Immunodiagnosis of tuberculous meningitis: rapid detection of mycobacterial antigens in cerebrospinal fluid by reverse passive hemagglutination assay and their characterization by Western blotting

Muralidhar K. Katti *

Immunology Laboratory, Department of Microbiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram - 695 011, India

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Abstract

Tuberculous meningitis (TBM) is one of the commonest chronic infections of the central nervous system (CNS). Diagnosis of TBM has been a problem as it causes various clinical manifestations which can be confused with those of other chronic infections of the CNS such as neurocysticercosis (NCC), neurobrucellosis and cryptococcal menigitis, that are prevalent in many underdeveloped and developing countries. Differential diagnosis of TBM can be made by detecting circulating mycobacterial antigens in CSF by immunoassays. In this study, a reverse passive hemagglutination (RPHA) has been developed using rabbit antimycobacterial IgG for detection of circulating mycobacterial antigens in CSFs from chronic infections of the CNS in order to develop a rapid, simple, sensitive and cost-effective method. Circulating mycobacterial antigens were characterized by immunoblot assay. The sensitivity limit of RPHA was 400 ng ml \(^{-1}\). RPHA was specific as antimycobacterial IgG did not show any reaction with porcine *Cysticercus cellulosae* which was used as a control antigen. RPHA could detect mycobacterial antigens in CSF at a sensitivity level of 94.11% with a specificity of 99.0%. Immunoblot analysis of RPHA positive CSFs revealed predominantly 30–32 kDa and 71 kDa antigens whilst 6, 86, 120, 96 and 110 kDa showed varied degree of reactivity. Antigens of masses 30–32 and 71 kDa were absent in culture filtrate of *Mycobacterium tuberculosis* H37Rv grown in Proskauer–Beck liquid medium. RPHA is a rapid, simple and sensitive immunological method with a long shelf life of 6–8 weeks if stabilized coated erythrocytes are stored at +4°C. RPHA could be used as an additional immunodiagnostic tool in both differential diagnosis and prognosis of TBM. Immunoblot results indicate that 30–32 kDa and 71 kDa antigens are cell wall derived.

Keywords: CSF; Tuberculous meningitis; Immunodiagnosis; RPHA; Western blotting; *Mycobacterium tuberculosis*

1. Introduction

Tuberculous meningitis (TBM) is one of the commonest and serious forms of extra pulmonary tuberculosis (TB) prevalent in many developing and underdeveloped countries [1]. The diagnosis of TBM has been problematic as it causes various clinical manifestations that can be confused with those of other chronic diseases of the central nervous system (CNS) such as neurocysticercosis (NCC), cryptococcal and carcinomatous menigitis. TBM is often diagnosed by clinical criteria combined with imaging techniques (computed tomography (CT) and magnetic resonance (MRI)). The laboratory 'gold standard' for definitive diagnosis of TBM is by demonstrating acid-fast bacilli either in direct smear or culture [1]. Since these methods are insensitive (smear technique) and time consuming (culture technique), additional immunodiagnostic methods such as antigen and or antibody demonstration in CSF samples have become necessary [2]. Several immunoassays have been described for detection of antimycobacterial antibodies [3–5] and mycobacterial antigens [6–9] in CSFs of TBM patients with varied degrees of success.

In this study, a reverse passive hemagglutination (RPHA) assay has been developed and applied to different groups of CSF samples using rabbit antimycobacterial...
IgG (polyclonal antibody) to detect circulating mycobacterial antigens in order to develop a simple, rapid and sensitive antigen detection immunoassay in differential diagnosis of TBM. Further circulating mycobacterial antigens of CSFs were analyzed by Western blot assay that could have importance in use as diagnostic markers for TBM.

2. Materials and methods

2.1. Antigens

2.1.1. Mycobacterium tuberculosis sonicate extract (MTSE)

The MTSE was prepared as described elsewhere [10]. Briefly, *M. tuberculosis* H37Rv strain obtained from National Tuberculosis Institute (NTI) Bangalore, India, was harvested in phosphate buffered saline (PBS), pH 7.2, from a culture grown to its mid-log phase, on Lowenstein–Jensen medium. The bacterial pellet was washed, heat killed at 60°C for 1 h and subjected to ultrasonication (15% pulse, 150 W) in an ice bath. The sonicate was centrifuged at high speed (17 000 × g) at +4°C for 30–40 min. The protein content of supernatant (MTSE) was estimated with 33% ammonium sulfate, dialyzed and stored frozen (−20°C) until use.

2.1.2. Culture filtrate antigens of *M. tuberculosis* H37Rv (CFA)

Briefly, *M. tuberculosis* H37Rv was grown to its late log phase in Proskeur–Beck liquid medium at 37°C for 4–6 weeks. The culture filtrate was collected by membrane filtration using a pore size of 0.22 μ. The CFA was concentrated, dialyzed and stored frozen (−20°C) until use. CFA was used to study the antigenic relationship between antigens of cell wall and secreted/metabolic products of *M. tuberculosis*.

2.1.3. Porcine whole cyst sonicate extract (PCSE)

This was prepared by ultrasonic treatment as described elsewhere [12]. PCSE was used as a control antigen to assess the specificity of immunoassays since neurocysticercosis is the commonest parasitic disease of the CNS in this part of the world.

2.2. Antibodies

2.2.1. Antimycobacterial IgG

A weight of 1.5–2.0 mg of MTSE protein was injected intradermally to rabbits at multiple sites on days 1, 8, 15, 43 and 58. Sera were collected on days 0 (preimmune serum), 7, 14, 21, 50 and 65 after the start of immunization. Hyperimmune immunoglobulin were fractionated with 33% ammonium sulfate, dialyzed and estimated for protein content [11] and stored in aliquots at −20°C.

2.2.2. CSF

A total of 182 CSF samples were obtained from patients and controls as follows. Group I: TBM (*n* = 51), the diagnosis of TBM was established on the basis of clinical features plus one or more of the following criteria: CSF parameters (pleocytosis, elevated protein and low glucose level) and neuroimaging evidence of hydrocephalus and basilar exudates [13]. Group II: controls which included (a) non-infectious non-neurological conditions (*n* = 29), CSFs obtained during spinal anesthesia from healthy individuals; (b) infectious neurological conditions (*n* = 10) including culture proven pyogenic meningitis, cryptococcal meningitis, serologically confirmed viral meningitis and surgically proven neurocysticercosis; and (c) non-infectious neurological conditions (*n* = 92) such as disc prolapse, carotid insufficiency, carcinomatous meningitis.

2.2.3. RPHA test

Coupling of antimycobacterial immunoglobulin on to chymotrypsin treated sheep erythrocytes (SRBC) using chromium chloride (CrCl₂) was done as described earlier [14]. Stabilization of coupled cells was done by the method of Cranage et al. [15]. Briefly, to a 50 μl of chymotryptsinized sheep erythrocytes, 50 μl of antimycobacterial IgG (500 μg ml⁻¹) was dispensed, while vortexing 80 μl of 0.033% CrCl₂, pH 5.0 and 20 μl of HEPES buffer, pH 5.0 were added. After 1 h of incubation at room temperature on a rotator, the coated cells were washed three times with PBS, pH 7.2 and stabilized with 0.1% glutaraldehyde for 1 h at room temperature. 1% of stabilized coated cells were used for detection of mycobacterial antigen.

Control cells were prepared using IgG from normal rabbit serum. Coupled, stabilized cells were stored at +4°C.

The sensitivity of RPHA was determined by titrating various concentrations of MTSE (0.062–128 μg ml⁻¹) in 25 μl volumes in U-bottom microtiter plates (Dynatech, USA) containing 1% BSA in 25 μl of PBS (diluent). A total of 25 μl of coated cells were dispensed per well and shaken vigorously for 2 min and hemagglutination patterns scored according to Stavitsky [16] after 2 h of incubation at room temperature.

CSFs were absorbed prior to titration as above with equal volumes of 10% control cells overnight at +4°C.

The specificity of RPHA was assessed by titrating 50 μg ml⁻¹ of control antigen (PCSE).

2.2.4. Western blotting

Test and control CSFs were separated in 10% SDS–PAGE [17] and electroblotted [18] onto nitrocellulose (NC). They were probed with 1:100 dilution of antimycobacterial IgG at +4°C overnight in PBS, pH 7.2 with 2% BSA, followed by antirabbit IgG peroxidase (1:500, Gen- ei, India) in a diluent containing 2% normal rabbit serum.
3. Results

3.1. RPHA

The lowest levels of soluble MTSE antigens detected were 400 ng ml⁻¹. The test was found to be specific as control antigen (PCSE) tested showed no reactivity. For RPHA, a visual reaction at the dilution of 1:4 was chosen as positive for mycobacterial antigen. Results of RPHA obtained in CSFs of various groups are shown in Table 1. Soluble antigens of *M. tuberculosis* were demonstrable in 94.11% of the cases by RPHA. The specificity level of this assay as assessed against CSFs from control individuals was 99.0%.

3.2. Western blotting

Western blotting analysis of a CSF pooled from clinically confirmed TBM patients revealed three major circulating mycobacterial antigens with masses of 30–32 kDa, 71 kDa and 120 kDa. They were not detectable in pooled CSFs from the spinal anesthesia control group (Fig. 1). Immunoblot analysis of RPHA positive CSFs revealed the presence of mycobacterial antigens with masses of 30–32 kDa consistently (Fig. 2, Table 2) and other antigens with masses of 71 kDa, 6 kDa, 120 kDa and 86 kDa with varied degree of reactivity whereas individual CSFs from a control group of patients (spinal anesthesia and infectious neurological conditions) and control antigen (PCSE) showed no reactivity. However one of 99 CSFs from the non-infectious neurological control group showed 110 kDa and 86 kDa mycobacterial antigens (Table 2). Results of the study would indicate that mycobacterial antigens of 30–32 kDa and 71 kDa seem to have been derived from...
bacterial cell wall, as they were not seen in culture filtrate antigen (Fig. 3).

4. Discussion

In the absence of bacterial evidence in CSF, the detection of specific mycobacterial antibody and/or antigen would be of a great value in diagnosis of TBM [9]. Detection and quantitation of mycobacterial antigens (secreted or the components of organism per se) would be superior to antibody detection in differential diagnosis and prognosis of the disease. The relative merits of antigen detection versus antibody analysis have been reviewed [19]. Previous studies have shown encouraging results using polyclonal immunoglobulin to components of *M. tuberculosis* for the detection of mycobacterial antigens in CSFs of TBM by latex agglutination [20] and by tandem immunoassay [7]. In this study, the rabbit mycobacterial IgG coupled to sheep erythrocytes (RPHA) has been used to test for mycobacterial antigens in CSFs of chronic meningitis patients and control groups of patients. Because RPHA is a simple, rapid and sensitive immunological method with a long shelf life of 6–8 weeks when stabilized coated erythrocytes are stored at 4°C, it does not require any sophisticated equipment. Non-specific binding of human (CSF/serum) and rabbit proteins and heterophile activity of sheep erythrocytes in human CSFs were eliminated by absorbing each CSF with 10% control cells in RPHA, while in immunoblotting blotted membranes were incubated with 2% normal rabbit serum. The sensitivity limit of RPHA was 400 ng ml$^{-1}$ while it was 45 ng ml$^{-1}$ in our previous studies [21]. Variation in the sensitivity limit of RPHA may be due to differences in titers of antibodies formed against each antigenic component in different hosts. RPHA appears to be highly specific as antimycobacterial antibody coupled erythrocytes did not react with control antigens. The sensitivity of RPHA in CSFs of TBM was 94.11% with a specificity of 99%. Non-detection of mycobacterial antigens in three cases of TBM may be because of high titered antibody masking the antigen [6]. The sensitivity of RPHA using monoclonal antibody was 88% in culture proven TBM cases [6]. The specificity of any immunoassay including RPHA would depend upon the type of specific probe employed in the system [22].

In immunoblotting, antigens of masses 30–32 kDa, 71 kDa and 120 kDa could be characterized (Fig. 1). The presence of these antigens in CSFs of TBM patients consistently, their absence in control groups of CSFs and in control antigens (PCSE) would indicate that these antigens are specific to *M. tuberculosis* and could be considered as a diagnostic marker for TBM (Figs. 2 and 3, Table 2). Results of this study indicate that 30–32 kDa and 71 kDa antigens were cell wall associated and not a secretory product as their presence was not observed in culture filtrate antigens of *M. tuberculosis* when grown in Proskauger–Beck medium (Fig. 3). This may be due to physicochemical differences between secreted and intracellular proteins with regard to charge properties [23]. Besides diagnostic importance, 30–32 kDa antigens seem to have an important role in pathogenesis of tuberculosis [24] as that has been known to bind fibronectin [25]. 30–32 kDa and

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**Fig. 3.** Immunoblot analysis of mycobacterial antigens: 30–32 kDa circulating antigens in CSFs of TBM patients (lanes 1 and 2); absence of mycobacterial antigens in a control group of spinal anesthesia CSFs (lane 3); absence of 30–32 kDa circulating antigens in culture filtrate antigen *M. tuberculosis* (lane 4); non-reactivity of antimycobacterial IgG with control antigens, PCSE (lane 5); and MTSE was used as positive control antigen (lane 6).

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**Table 1**

Detection of antigens of *M. tuberculosis* in CSFs of chronic meningitis patients and controls by RPHA using rabbit antimycobacterial antibody

<table>
<thead>
<tr>
<th>Group</th>
<th>Number tested</th>
<th>Number of positive responses*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic meningitis (Group I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBM</td>
<td>51</td>
<td>48</td>
</tr>
<tr>
<td>Controls (Group II)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Non-infectious non-neurological conditions</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>(b) Infectious neurological condition</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>(c) Non-infectious neurological condition</td>
<td>92</td>
<td>1</td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td>94.11%</td>
</tr>
<tr>
<td>Specificity</td>
<td></td>
<td>99.1%</td>
</tr>
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*A visual reaction at a dilution of 1:4 was considered as positive.*
71 kDa antigens have been shown to induce protective immunity in the host [26]. Thus RPHA is sensitive enough to detect major mycobacterial antigens including 30–32 kDa and 71 kDa (Table 1) as evidenced by Western blotting. The merits of RPHA are that it is simple, rapid and cost-effective with a long shelf life of 6–8 weeks if stabilized. Coated cells are stored at +4°C. Therefore RPHA could be used as an additional immunodiagnostic tool in the differential diagnosis of TBM.

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References


