detection of mycobacterial antigen in cerebrospinal fluid (CSF) by an inhibition enzyme-linked immunosorbent assay (ELISA). *M. tuberculosis* was cultured from CSF of 14 out of 70 patients with a clinical diagnosis of tuberculous meningitis (TBM). Mycobacterial antigens were demonstrated in CSF specimens by inhibition ELISA in all 14 culture-positive patients with antigen concentrations of 14.5–295 ng/ml (mean 158.8 ng/ml). Thus there was positive correlation between the detection of mycobacterial antigen and isolation of *M. tuberculosis*. Based on this observation, 56 CSF specimens from culture-negative patients with clinically diagnosed TBM were examined for mycobacterial antigen and the data were compared with those from culture positive patients. ELISA gave positive results in 38 specimens, with antigen levels of 12.5–280 ng/ml (mean 152.6 ng/ml). In 70 CSF specimens from patients with non-tuberculous neurological disease (control group), ELISA results were negative. Thus, detection of mycobacterial antigen in CSF specimens by inhibition ELISA had a specificity of 100% and a sensitivity of 67.8% for the diagnosis of TBM and is of potential value in the laboratory diagnosis of TBM.

Introduction

Tuberculous meningitis (TBM) is one of the common infectious diseases among patients hospitalised for neurological diseases. The diagnosis of TBM is usually based on clinical features, supported by cytological and biochemical examination of cerebrospinal fluid (CSF) specimens. However, atypical clinical manifestations of TBM can pose diagnostic difficulties. At times, meningitis due to other agents may closely mimic the clinical presentation of TBM. Demonstration of the causative agent, *Mycobacterium tuberculosis*, in CSF specimens by bacteriological culture requires several weeks, and positive cultures are infrequent. Early diagnosis and effective chemotherapy would reduce the current mortality and morbidity rates in patients with TBM, particularly in developing countries.

During the past decade, several immunodiagnostic assays have been reported as an adjunct in the diagnosis of TBM.^{1–8} These studies have described

either the detection of circulating mycobacterial antigens or the demonstration of specific antibodies in CSF specimens from patients with TBM and have emphasised their diagnostic significance. To investigate the predictive value (percentage of patients correctly classified among all tested patients) and specificity (percentage of negative results among the control subjects), we describe here an inhibition enzyme-linked immunosorbent assay (ELISA) for the detection of mycobacterial antigen in CSF specimens from 70 patients with a clinical diagnosis of TBM, 56 of whom were culture negative. CSF specimens obtained from 70 patients with non-tuberculous neurological diseases formed the control group. The potential application of the assay in the laboratory diagnosis of TBM was assessed.

Materials and methods

Patients and specimens

In this retrospective study, inhibition ELISA for the

detection of mycobacterial antigen in CSF was done on samples from 70 patients with a clinical diagnosis of TBM. Since this Institution is a referral centre for neurological diseases, the time from the onset of meningitis to admission into the in-patient neurology unit varied. In these patients, a diagnosis of TBM was considered on the basis of clinical manifestations, elevated protein and lymphocytosis in CSF specimens, and clinical response to antituberculosis chemotherapy. Multiple (two-to-three) CSF specimens were taken and *M. tuberculosis* was demonstrated by bacteriological culture in the CSF of 14 patients. In the remaining 56 patients, repeated CSF cultures for *M. tuberculosis*, fungi and bacteria (including anaerobes) gave negative results. All of these patients had received antituberculosis chemotherapy for 2-6 weeks during their hospital stay. CSF specimens from 70 patients with non-tuberculous neurological diseases formed the control group. Inhibition ELISA was performed on the CSF specimens⁹ and repeated on two different occasions to assess the reproducibility of the assay.

Antigen

A cell-free culture filtrate of *M. tuberculosis* H₃₇Ra (supplied by the Tuberculosis Research Centre, Madras) was used as the antigen. Cultures were grown on Proskauer-Beck medium (Hi-Media, Bombay, India) for 8-10 weeks. At optimum growth, the culture was initially clarified by filtration through Whatman paper IV and finally through a 0.45- μ m pore-size membrane (Millipore). The cell-free culture filtrate was then dialysed several times against distilled water, and concentrated with an Amicon ultrafiltration unit (Amicon, Witten, Germany). The protein content of the culture filtrate was determined, and the filtrate was dispensed at a concentration of 2 mg/ml in small volumes and stored at -20°C until used in the assay.

Antiserum

Polyvalent antiserum was raised in adult rabbits by subcutaneous and intra-muscular injections of 2 mg of antigen, plus 2.5 mg of autoclaved dried bacillary sediment of *M. tuberculosis* H₃₇Ra, mixed thoroughly in 2 ml of incomplete Freund's adjuvant. A course of five injections was administered. Immuno-electrophoresis demonstrated multiple precipitin arcs with this antiserum against culture-filtrate antigen.

Inhibition ELISA procedure

Before patient sampling, the assay was standardised by incubating different concentrations of antigen (1-1000 ng/ml) in phosphate-buffered saline (PBS) with an equal volume of polyvalent antiserum (used at a dilution of 1 in 5000) for 12 h at 4°C. This antigen-antibody complex was centrifuged at 5000 rpm for 10 min and 200 μ l of clear supernate was then transferred to each

well of an ELISA microtitration plate (Dynatech Inc., Alexandria, VA, USA). The microtitration plate was presensitised with culture filtrate antigen (2 μ g/well in carbonate bicarbonate buffer, pH 9.6) and subsequently quenched with bovine serum albumin (1%). After incubation for 12 h, the plates were washed with Tween-20 0.05% in PBS, and 200 μ l of a 1 in 40 000 dilution of anti-rabbit IgG biotin-conjugate (Sigma) was added to each well and incubated for 2 h at room temperature. The microtitration plates were washed thoroughly with Tween-20 0.05% in PBS and 200 μ l of a 1 in 200 dilution of avidin-alkaline phosphatase (Sigma) was added to each well and incubated for 2 h. The colour reaction was developed by the addition of 200 μ l of *p*-nitrophenyl phosphate (1 mg in 1 ml of diethanolamine 10% buffer, pH 9.8) and stopped after 30 min by the addition of 50 μ l of 3 N NaOH. The control wells in the standardisation inhibition ELISA procedure contained antigen, polyvalent antiserum, antirabbit IgG-biotin conjugate, avidin-alkaline phosphatase and substrate. The assay was repeated six times at different concentration of the antigen. The mean absorbance in the control and test wells (table I) were recorded at 405 nm with an automated ELISA reader (Titertek Multiscan, Flow Laboratories). The difference between the absorbance recorded in the control and test wells was plotted against the log value of the antigen concentration. A linear relationship was obtained, and was reproducible over several experiments.

The CSF specimens (between 1 ml and 3 ml) from patients with TBM and from the control group were assayed for mycobacterial antigen under identical laboratory conditions to those described in the standardisation procedure. Specimens were centrifuged (under aseptic conditions) at 1500 rpm for 15 min. Supernatant CSF was concentrated by ultrafiltration and inactivated at 56°C for 1 h to remove the IgG activity. For every CSF specimen, the assay was performed in duplicate and the mean absorbance was calculated. Mycobacterial antigen in CSF specimens in the TBM and control groups was measured directly from the standard graph. The centrifuged deposits were inoculated on to Lowenstein-Jensen medium to culture *M. tuberculosis*.

Results

Table I shows that the range of absorbance values was 0.46-0.25 over an antigen range of 1.0-1000 ng/ml. It is apparent that with the increasing concentrations of the antigen, there is a decrease in the absorbance which clearly indicates competitive inhibition of unabsorbed antigen. The absorbance in the control and absorbance at an antigen concentration of 1 ng/ml were similar but a clear difference in the absorbance was evident at an antigen concentration of 5 ng/ml.

Results obtained with the inhibition ELISA for the quantitation of mycobacterial antigens in CSF specimens from culture-positive and culture-negative patients with TBM, and in the control group,

Table I. Difference in the absorbance between control and culture-filtrate antigens in standardisation procedure for inhibition ELISA

Antigen concentration (ng/ml)	Mean (SEM) absorbance at 405 nm*	Difference in absorbance
Control (0)	0.460 (0.005)	—
1	0.460 (0.005)	0
5	0.435 (0.007)	0.025
10	0.414 (0.015)	0.046
100	0.355 (0.016)	0.105
500	0.307 (0.019)	0.153
1000	0.250 (0.007)	0.210

*Mean of six experiments.

are shown in tables II and III. No false-negative results were recorded in 14 culture positive patients, and the concentration of mycobacterial antigen in CSF specimens was in the range 14.5–295 ng/ml (mean 158.8 ng/ml). In 38 out of 56 culture-negative patients with TBM, the antigen concentration in CSF was in the range 12.5–280 ng/ml (mean 152.6 ng/ml). The antigen concentration in culture-positive and culture-negative patients is not statistically different ($p > 0.05$). In the remaining 18 patients the concentration of mycobacterial antigen in CSF was in the range 1.2–3.8 ng/ml (mean 1.45 ng, SD 1.6). In the 70 CSF specimens from the control group, the mean antigen concentration was 1.45 (SD 1.3). A test was considered positive if the antigen concentration was greater than 5.35 ng/ml (mean + 3 SD). By this criterion, the assay gave 100% specificity and 67.8% sensitivity for the diagnosis of TBM.

Bacteriological culture for isolation of *M. tuberculosis* from CSF specimens in patients with TBM gave a sensitivity of 20%. Furthermore, *M. tuber-*

culosis could be isolated only in the first CSF specimen and the cultures were negative in the subsequent specimens from the same patient. In contrast, mycobacterial antigen could be demonstrated in all the CSF specimens obtained in a patient with TBM. While the culture procedure required 4–6 weeks, the results of inhibition ELISA were available within 48 h after the receipt of the specimen in the laboratory.

Discussion

Absolute criteria to establish the diagnosis of TBM depend upon the demonstration of its causative agent, *M. tuberculosis*, in CSF specimens, either by direct Ziehl-Neelsen stained cytospin smears or by culture techniques. Isolation of *M. tuberculosis* from CSF specimens by conventional culture techniques is not only time-consuming but also infrequent. In this study, *M. tuberculosis* could be isolated from only 20% of patients with TBM. There could be several reasons for the infrequent demonstration of *M. tuberculosis* in CSF specimens in patients with TBM. The specimens from these patients are usually obtained through the lumbar route and lumbar CSF contains low concentrations of *M. tuberculosis* compared to ventricular or cisternal CSF. Secondly, *M. tuberculosis* organisms are embedded in the dense exudate, either in the basal cisterns or in the leptomeninges, and the exudate forms a 'barrier' for *M. tuberculosis* to circulate in the lumbar CSF. Thirdly, and perhaps most importantly, the vast majority of patients with TBM have received a course of anti-tuberculosis chemotherapy before being referred to the specialised centres for neurological diseases. CSF specimens in partially treated patients with TBM seldom contain acid-fast bacilli and hence cultures are invariably negative. In the absence of any definitive

Table II. Mycobacterial antigen concentrations in CSF of patients with tuberculous and non-tuberculous meningitis

Patient group (n)	Number of patients with antigen concentrations		Antigen concentration (ng/ml) in CSF	
	< 5 mg/ml	≥ 5 mg/ml	Range	Mean
Tuberculous meningitis (70)				
culture-positive (14)	0	14	14.5–295	158.8
culture-negative (56)	18	38	12.5–280*	152.6*
Non-tuberculous meningitis (70)	70	0	1.15–3.77	1.45

*In the antigen-positive patients.

Table III. Comparison of culture of *M. tuberculosis* and detection of mycobacterial antigen in CSF in patients with tuberculous meningitis

Culture result	Number of CSF samples that gave ELISA results		Total
	Positive	Negative	
Positive	14	0	14*
Negative	38	18	56
Total	52†	18	70

*Sensitivity 20% for culture.

†Sensitivity 67.8% for ELISA.

diagnostic criteria, an alternative laboratory marker to substantiate the clinical diagnosis of TBM is required. In this study we have determined the specificity and sensitivity of an inhibition ELISA for the detection of mycobacterial antigens. CSF specimens from 56 culture-negative patients with TBM were compared in terms of antigen levels with 14 culture-positive patients. In 38 CSF specimens from culture negative patients the antigen was demonstrated in significant concentrations, giving a predictive value of 67.8%. The antigen concentration in all 70 CSF specimens from the control group was below the 'cut-off' level (5.35 ng/ml) and therefore, was considered negative, giving a specificity of 100%.

Earlier published reports on the detection of mycobacterial antigen in CSF specimens in patients with TBM have used a variety of techniques, including sandwich ELISA^{1,3,10} a latex-particle

agglutination test,² and a reverse passive-haemagglutination test.⁶ There are few published reports on inhibition ELISA for the quantitation of mycobacterial antigen in CSF in patients with TBM. Bal *et al.*¹¹ used such an assay in their study and reported occasional false positive results. The present assay system differed in methodology in that we used an avidin-biotin system to improve the specificity of the assay and hence eliminate false-positive results in non-tuberculous subjects.

The inhibition ELISA is reproducible and the results are available within 48 h after the receipt of the CSF specimens in the laboratory. Inconsistent or gross variation in the results are seldom observed. However, it should be emphasised that standardisation of the assay with culture-filtrate antigen is essential and a standard linear graph must be obtained every time before testing CSF specimens, in order to determine the antigen concentrations. This eliminates technical errors and improves the reliability of the assay.

In conclusion, we advocate the use of the inhibition ELISA for the specific laboratory diagnosis of TBM, particularly in developing countries where the reported incidence and prevalence of TBM is still high. The assay should be considered as an alternative method to diagnose TBM particularly when bacteriological cultures are negative for *M. tuberculosis* in CSF specimens. We are currently assessing the value of the technique in the routine diagnosis of TBM.

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