Assessment of antibody responses to antigens of *Mycobacterium tuberculosis* and *Cysticercus cellulosae* in cerebrospinal fluid of chronic meningitis patients for definitive diagnosis as TBM/NCC by passive hemagglutination and immunoblot assays

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Abstract

Tanned sheep erythrocytes stabilized with pyruvic aldehyde and glutaraldehyde, called double-aldehyde-stabilized cells, were used to standardize passive hemagglutination assay (PHA) for detection of antibody responses to sonicate extract of *Mycobacterium tuberculosis* and *Cysticercus cellulosae* soluble antigens. PHA was performed in the following groups of cerebrospinal fluid (CSF) samples: group I – chronic infections of the central nervous system with the possible diagnosis of tuberculous meningitis (TBM), tuberculoma and neurocysticercosis (NCC) (*n* = 88), and group II – controls which included (a) non-infectious non-neurological conditions (*n* = 30), (b) infectious neurological conditions (*n* = 21) and (c) non-infectious neurological conditions (*n* = 133). PHA could detect antimycobacterial antibodies at the sensitivity level of 80.76% with a specificity of 92.4% and anti-cysticercal antibodies with a sensitivity of 100% and specificity of 92.94%. However, in 6.33% (i.e. 14/221) of group I and group II (c) CSFs both anti-mycobacterial and anti-cysticercal antibodies were detected. Immunoblot analysis of CSFs derived from TBM patients reacted predominantly to 120-kDa, 96-kDa, 65-kDa, 38-kDa, 26-kDa, 23-kDa, 19-kDa and 12–14-kDa and 4–6-kDa antigens of *M. tuberculosis* sonicate extract (MTSE), whilst CSFs of proven NCC reacted to 110-kDa, 96-kDa, 80-kDa, 66–68-kDa, 52-kDa and 26–28-kDa antigens of porcine whole cyst sonicate extract (PCSE). On immunoblot analysis, some of the CSFs of TBM patients were PHA positive for both MTSE and PCSE showed antibody reactivity to 70-kDa and 10-kDa antigens of *C. cellulosae*. Similarly CSF antibody of some Guillain Barre syndrome and myeloradiculopathy patients reacted with cysticercal antigens. But per se no cross-reactivity between MTSE and anti-cysticercal antibodies and vice-versa were observed. However, findings of this study should alert laboratory personnel especially in endemic areas to be extra careful in interpretation of antibody detection results. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Tuberculous meningitis; Neurocysticercosis; Passive hemagglutination; Enzyme-linked immunosorbent assay; Immunoblot assay; Immunodiagnosis

1. Introduction

Chronic infections of the central nervous system (CNS) that are prevalent in under-developed and developing countries are either of bacterial (neurotuberculosis, neurobrucellosis, neurosyphilis), parasitic (neurocysticercosis (NCC), toxoplasmosis), fungal (cryptococcal meningitis) or viral (HIV-1, HTLV-III) origin [1]. Clinicians often have difficulty in differential diagnosis of chronic meningitis in determining whether it is tuberculous meningitis (TBM), NCC, neurobrucellosis or cryptococcal meningitis, because these infections are highly endemic in many under-developed and developing countries, and various clinical manifestations of TBM overlap with those of other diseases of the CNS [2]. Because of insensitive (Zeil–Nelson staining for acid-fast bacilli) and time consuming (culture) techniques, an etiological confirmation is essential for further management and that can be achieved by immunological methods. Several immunoassays have been described for the detection of antibody and antigen for diagnosis of TBM and NCC [3–9]. However, immunodiag-
nostic systems also suffer from serious problems of offering conclusive diagnosis as TBM or NCC, when sonicate extracts of Mycobacterium tuberculosis and Cysticercus cel-
losae were used either in enzyme-linked immunosorbent assay (ELISA), agglutination or immunoblot test systems.

In this study we report our observation on cerebrospinal fluid (CSF) antibody reactivity to antigens of both M. tuberculosis and C. cellulosae in some of the TBM patients and not in all TBM cases, by passive hemagglutination assay (PHA), and their analysis by immunoblot assays. Double-aldehyde-stabilized (DAS) cells were used in PHA after the treatment of sheep erythrocytes with pyruvic aldehyde and glutaraldehyde, as it offers several notable advantages such as rapidity, simplicity, cost-effectiveness, long shelf-life and stability of sensitized and unsensitized DAS cells, non-susceptibility to lysis, non-leaching of bound antigens from cells, increased agglutinable property of cells and a high degree of sensitivity [10,11].

2. Materials and methods

2.1. Antigens

2.1.1. M. tuberculosis sonicate extract (MTSE)

MTSE was prepared as described elsewhere [12]. Briefly, M. tuberculosis strain H37Rv 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-exponential 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 17,000 g at 4°C for 30–40 min. The protein content of the supernatant was estimated from band positions relative to molecular mass markers.

2.1.2. Porcine whole cyst sonicate extract (PCSE)

This was prepared by ultrasonic treatment as described elsewhere [8]. Briefly, non-ruptured whole cysts carefully dissected from infested pork were washed, homogenized in PBS, pH 7.2, ultrasonicated and centrifuged at high speed (17,000 × g) at 4°C for 30–40 min. The protein content of the supernatant was estimated by the Bradford method [13]. The supernatant was aliquoted and stored frozen (−20°C) until use.

2.2. Antibodies

2.2.1. Hyper immune sera

Rabbit antisera were raised against MTSE and PCSE by injecting rabbits intradermally with 1.5 mg antigen of each, bleeding the animals 8–10 days later and purifying IgG from sera.

2.2.2. CSF

A total of 272 CSFs were obtained from patients and controls as follows: group I: chronic infections of the CNS as TBM/NCC (n = 88) based on clinical findings and one or more of the following criteria: (i) CSF parameters (cells, protein and glucose levels), (ii) neuro-imaging and (iii) evidence of concomitant extra neurological tuberculosis/cysticercal localization [1]. Group II: controls which included (a) non-infectious non-neurological conditions (n = 30) – CSFs obtained during spinal anesthesia from healthy individuals, (b) infectious neurological conditions (n = 21), including culture diagnosed pyogenic meningitis and serologically confirmed viral meningitis and (c) non-infectious neurological conditions (n = 133), such as disc prolapse, carotid insufficiency, Guillain Barre syndrome (GBS).

2.3. Immunoassays

2.3.1. PHA

DAS cells were prepared by treating sheep erythrocytes with tannic acid, pyruvic aldehyde and glutaraldehyde as described elsewhere [14]. Defined amounts (200 μg) of MTSE and PCSE were coupled to DAS cells, which were titrated against serial two-fold dilutions in PBS–bovine serum albumin of CSFs, starting at a dilution of 1:4. Hemagglutination reactions were read as described by Sta-vitsky [15]. All CSFs were pre-absorbed with an equal volume of 20% unsensitized DAS cells at 4°C overnight.

2.3.2. Immunoblot assays

MTSE and PCSE were separated in a 10% homogeneous gel [16] and electro-blotted onto nitrocellulose (NC) membranes [17]. Control and test CSFs were diluted 1:10 in diluent and incubated with NC strips bearing MTSE and PCSE antigens for 2 h. Diamine benzidine tetrahydrochloride was used to develop the strips and protein masses were estimated from band positions relative to molecular mass markers.

3. Results

The results of PHA using MTSE and PCSE preparations are shown in Table 1. A visual reaction at the dilution of 1:4 was considered as positive response since CSFs of control group II (a) did not show agglutination reactions. Our case records showed 26 out of 88 (group I) patients were confirmed as TBM and three as NCC. PHA could detect anti-mycobacterial antibodies in CSFs of TBM patients at the sensitivity level of 80.76% with a specificity of 92.4%, and anti-cysticercal antibodies in all three patients (100%) with a specificity of 92.94%. Reactivity to both MTSE and PCSE was observed in 8/88 (9.91%) of group I and 6/133 (4.51%) of group II (c).
CSFs (Table 1). Of these eight patients, five were diagnosed as TBM and three as myelopathy.

Immunoblot analysis of CSFs derived from TBM patients recognized predominantly 120-kDa, 65-kDa, 23-kDa, 19-kDa, 14-kDa, 12-kDa and 4-6-kDa antigens of MTSE whereas CSF antibody of NCC patients showed reactivity to 110-kDa, 96-kDa, 80-kDa, 52-kDa and 29-kDa antigens of PCSE (Fig. 1A,B). Immunoblot analysis of CSFs of group II including the case of GBS and myelopathy recognized 70-kDa and 10-kDa antigens of PCSE (Fig. 2). Some of the TBM patients that were positive for both MTSE and PCSE by PHA showed antibody reactivity to antigens of C. cellulosae (Fig. 3).

4. Discussion

With the advent of newer sensitive techniques like enzyme immunoassays, radioimmunoassay, the use of (hem)agglutination assays has been limited or neglected. At the same time hemagglutination assays have interested some researchers with the availability of chemicals like diazotizing compounds or aldehydes to treat erythrocytes [10,14]. Earlier studies have demonstrated the effects of treatment of erythrocytes with chemicals like tannic acid resulting in aggregation by reducing the surface potential of erythrocytes [18] and aldehydes making the cell surface anionic by elimination of amino groups rendering the cell more lipophilic. Their effects were thought to increase the affinity of the cells for more protein through salt-like or non-ionic forces [10,19,20].

In view of the problems associated with the clinical diagnosis of chronic infections of the CNS as TBM and NCC, it was decided to investigate antibody responses to both mycobacterial and cysticercal antigens. Since the hosts’ response is heterogeneous total sonicate extract (soluble) antigens of both MTSE and PCSE were used. PHA in this study has been found to be specific as none of the control CSFs (group II a) showed agglutination

| Number tested | Antibody response to | MTSE | PCSE | MTSE and PCSE |
|---------------|----------------------|------|------|---------------|
| Group I: chronic infections of the CNS | 88 | 36 | 9 | 8 |
| TBM-confirmed | 26 | 21 | 0 | 5 |
| NCC-confirmed | 3 | 0 | 3 | 0 |
| Group II: controls | | | | |
| (a) Non-infectious non-neurological | 30 | 0 | 0 | 0 |
| (b) Infectious neurological | 21 | 2 | 0 | 0 |
| (c) Non-infectious neurological | 133 | 6 | 7 | 6 |

Sensitivity

- TBM 80.76%
- NCC 100.0%

Specificity

- TBM 92.4%
- NCC 92.97%

Fig. 1. Immunoblot analysis of CSFs derived from TBM (A) and NCC (B) patients.
reactions (Table 1) nor was there any true cross-reactivity between anti-mycobacterial antibody and that of PCSE, or vice-versa [12]. Therefore positive responses obtained in this study are true positives (Table 1). An interesting observation was the presence of anti-cysticercal antibodies in CSFs of five TBM patients (Table 1) whereas no anti-mycobacterial antibody reactivity was observed in CSFs of confirmed NCC. Similarly both anti-mycobacterial and cysticercal antibodies were reactive in some of the CSFs of control group patients (group II c) (Table 1) including GBS, peripheral neuropathy. Even by ELISA these antibodies were detected [21]. Possible explanations for this phenomenon are (i) transudation or exudation of parasitic/bacterial antibodies from systemic circulation through inflamed blood–brain barrier to the CNS [22] and (ii) induction of an immune response to stress proteins of mycobacteria [23], that may have structural homology with that of eukaryotic stress proteins resulting in positive signals.

Immunoblot assay was used to study individual antigen–antibody reactions among PHA positive CSFs especially those that were positive for both MTSE and PCSE. CSF antibody reactivity of TBM and NCC patients showed a higher specificity with low molecular masses of MTSE (6 kDa, 12–14 kDa, 19 kDa, 23 kDa) and high molecular masses of PCSE (66–68 kDa, 80 kDa, 96 kDa and ≥110 kDa) respectively Fig. 1. Previously three different 10-kDa antigens of C. cellulosae derived from vesicular fluid [24] and scolex [25] were claimed to be specific in immunodiagnosis of NCC. In the present study, CSFs of control group II (c) and some of the TBM patients also showed immune reactivity to 10-kDa and 70-kDa antigens of PCSE (Figs. 2 and 3). This suggests that these antigens may be stress or heat shock proteins (hsp) that are conserved across many genera [26] and 10-kDa hsp is abundantly expressed in M. tuberculosis [23]. Results of this study indicate that if antibody detection for MTSE or PCSE alone is performed individually, that may misdiagnose or result in inconclusive diagnosis. For diagnostic accuracy, it is necessary to look for antibody responses simultaneously to purified specific immunodiagnostic antigens of both M. tuberculosis and C. cellulosae, and check for circulating mycobacterial, cysticercal and cryptococcal antigens in CSFs. A battery of immunodiagnostic tests would certainly be an added advantage in differential diagnosis of chronic infections of the CNS such as TBM, NCC or cryptococcal meningitis especially in endemic areas of under-developed and developing countries.

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