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**Abstract**

Tuberculous meningitis cases were analyzed by an immunoblotting test based on Mycobacterium bovis BCG antigen complex A60. Anti-A60 immunoglobulin G (IgG) in cerebrospinal fluid (CSF) allowed early diagnosis, and concentrations decreased after recovery. In primary meningitis forms, anti-A60 IgGs were intrathecally synthesized and specific oligoclonal IgGs were present in CSF. In meningeal complications of pulmonary tuberculosis, there were matching titers of anti-A60 IgG in blood and CSF (mirror pattern). Correlation between CSF-restricted patterns and CSF pleocytosis was shown.

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Analysis of Tuberculous Meningitis Cases by an Immunoblotting Assay Based on a Mycobacterial Antigen Complex

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A recent increase in tuberculosis incidence and complications has been registered in connection with the spread of antibiotic resistance and AIDS. Tuberculous meningitis is a complication invariably lethal without early therapy (10, 33); however, identification of Mycobacterium tuberculosis in the cerebrospinal fluid (CSF) is a rare event, and in vitro culture is a lengthy process. Detection of PCR-amplified mycobacterial DNA by nucleic acid probes (32, 40) and of anticytobacterial immunoglobulins in CSF by radioimmunoassays (30), enzymometric methods (enzyme-linked immunosorbent assay) (1, 6, 7, 15–17, 25–27, 29, 31, 34, 38, 39, 44, 46), and latex agglutination (36) have been explored insufficiently at the clinical level. Literature in this field has been extensively reviewed (8, 12, 22, 35, 45).

The thermostable macromolecular antigen complexes from mycobacteria (12, 20) (A60 of Mycobacterium bovis and A36 of Mycobacterium paratuberculosis) elicit humoral and cellular immune reactions (3, 13) and are immunodominant in mycobacteria (12). A60- and A36-based immunoassays have been applied to diagnosis of tuberculosis (2, 9, 12, 21, 23, 24) and paratuberculosis (18), respectively. We have previously explored the use of immunoassays in meningitis (4, 5) and described an A60-based immunoblotting procedure for analysis of the CSF of tuberculous meningitis patients (41). By this method, a very large proportion of anticytobacterial immunoglobulin G (IgG) in patients’ CSF was shown to be directed against A60 (41). This study is complemented by the present work, in which samples of blood and CSF from patients with proven or suspected tuberculous meningitis were subjected to A60 immunoblotting analysis at different phases of the disease.

Our study was carried out on 80 CSF samples from 32 Chinese patients (19 females and 13 males) of various ages (between 5 months and 66 years): 15 patients with proven tuberculous meningitis (positive Ziehl-Neelsen staining and/or CSF culture) and 17 patients with suspected tuberculous meningitis (negative CSF culture). Some patients (25 of 32) had radiographically confirmed pulmonary tuberculosis with open lesions (18 of 25 exhibited sputum positivity). Only one individual had been vaccinated with M. bovis BCG and was positive in purified protein derivative cutaneous testing. Subjected to standard chemotherapy, two patients died, whereas the others recovered either completely (20 of 32) or partly (10 of 32) (residual sequelae and/or impairment of blood-CSF barrier). Standard CSF analyses (cell number and glucose) were performed periodically. A control group included 2 healthy persons and 15 nontuberculous patients (1 leukemia, 1 lupus erythematosus, 1 Guillain-Barré syndrome, 1 cerebral cysticercosis, 4 meningococcal meningitis, 2 viral meningitis, 2 hypertensive headache, 2 ischemic stroke, 1 cerebral tumor). Cases of uncomplicated miliary tuberculosis or miliary tuberculosis complicated with tuberculous meningitis were included in our study.

At different times (up to 14 years after the clinical onset), paired samples of CSF and serum were collected and kept frozen at −20°C. Protein content was determined by a nephelometric micromethod using benzenthonium chloride in microtiter plate wells (37), and IgG levels were determined by immunoturbidimetry (Behring, Marburg, Germany). Serum samples were diluted with H2O to the same IgG concentration as the paired CSF samples. Aliquots (10 μl) of CSF and diluted sera were applied side by side on agarose gel plates (pH gradient, 3.5 to 9.5), which were prepared in distilled water with 0.36 g of Agarose-IEF, 2 ml of Ampholine (Pharmacia, Uppsala, Sweden), and 4.3 g of sorbitol (Merck, Darmstadt, Germany). Samples were isoelectrically focused for 70 min at 10°C in an electrophoretic unit (Multiphor; LKB, Uppsala, Sweden).

Gels were blotted (40 min, 10°C) onto A60-loaded polyvinylidine difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, Mass.) under a uniform weight of 1 kg. A60-PVDF membranes were prepared as follows. PVDF membranes were successively steeped in methanol and water, incubated overnight with A60 (100 μg/ml), and rinsed three times for 20 min with TBST buffer (500 mM NaCl, 20 mM Tris-HCl [pH 7.5] containing 0.05% Tween 20). A60 prepared from the cytoplasm of M. bovis BCG (Pasteur Institute, Brussels, Belgium) by exclusion gel chromatography on Sepharose 6B columns (Pharmacia), as described previously (14), was identified by two-dimensional immunoelectrophoresis according to an established reference system (11) and quantitated by spectrophotometric measurement of its protein content (20). Immunoblots were washed with TBST buffer and fixed with glutaraldehyde (0.25% solution in TBST buffer, 20 min, 4°C) (28). After repeated washings with buffer, membranes were incubated with alkaline phosphatase-labeled anti-
human IgG (Bio-Rad, Richmond, Calif.) 1/1,500 dilution in TBST buffer containing 0.33% defatted milk, 60-min incubation at room temperature), followed by three 5-min washings with buffer and staining with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad).

Samples from controls (healthy persons and persons with nontuberculous neurological diseases) did not contain anti-A60 IgG. Immunoblots from all tuberculous meningitis patients invariably bore stained bands of anti-mycobacterial IgG.

In most cases of tuberculous meningitis complicating miliary and postprimary pulmonary tuberculosis, the intensities of stained bands in paired CSF and serum samples (taken from the same individual at the same time) were comparable (mirror patterns in Fig. 1). However, in some patients (primary tuberculous meningitis without severe lung complications), the intensity of stained bands in CSF was higher than that of the paired serum sample, thus indicating an intrathecal synthesis of specific immunoglobulins. In the latter case, CSF-restricted oligoclonal bands (Fig. 2, arrows) were also present, an additional proof of in situ antitycobacterial IgG formation. Similar observations, in cases of viral meningoencephalitis, have been related previously (19, 42, 43). Samples taken with increasing frequency from the same subject showed A60-specific IgG bands of increasing intensities, which disappeared, in parallel with CSF pleocytosis, after recovery, in agreement with previous reports (39, 41). However, in a few instances (severe meningitis with permanent sequelae), high levels of antitycobacterial IgG were still present 2 years after disease onset (Fig. 3).

FIG. 1. A60 immunoblotting analysis of CSF and sera from meningitis patients. Immunoblots of A60-specific IgG from paired samples of CSF and serum (SER), diluted to the same IgG concentration, after isoelectric focusing in agarose gel and immunoaffinity transfer to PVDF sheets coated with A60. Two samples from patients with purulent meningitis (PM) and viral meningitis (VM) were used as controls. In lanes 2 (tuberculous meningitis complicated by postprimary tuberculosis) and 3 (tuberculous meningitis complicating miliary tuberculosis), A60-specific IgG oligoclonal bands of similar intensities were stained in CSF and serum (mirror pattern). In lanes 4 and 6 (tuberculous meningitis alone) and lane 5 (tuberculous meningitis complicating miliary tuberculosis), A60-specific CSF-restricted oligoclonal IgG indicated an intrathecal synthesis.

FIG. 2. A60 immunoblots of CSF and serum samples taken from a patient at different stages of disease. Immunoblots of A60-specific IgG from paired samples of CSF and serum (SER), diluted to the same IgG concentration, after isoelectric focusing in agarose gel and transfer by immunoaffinity to PVDF sheets coated with A60. Paired samples were collected, at days 9, 16, and 37 after clinical onset, from a patient with tuberculous meningitis complicating miliary tuberculosis. The total amounts of IgG in gels were 19.8, 6.5, and 7.3 μg, respectively. The greater intensity of staining of some oligoclonal IgG antibodies in the CSF indicated an intrathecal synthesis (arrows).

FIG. 3. A60 immunoblots of CSF and serum samples taken from a patient about 2 years after onset of disease. When admitted to the hospital, the patient presented with severe meningitis and, after recovery, permanent sequelae. The greater intensity of staining of some oligoclonal IgG antibodies in the CSF and the characteristic CSF restriction of these bands (arrows) indicated an intrathecal synthesis of antitycobacterial immunoglobulins.
CSF pleocytosis (Table 2). Most samples (18 of 21) with normal cell numbers in CSF had similar anti-A60 IgG titers in blood and serum (mirror pattern), whereas in samples with pleocytosis 27 of 56 displayed CSF-restricted anti-A60 IgG (intrathecal synthesis) \( (P < 0.01) \). In this connection, it ought to be mentioned that, while all of the analyzed adult cases of primary meningitis presented with intrathecal synthesis of antityphococcal antibodies, newborns (Table 1, first row) displayed mirror patterns.

Since mycobacterial staining in CSF is invariably negative and culture positivitity can be demonstrated, after long delays, in only a minority of cases, early diagnosis of tuberculous meningitis (which is essential for recovery) ultimately relies on sensitive and fast molecular biology techniques. Hybridization of labeled probes with segments of the mycobacterial genome, duly amplified by PCR, is presently investigated in several laboratories, including ours. An alternative procedure is the serological identification, in the CSF, of antityphococcal immunoglobulins. The handicap of the PCR probe approach is the expansion of contaminating signals. The immunoblot technique, which exploits the clonal expansion of specific B lymphocytes, is less prone to contamination problems. In addition, appearance and disappearance of specific oligoclonal IgG species during the course of the disease may provide precious information about the efficiency of chemotherapeutic intervention.

The following conclusions can be drawn from our data. (i) Anti-A60 CSF IgG is an early symptom of tuberculous meningitis. (ii) Anti-A60 IgG titers decrease in parallel with pleocytosis upon successful therapy. (iii) Matching anti-A60 IgGs in blood and serum (mirror pattern) are common in cases of meningeal complications of pulmonary tuberculosis. (iv) CSF-restricted anti-A60 IgGs (intrathecal synthesis) are specific to primary tuberculous meningitis and meningeal complications of miliary tuberculosis.

**REFERENCES**


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**TABLE 1. Clinical data and anti-A60 antibody production in CSF of tuberculous meningitis patients**

<table>
<thead>
<tr>
<th>Groups (no. of cases)</th>
<th>Microbiological analysis*</th>
<th>PPD + skin test (no. positive)</th>
<th>No. (%) showing intrathecal synthesis of anti-A60 antibodies</th>
<th>No. showing mirror pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSF + Sputum +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuberculous meningitis alone (7)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>5 bacteriologically proven</td>
<td>5</td>
<td>0</td>
<td>5 (71)</td>
<td>2</td>
</tr>
<tr>
<td>2 clinically suspected</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Primary pulmonary tuberculosis + tuberculous meningitis (3)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1 bacteriologically proven</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2 clinically suspected</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Miliary tuberculosis + tuberculous meningitis (18)</td>
<td>8</td>
<td>4</td>
<td>8 (44)</td>
<td>10</td>
</tr>
<tr>
<td>8 bacteriologically proven</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>10 clinically suspected</td>
<td>0</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Postprimary pulmonary tuberculosis + tuberculous meningitis (4)</td>
<td>1</td>
<td>1</td>
<td>0 (0)</td>
<td>4</td>
</tr>
<tr>
<td>1 bacteriologically proven</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3 clinically suspected</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* Number of cases positive. Microbiological positivity in CSF was obtained by culture. Some of the culture-positive sputum samples were also identified by staining.

* PPD, purified protein derivative.

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**TABLE 2. Correlation between CSF pleocytosis and intrathecal synthesis of anti-A60 antibodies**

<table>
<thead>
<tr>
<th>No. of cells in CSF (cells/μL)*</th>
<th>No. of samples with the following immunoblotting pattern:</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mirror pattern</td>
<td>CSF-restricted pattern</td>
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<tr>
<td>≤8</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>&gt;8</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>30</td>
</tr>
</tbody>
</table>

* Correlation between the CSF-restricted pattern and pleocytosis was found to be significant (two-tailed P value, 0.071141) according to Fisher's Exact Test.

* The normal range of cells in control CSF samples is 1 to 8 cells per μL.


