Immunocytochemical Method for Early Laboratory Diagnosis of Tuberculous Meningitis

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A simple immunocytochemical method was standardized for the direct demonstration of mycobacterial antigen in cerebrospinal fluid (CSF) specimens of patients with tuberculous meningitis (TBM). CSF-cytospin smears were prepared from 22 patients with a clinical diagnosis of TBM and also from an equal number of patients with nontuberculous neurological diseases (disease control). Immunocytochemical demonstration of mycobacterial antigens in the cytoplasm of monocytoid cells was attempted, by using rabbit immunoglobulin G to Mycobacterium tuberculosis as the primary antibody. Of the 22 CSF-cytospin smears from TBM patients, 16 showed positive immunostaining, while all of the CSF-cytospin smears from the disease control showed negative immunostaining for mycobacterial antigen. The technical aspects of this immunocytological method for the demonstration of mycobacterial antigens are simple, rapid, and reproducible, as well as specific, and therefore can be applied for the early diagnosis of TBM, particularly in patients in whom bacteriological methods did not demonstrate the presence of M. tuberculosis in the CSF.

MATERIALS AND METHODS

The Sree Chitra Tirunal Institute for Medical Sciences and Technology (referred to here as “the Institute”), located in the Kerala State, is the major tertiary referral center for neurological diseases in Thiruvananthapuram, India. In 2000, 22 patients with a clinical diagnosis of TBM were referred to the Institute from several outpatient hospitals in the Kerala State. Prior to admission, most of these patients (20 of 22) had received ATT for periods ranging between 2 and 7 weeks (rifampin at 450 mg, isoniazid at 300 mg, streptomycin at 500 mg, and ethambutol at 50 mg daily). At the Institute, the diagnosis of TBM in these patients is based on relevant clinical features such as neck rigidity, positive Kernig’s sign, and compatible neuroradiological evidences of basal exudates in magnetic resonance image scan. None of these patients had clinical or radiological evidence of tuberculosis in the lungs. At the Institute, CSF analysis is one of the laboratory investigations performed in the management of patients with meningitis. CSF obtained from the lumbar region was collected from all of these patients and was analyzed by routine cytological, biochemical, microbiological, and immunological methods.

The biochemical parameters in all of the CSF samples showed elevated protein (70 to 900 mg/100 ml) and reduced glucose concentration (10 to 30 mg/100 ml). A cytopsin (Cytoprep; Wescor, USA) was used for the CSF cytological studies. Three cytopsin smears from each CSF sample were prepared. One smear was stained with hematoxylin and eosin and then examined by microscopy. Of 22 CSF smears from TBM patients, 16 showed a mixture of lymphocytes, plasma-cytoid lymphocytes, and monocytoid cells. The number of monocytoid cells ranged between 10 to 65 in individual CSF-cytospin smears. In six TBM patients, the CSF-cytospin smear showed only occasional lymphocytes and monocytoid cells were not present. The second cytopsin smear from each of the 22 TBM patients was fixed in cold acetone and used for immunocytochemical studies. M. tuberculosis was isolated in the CSF samples from 3 of 22 TBM patients by
culture and, in 19 patients, the CSF cultures were repeated twice but they did not grow M. tuberculosis. Acid-fast bacilli were not demonstrated in any of the 22 CSF smears by the Ziehl-Neelsen staining method. CSF specimens from 22 patients with nontuberculous neurological diseases were selected as disease controls. The 22 patients in the disease control group were grouped as follows: 5 had bacterial meningitis due to Haemophilus influenzae (n = 3) or Nisseria meningitidis (n = 2), 5 had partially treated pyogenic meningitis, 2 had cryptococcal meningitis, and 10 had chronic meningitis. The CSF samples from the disease control group were similarly subjected to cytological, biochemical, microbiological, and immunocytological analyses. The CSF samples from the disease control group also showed elevated proteins (60 to 750 mg/100 ml) and reduced glucose concentrations (5 to 30 mg/100 ml). The CSF-cytospin smears in the disease control group showed a mixture of neutrophils, lymphocytes, and monocytoid cells in 18 of 22 CSF-cytospin smears. The number of monocytoid cells in the patients ranged between 0 to 8 cells. In four patients, CSF-cytospin smears showed only a few lymphocytes (<10/mm³) and no monocytoid cells.

**Immunocytocchemical method for the demonstration of mycobacterial antigens.** The cytospin smears from the TBM and disease control groups were simultaneously stained by the immunocytochemical method to demonstrate the presence of mycobacterial antigens. Briefly, the acetone-fixed CSF smears were washed several times with 0.05 M Tris-buffered saline with Tween 20 (pH 7.6) (TBS-T). CSF-cytospin smears were then treated with 3% H₂O₂ for 5 min and washed thrice in 0.05 M TBS-T. Smears were then incubated with primary antibody (20 μg of polyvalent rabbit IgG to M. tuberculosis/ml [10]) for 1 h at 37°C. Subsequently, the smears were incubated with the anti-rabbit IgG-biotin conjugate and streptavidin horseradish peroxidase (Dako LSAB2 System) for 45 min each at room temperature. After that, the smears were washed thoroughly with TBS-T. Smears were then incubated for 10 min at room temperature in a substrate, consisting of diaminobenzidine tetrachloride (10 mg dissolved in 5 ml of 0.05 M TBS-T and 5 ml of 3% H₂O₂). Finally, the smears were counterstained with Harris hematoxylin, dehydrated, cleared in xylene, mounted in Permount (Sigma Chemical Co.), and visualized under a microscope.

**RESULTS**

Of 22 CSF-cytospin smears from TBM patients, 16 showed a mixture of lymphocytes and monocytoid cells (Fig. 1). Approximately, 15% monocytoid cells and lymphocytes showed degenerative changes in their cytoplasm. All of the well-preserved monocyes in the smear showed positive immunostaining for mycobacterial antigens in the form of brownish red granules in the cytoplasm (Fig. 2). About 70 to 80% of monocytoid cells in the smears showed positive immunostaining or mycobacterial antigens. Besides this, aggregates of immunostained extracellular brownish material was also seen in the smears. In six CSF smears of TBM patients, the immunostaining was negative because in these cases the smears showed only a few lymphocytes. Positive immunobinding in the monocytoid cells was also seen in the three TBM CSF samples in which M. tuberculosis was isolated by culture. In order to evaluate the reproducibility of the assay, immunostaining was repeated on the third CSF-cytospin smear in the same patient. There was no variation in the immunostaining pattern. All of the 22 patients received ATT based on the results of the immunocytochemical staining. A total of 16 patients had optimal neurological recovery, and in 6 patients the neurological recovery was suboptimal. None of these CSF-cytospin smears from the patients in the disease control group showed positive immunostaining, indicating that nonspecific immunostaining did not occur by this technique.

**DISCUSSION**

TBM is a potentially curable infectious disease of the CNS, and thus there is a need to design an alternative diagnostic method to the conventional microbiological method for the early laboratory diagnosis of TBM so that an effective therapeutic modality can be instituted quickly in patients with TBM. To meet the above objective, we devised a simple immunocytochemical method. The fundamental principle of this assay is that the CSF in patients with TBM during active stages of the disease contains monocytoid cells (macrophages) and lymphocytes. The function of these monocytes is to phagocytose the tubercle bacilli and process the antigenic component of the bacilli. Thus, the cytoplasm of the monocytes in patients with
TBM during the active stages contain mycobacterial antigen. The presence of mycobacterial antigens in these monocytes has been demonstrated by an immunocytochemical method in this study. We used rabbit IgG to *M. tuberculosis* as the primary antibody to demonstrate mycobacterial antigens in the CSF smears. Of 22 CSF-cytospin smears from patients with TBM, 16 showed positive immunostaining, while cytospin smears from 6 TBM patients yielded negative immunostaining because there was a paucity of monocytypoid cells in these CSF smears. There was no false-positive immunostaining in the CSF smears from the disease control group.

Earlier immunoassays described in the literature for the detection of mycobacterial antigen in the CSF of patients with TBM include the latex agglutination test with anti-plasma membrane antibody (5), a sandwich enzyme-linked immunosorbent assay (ELISA) with anti-BCG antibody (12), and an inhibition ELISA with polyvalent antibody against *M. tuberculosis* (2). In our earlier study, we also used a Dot-Ilba to detect a 14-kDa mycobacterial antigen in the CSF of patients with TBM (13). The goal of these earlier studies was to detect the circulating mycobacterial antigens in the CSF of patients with TBM. In the present study, however, we have demonstrated the presence of mycobacterial antigens in the monocytopoid cells of the CSF. This method carries a sensitivity of 72.5% (16 of 22) and a specificity of 100%. The technical part of the assay is much more simple than the methodology described in earlier studies. The result of this assay can be easily visualized under the microscope and can be obtained within 5 h of the receipt of CSF samples in the laboratory. The presence of an adequate number of monocytopoid cells (>5/high-power field) in the CSF is essential for immunostaining, and this should be ascertained in the intimal hematoxylin-and-eosin-stained smear. A positive result obtained by this immunocytochemical method has potential diagnostic application in patients with TBM. Hitherto, a similar study has not been described in the literature. We therefore consider this newer diagnostic approach to have potential application for the laboratory diagnosis of TBM, particularly in patients for whom bacteriological methods did not confirm the diagnosis.

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