

DIAGNOSIS OF TUBERCULOUS MENINGITIS BY ELISA USING SEROLOGICALLY ACTIVE GLYCOLIPID ANTIGENS

Ümit Bilge DOĞAN, M.D., Hasan Salih AKSU, M.D.

Çukurova University, Faculty of Medicine, Department of Internal Medicine, Adana, Turkey
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SUMMARY : Culture and identification of *Mycobacterium tuberculosis* in cerebrospinal fluid (CSF) of patients with tuberculous meningitis (TBM) is slow, and prognosis depends on early diagnosis and treatment. A rapid and reliable test to detect mycobacterial antigens in body fluids is therefore needed. In this study, the sensitivity of the enzyme-linked immunosorbent assay (ELISA) with serologically active glycolipid (SAG) antigens was found 100 % in the CSF of 7 culture positive and 19 culture negative patients with TBM. The specificity of the assay was found 95 % in the CSF of 20 patients with non-tuberculous neurological diseases (control group). In conclusion, we think that SAG antigens may be sensitive and specific as other mycobacterial antigens and even superior to the others by its rapid and practical extraction in the laboratory diagnosis of TBM.

Key Words : Glycolipids, Tuberculous Meningitis, ELISA.

INTRODUCTION

Tuberculous meningitis (TBM) remains a common disease in developing countries, and the prognosis is closely related to the stage of the illness at the initiation of treatment (6). Culture and identification of *Mycobacterium tuberculosis* in CSF from patients with TBM is slow and tedious. Results are not available for 2-4 weeks with routine methods (19), or for 1 week with other assays such as selected ion monitoring (11), and a rapid and reliable test to detect mycobacterial antigens in body fluids is therefore needed. A rapid serologic test in the diagnosis of tuberculosis has been an area of active research since the report of Brown and Petrof in 1918 (2). A variety of methods have been investigated, including hemagglutination (4, 5), precipitation in gels (13), agglutination tests (10, 20), and ELISA (3). Among serological tools, ELI-

SA has been the method of choice for detecting serum antibodies and mycobacterial antigens from clinical specimens because of its high sensitivity, simplicity, reproducibility and versatility in screening a large number of specimens.

Among mycobacterial antigens, purified protein derivative (PPD) and BCG antigens are not very specific for TBM (14, 17). Results of studies using inhibition (7, 16) and indirect ELISA (7, 15) with antigen-5 give a higher specificity. Although the detection of mycobacterial antigens from CSF specimens using polyclonal or monoclonal antibodies gives more direct evidence for TBM, the information by the assays is not sufficient to determine whether these immunological tools can be used in clinical laboratories for the diagnosis of TBM. Also polymerase chain reaction (PCR) technology for the detection of mycobacterium

tuberculosis DNA from clinical specimens may be impractical to be used everywhere.

In this study, a more practically and rapidly extracted glycolipid antigen of mycobacterium tuberculosis was used for ELISA to diagnose TBM.

METHODS

Patient selection : CSF samples were obtained from 46 subjects. Group I, consisting of 26 patients with TBM, was divided into group Ia, seven patients with bacteriologically confirmed disease, and group Ib, 19 patients with the presumptive diagnosis of TBM; supported by a CSF glucose level of < 40 mg/dl, protein > 40 mg/dl, pleocytosis with lymphocytosis, negative culture for other bacteria and fungi, plus two or more of the following : a clinical picture of TBM, positive tuberculin skin test, evidence of tuberculosis in other sites, or a clinical response to treatment. Group II consisted of 20 patients with non-tuberculous neurological diseases. In group I and II, mean ages were 12 ± 11 , 16 ± 14 and male female ratios were 1.6 and 1.5, respectively. Both groups were comparable in age and sex. CSF samples were obtained before the anti-tuberculosis treatment.

Antigen preparation : Mycobacterium tuberculosis colonies cultured from Lowenstein-Jensen medium were inactivated in 20 ml of acetone solution for 24 hours at room temperature. Inactivated bacterial cells were exposed three times to boiling acetone (with the help of water bath at 80-90°C) and then filtered. Glycolipids were extracted from bacterial cells with methanol and chloroform as described by Reggiardo et al. (18). The extracts were freed of tuberculoproteins and polysaccharides according to Pangborn and McKinney (12). Purification was done by silicic acid thin layer chromatography using silikagel G commercial plates (E. Merck, Darmstadt, W. Germany) as described by Reggiardo et. al. with a solvent consisting of chloroform, methanol, water, (65:35:8). Cardiolipin (Bovine, Sigma, USA) was used as a standard reference compound for comparison of reference values of lipid spots on the chromatography. Even a small amount of glycolipid antigen purified by this technique was shown to be serologically active by the same authors. The glycolipid antigens were concentrated to 0.5 mg/ml by ultrafiltration, and finally stored frozen (-80°C) until used.

Conjugate : Horse Radish-peroxidase (HRPO) linked anti-human globulin (IgG fraction, rabbit) conjugate was prepared as described by Nakane (9).

ELISA : Solid phase (active microplate) was coated with glycolipid antigen for this study. 200 μ l of antigen suspension in PBS were added to each well of microplates and incubated overnight (16 hr) at 4°C. Excess conjugate was washed out with PBS (1% Bovine Serum Albumin-1% Tween 20) solution. It was then incubated with 1% of gelatin solution at room temperature. Microplates were washed with PBS again.

100 μ l of 1/100 diluted CSF samples were added to all the wells of coated microplates but one which was used as a reference. Microplates were incubated at 37°C for two hours. Then microplates were washed with PBS. 100 μ l of 1/1000 PBS diluted HRPO linked anti-human globulin (IgG) was added to each well and incubated for 1 hour at 37°C. Microplates were washed 6 times with PBS solution before each addition.

For reaction formation, 100 μ l of substrate solution of O-fenilendiamin 0.04 % + H₂O₂ 0.012 % phosphate citrate buffer with pH = 5 were added to all the wells. The plates were incubated at room temperature for 20 minutes. The reactions were stopped using 100 μ l of 2N sulfuric acid. Absorbance at 490 nm was measured by spectrophotometer. The well without CSF was used as a reference and labeled as zero. Each sample was run in duplicate.

RESULTS

The mean absorbance of the 26 samples from patients with TBM (group I) was 1.372 ± 0.507 . Group Ia and group Ib gave similar ELISA values. In group II, the mean absorbance was 0.333 ± 0.178 . The mean absorbance in group I was significantly higher than that in group II ($p < 0.001$). Test results are shown on figure-1.

Cut off absorption value was found to be 0.550 by using logistic regression analysis. Sensitivity and specificity of this test, using 0.550 as the cut off value, were found to be 100 % and 95 %, respectively. Positive and negative predictive values were 96 % and 100 %, respectively. There was one false-positive result among 20 patients with non-tuberculous neurological diseases.

DISCUSSION

The diagnosis of TBM is usually made on the basis of the history of exposure, clinical picture, characteristic CSF, positive tuberculin skin test, and the culture of *M. tuberculosis* in CSF (1, 8). However, cultures are positive in only 55-75 % of patients (1). A rapid and reliable serologic test to detect mycobacterial antigens in body fluids is therefore needed. ELISA is a simple and rapid method for detecting mycobacterial antigens from clinical specimens.

ELISA may be the first choice at that point, but a major problem presenting difficulty for widespread use of ELISA is the false positive results caused by active and passive immunization. However this problem may be eliminated by comparing CSF of TBM patients with appropriate control group.

To eliminate this problem we formed two study groups and a control group for comparison. In group-Ia and group-Ib, ELISA gave similar values. Sensitivity was found to be 100 % and specificity was found to be 95 % as compared with group-II suggesting that this test may safely be used in differentiation of TBM from non-tuberculous neurological diseases. Also, the high sensitivity and specificity obtained in culture negative patients suggest that this test will be valuable in the diagnosis of this hardly recognized patient group.

Results of this study are similar to other studies which using purified antigens. We therefore think that practically and rapidly extracted glycolipid antigen of mycobacterium tuberculosis may be safely used in ELISA to diagnose TBM. This method is cheaper too. However, further clinical studies are needed to compare this serologic study with non-tuberculous meningitis as the antibody levels may be correlated with CSF protein and pleocytosis.

Correspondence to : Dr. Ümit Bilge DOĞAN
Ataç 2 Sok. 55/8
Yenişehir
06420 ANKARA - TÜRKİYE
Phone : 312 - 433 67 79

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