

## Detection of Antibody to *Mycobacterium tuberculosis* Protein Antigens in the Cerebrospinal Fluid of Patients with Tuberculous Meningitis

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Antibodies against *Mycobacterium tuberculosis* antigens were detected by enzyme-linked immunosorbent assay in cerebrospinal fluid (CSF) samples obtained from 442 patients with tuberculous meningitis (TBM) and 102 control patients. Antibodies were found in the CSF of 87% of patients with clinical (culture-negative) TBM, 72% of patients with culture-positive TBM, and 65% of patients with autopsy-proven TBM. That anti-*M. tuberculosis* antibodies were detected in the CSF of patients with clinically diagnosed cases more frequently than in patients with culture-positive cases suggests that the detection of antibodies in CSF tends to decrease as bacillary load increases. Of the patients with clinical TBM who were coinfecting with human immunodeficiency virus (HIV), 70% exhibited anti-*M. tuberculosis* antibody in CSF, which suggests that antibody responses in this group were substantially weaker than those in HIV-negative patients with clinical TBM. Some groups showed a stronger response to certain antigens, which suggests that antigen recognition patterns may be specific for the stage of disease.

Tuberculous meningitis (TBM) is the most common clinical manifestation of involvement of the central nervous system (CNS) in tuberculosis [1]. Before the era of human immunodeficiency virus (HIV), TBM affected mostly children, in particular those living in developing countries. In those countries, HIV infection has made young adults the target population for TBM [2]. Timely diagnosis is key to the management of TBM: the case-fatality rate for untreated TBM is almost 100%, and delays in treatment often lead to permanent neurologic damage [3, 4].

Early diagnosis of TBM remains a challenge. Suspicion of TBM arises from the patient's history and clinical symptoms (headache, vomiting, and fever), and the diagnosis is established by examination of the cerebrospinal fluid (CSF). Although cer-

tain CSF abnormalities (lymphocytosis, elevated protein, and reduced glucose levels) are suggestive of TBM, definitive diagnosis requires detection of tubercle bacilli in the CSF by smear examination or bacterial culture. Unfortunately, bacteriologic confirmation is often difficult. Acid-fast bacilli are detected in 10%–80% of cases, but the highest values are achieved only when large-volume serial CSF samples are available for testing. Culture of the CSF for tubercle bacilli is slow and insensitive, and culture results are positive in 25%–70% of clinically diagnosed cases [2]. A new generation of diagnostic tests that are based on bacterial nucleic acid amplification identifies  $\leq 60\%$  of TBM cases [3] and tends to be too costly for use in low-income settings. Thus, none of the existing methods satisfies the requirements for timely diagnosis of TBM, especially in developing countries.

Antibody detection is a promising venue for diagnosis of TBM, because antibodies against *Mycobacterium tuberculosis* antigens are found in the CSF of patients with TBM [5–7]. Previous work in which a few defined mycobacterial antigens were used detected antibodies in  $\sim 60\%$  of TBM cases [6], demonstrating that the method has a potential, albeit limited, diagnostic usefulness. Because antigen recognition by serum antibodies varies highly from person to person [8], the design of serodiagnostic tests for tuberculosis needs to be based on multi-antigen cocktails. We undertook the present study to determine whether the antibody repertoire in the CSF of TBM patients

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Cerebrospinal fluid samples were obtained for routine diagnostic analysis from patients admitted to the National Institute of Mental Health and Neurosciences in Bangalore, India, in accordance with guidelines of the Institutional Ethics Committee.

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is heterogeneous. Our objective was to determine the usefulness of antibody-detection methods that are based on multiple antigens for the diagnosis of TBM.

## Methods

**Study design.** The present study was conducted retrospectively using stored CSF samples obtained for routine diagnostic analysis from 544 patients admitted between January 1995 and December 1999 to the National Institute of Mental Health and Neurosciences (NIMHANS) in Bangalore, India, a tertiary care center for neurologic and psychiatric diseases. A few CSF samples from autopsy-proven TBM cases were obtained from the Human Brain Tissue Repository, a facility of the Department of Neuropathology at NIMHANS. Patient serum samples were not available.

The CSF samples, which were stored at  $-80^{\circ}\text{C}$ , had been obtained from patients assigned on the basis of diagnostic criteria to the following 4 groups: the clinical TBM group (301 patients), the culture-positive TBM group (264 patients), the autopsy-proven TBM group (72 cases), and the control group (102 patients). For the first group, the diagnosis of clinical TBM was based on clinical findings only (i.e., headache, fever, vomiting of  $>3$  weeks' duration, pleocytosis and increased protein in the CSF, and basal exudate with or without hydrocephalus, as revealed by cranial computed tomographic scan). *M. tuberculosis* did not grow in cultures of CSF samples from this group. For the culture-positive TBM group, diagnosis was confirmed by growth of *M. tuberculosis* in CSF cultured on Lowenstein-Jensen medium. For the autopsy-proven TBM group, diagnosis was confirmed by gross and histopathologic examination of the brain (basal exudate, hazy meninges, meningeal tubercles, and epithelioid cell granulomas with Langerhans-type giant cells, with or without caseous necrosis in meninges and parenchyma). The control group consisted of patients who had undergone lumbar puncture for diagnosis of CNS diseases of noninfectious etiology (e.g., prolapsed intervertebral disk, multiple sclerosis, and demyelinating diseases). Patient interviews and examination of chest radiologic findings demonstrated that no patient in the control group had a history of pulmonary tuberculosis. The number of CSF samples analyzed in each patient group is presented in table 1.

**HIV antibody testing.** Serum samples were tested, according to guidelines developed by the National AIDS Control Organization, for the presence of antibodies to HIV-1 and HIV-2. Serum samples were tested first by ELISA, and reactive samples were further tested by a different ELISA test and by an RIA. The suppliers participating in the National AIDS Control program were Diagnostics Biochem, Innogenetics, Magiwell, and United Biotech. A sample was considered to be positive for HIV antibodies if it showed seroreactivity in all 3 assays. Western blot analysis was done on samples that yielded discrepant results.

**Antibody detection in CSF.** Six protein antigens of *M. tuberculosis* (ESAT-6, 14 kDa, MPT63, 19 kDa, MPT64, and 38 kDa) were selected as coating antigens for ELISA on the basis of previous serologic evaluation [8]. Antigens were purified to near homogeneity from recombinant *Escherichia coli* as described elsewhere [9]. Polystyrene 96-well microtiter plates (Costar) were coated overnight at  $4^{\circ}\text{C}$  with  $50\ \mu\text{L}$  of antigen solution in  $0.05\ M$  carbonate-

**Table 1.** Patient groups in a retrospective study done to determine the usefulness of antibody detection methods in the diagnosis of tuberculous meningitis (TBM).

Patient group	No. of patients ( <i>n</i> = 544)
Clinical TBM	301
HIV positive	37
HIV negative	264
Culture-positive TBM <sup>a</sup>	69
Autopsy-proven TBM	72
HIV positive	13
HIV negative	59
Control	102

NOTE. HIV, human immunodeficiency virus.

<sup>a</sup> Patients in this group were all HIV negative.

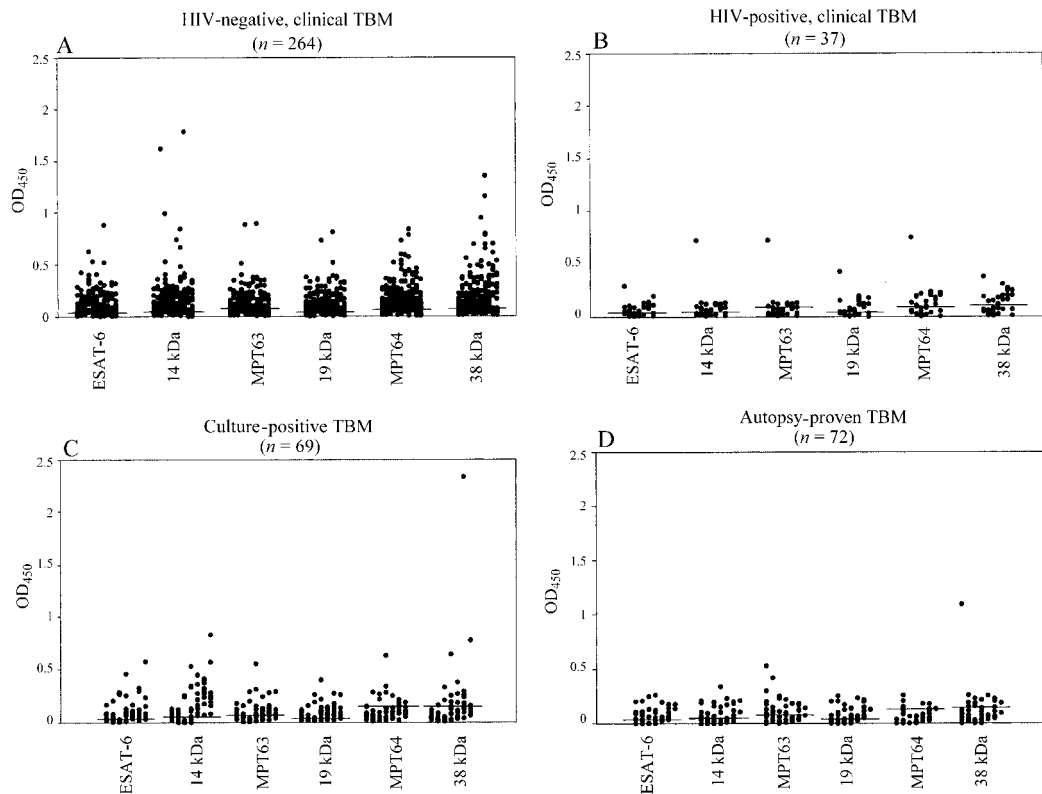
bicarbonate buffer (pH 9.6). Coating antigen concentrations were  $1\ \mu\text{g}/\text{mL}$  for ESAT-6, 19 kDa, and MPT64;  $0.2\ \mu\text{g}/\text{mL}$  for MPT63;  $2\ \mu\text{g}/\text{mL}$  for 38 kDa; and  $0.3\ \mu\text{g}/\text{mL}$  for 14 kDa. After coating, plates were blocked with 1% nonfat milk (Sigma) in  $0.15\ M$  PBS and 0.05% Tween 20 (PBS-T) for 1 h at room temperature. A CSF sample ( $50\ \mu\text{L}$ ) was added to each well at a 1:10 dilution in PBS-T containing 1% nonfat milk and incubated for 2 h at room temperature. After plates had been washed with PBS-T, they were incubated for 1 h at room temperature with rabbit anti-human IgG antibody conjugated with horseradish peroxidase (Dako) diluted 1:6000 in PBS-T. Plates were washed with PBS-T, and enzyme activity was assayed by incubation for 15 min at room temperature with  $50\ \mu\text{L}/\text{well}$  TBM peroxidase substrate (Bio-Rad). To stop the reaction,  $50\ \mu\text{L}$  of  $4\ N$  sulfuric acid was added to each well, and the optical density was measured at 450 nm ( $\text{OD}_{450}$ ) using an automatic microtiter plate reader (Spectra Shell; Tecan).

**Data analysis.** ELISA results were analyzed by using cutoff values equal to the mean  $\text{OD}_{450} + 3\ \text{SD}$  obtained with 102 CSF samples from control patients, as described in previous studies [8, 10]. For statistical analysis, differences in proportions were evaluated by a Pearson  $\chi^2$  test, without correction for continuity.

## Results

Using ELISA, we measured IgG antibodies to 6 *M. tuberculosis* antigens in the CSF of 442 patients with TBM and 102 control patients with noninfectious CNS disease. Raw data for all patients with TBM, along with the cutoff values obtained for each antigen, are shown in figure 1.

**Antibody detection in CSF specimens from patients with TBM and control patients.** All 102 CSF samples from control patients had  $\text{OD}_{450}$  readings that were below the cutoff point calculated for each antigen (figure 2). Figure 2 shows that, among patients with TBM, the highest proportion of antibody-positive CSF specimens (i.e., those with  $\text{OD}_{450}$  readings equal to or greater than the cutoff point for at least 1 antigen) was found in the group of HIV-negative patients with clinical TBM (228 [87%] of 264 patients). Patients with culture-positive TBM ranked next (50 [72%] of 69 patients), and patients with autopsy-proven TBM



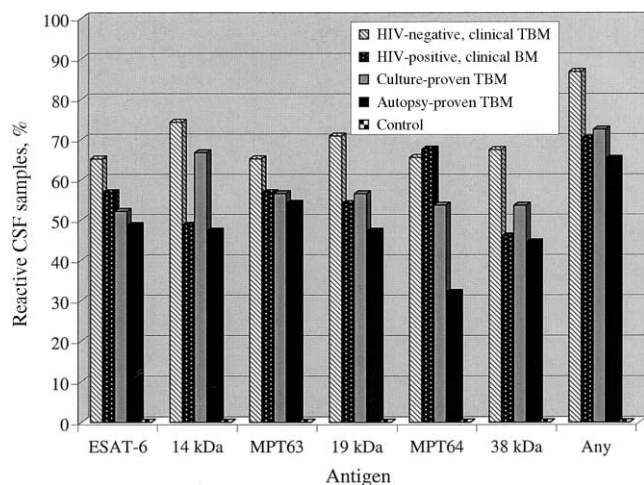
**Figure 1.** Levels of IgG antibodies in cerebrospinal fluid (CSF) samples from patients with tuberculous meningitis (TBM). CSF samples were analyzed by ELISA with 6 *Mycobacterium tuberculosis* antigens. Each data point represents 1 patient. Each panel represents 1 group of patients. The horizontal bar crossing each data set denotes the cutoff point determined by the mean optical density at 450 nm ( $OD_{450}$ ) + 3 SD obtained for each antigen in 102 CSF samples from control patients. The mean  $OD_{450}$  (SD) used for cutoff determinations was 0.019 (0.013) for ESAT-6, 0.02 (0.013) for 14 kDa, 0.023 (0.014) for MPT63, 0.02 (0.012) for 19 kDa, 0.026 (0.019) for MPT64, and 0.030 (0.018) for 38 kDa. *A*, Human immunodeficiency virus (HIV)-negative patients with clinical TBM; *B*, HIV-positive patients with clinical TBM; *C*, patients with culture-positive TBM; and *D*, patients with autopsy-proven TBM.

had the lowest proportion (47 [65%] of 72 patients). Differences in the proportions of patients who had antibody responses in the 3 groups of patients with TBM were statistically significant (HIV-negative patients with clinical TBM vs. patients with culture-positive TBM,  $P = .0026$ ; HIV-negative patients with clinical TBM vs. patients with autopsy-proven TBM,  $P < .001$ ). Analysis of data for patients with autopsy-proven TBM according to HIV status showed no significant difference in the proportion of antibody-positive CSF specimens among HIV-positive patients and that among HIV-negative patients (62% and 66%, respectively;  $P = .83$ ), either because the sample size was limited or because HIV coinfection did not further depress the immune systems of these already severely compromised patients. Thus, patients with autopsy-proven TBM were analyzed as a single group, regardless of HIV status, throughout the study.

*Effect of HIV coinfection on antibody responses in patients with clinical TBM.* In contrast to the findings noted above for the group of patients with autopsy-proven TBM, differences were found in CSF antibody levels and antigen recognition in the clinical TBM group when data were analyzed according to

HIV status. ELISA found an antibody response in 70% of the CSF samples from 37 patients with TBM who were coinfecting with HIV (figure 2). This value was lower than that obtained for CSF from HIV-negative patients with clinical TBM (87%), which indicates that HIV coinfection is associated with a significant reduction in detectable CSF antibodies in patients with clinical TBM ( $P = .008$ ). A less favored explanation of these results is that TBM was misdiagnosed in HIV-positive patients; TBM has been reported to have an unusual clinical, radiological, and pathological presentation in such patients by some authors [11] but not by others [4].

*Recognition of M. tuberculosis antigens by antibody in CSF.* Analysis of antigen recognition by antibodies in CSF showed that 52%–58% of the antibody-positive CSF samples from 3 patient groups (patients with culture-positive TBM, HIV-negative patients with clinical TBM, and HIV-positive patients with clinical TBM) reacted with all 6 antigens (figure 3). Notably, only 39% of patients with autopsy-proven TBM had antibodies to all 6 antigens, largely because reactivity with the MPT64 antigen was relatively low (figure 2). To ascertain whether CSF



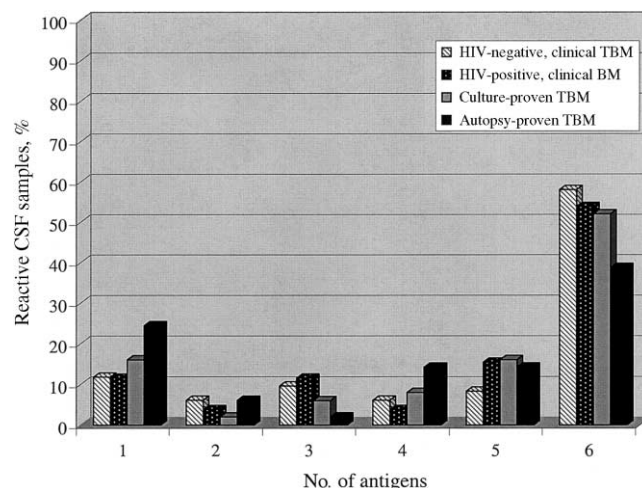
**Figure 2.** Antibody responses in cerebrospinal fluid (CSF) samples, by antigen, from patients with tuberculous meningitis (TBM). Data are the percentage of CSF samples (from patients in each of 5 groups) with antibody reactions (i.e., samples for which optical density readings were greater than or equal to the cutoff value for a particular antigen). HIV, human immunodeficiency virus.

samples from different groups of patients preferentially react with certain antigens, we conducted 2 sets of analyses. In one, we focused on CSF specimens that reacted to only 1 antigen (figure 3). The group of HIV-positive patients with clinical TBM was not included in the analysis, because only 3 patients in that group had antibody responses to a single antigen. No antigen preference was found among 26 HIV-negative patients with clinical TBM who had antibody responses to a single antigen. In contrast, a strong antigen preference was found among patients with culture-positive TBM who had antibody responses to a single antigen (5 [62%] of 8 patients had responses to the 14-kDa antigen) and among patients with autopsy-proven TBM who had responses to a single antigen (6 [50%] of 12 patients had responses to MPT63) (data not shown).

Similar patterns of antigen preference in patient groups were revealed by a second type of analysis, in which we determined, for each antibody-positive CSF specimen, which antigen resulted in the highest OD<sub>450</sub>. Results are shown in table 2. The proportion of patients with strong reactions against the 14-kDa antigen was much larger in the culture-positive TBM group (28 [56%] of 50 patients) than in the HIV-negative, clinical TBM group (38 [17%] of 228 patients) or the autopsy-proven TBM group (8 [17%] of 47 patients) ( $P = .038$ ). More patients in the autopsy-proven TBM group had strong reactions to the MPT63 antigen than in any other group (14 [30%] of 47 patients vs. 12 [5%] of 228 in the HIV-negative, clinical TBM group and 4 [8%] of 50 in the autopsy-proven TBM group;  $P = .054$ ). In contrast, the autopsy-proven TBM group had the lowest proportion of patients with strong anti-MPT64 reactions of all the groups (2 [4%] of 47 patients vs. 52 [23%] of 228 in the HIV-

negative, clinical TBM group and 6 [12%] of 50 in the autopsy-proven TBM group;  $P = .168$ ). Differences in the proportion of patients in each group who had strong reactions to the 38-kDa antigen, ESAT-6, and the 19-kDa antigen were not statistically significant (data not shown). Together with the data analysis presented in figure 2, these analyses show that the 14-kDa protein reacted most strongly with CSF from patients with culture-positive TBM and that MPT63 reacted most strongly and MPT64 reacted least strongly with CSF from patients with autopsy-proven TBM.

*Diagnostic usefulness of an antibody-detection assay.* There is a need for diagnostic tools for TBM that perform better than the existing methods. Because anti-*M. tuberculosis* antibodies were found in the CSF of large proportions of TBM patients (figure 2), we used the data generated in the present study to evaluate the potential usefulness of an antibody-detection assay for TBM in comparison with the current method for definitively diagnosing TBM (i.e., growth of *M. tuberculosis* on CSF cultures). For this analysis, we used data obtained for all patients who had received a clinical diagnosis of TBM, regardless of HIV status. As shown in table 1, our study included 370 patients who had received a diagnosis of TBM based on clinical findings. For 69 of these patients, cultures were positive for *M. tuberculosis* (culture-positive TBM in table 1), and for 301 patients, no microbiologic diagnosis was achieved (clinical TBM in table 1). A patient-by-patient comparison of antibody detection with culture methods showed that, of 370 patients who had received a clinical diagnosis of TBM, 304 (82%) had detectable antibody in CSF, and only 69 (19%) had positive culture results (table 3). Thus, diagnostic assays based on detection of specific an-



**Figure 3.** Antibody responses, by no. of antigens, in cerebrospinal fluid (CSF) samples from patients with tuberculous meningitis (TBM). Data are the proportion of CSF samples with antibody reactions to different nos. of antigens among all reactive CSF samples (i.e., those with optical density readings equal to or greater than the cutoff value). HIV, human immunodeficiency virus.

**Table 2.** Antigen recognition in cerebrospinal fluid (CSF) samples from patients with tuberculous meningitis (TBM).

Antigen	CSF samples for which a given antigen provoked the highest OD <sub>450</sub> , no. (% of all reactive samples)		
	From HIV-negative clinical TBM patients (n = 228)	From culture-positive TBM patients (n = 50)	From autopsy-proven TBM patients (n = 47)
ESAT-6	27 (12)	5 (10)	5 (11)
14 kDa	38 (17)	28 (56)	8 (17)
MPT63	12 (5)	4 (8)	14 (30)
19 kDa	26 (11)	3 (6)	6 (13)
MPT64	52 (23)	6 (12)	2 (4)
38 kDa	73 (32)	4 (8)	12 (25)

NOTE. Percentages were calculated by dividing the no. of samples for which a particular antigen provoked the strongest antibody response by the total no. of samples that had antibody responses to any antigen and multiplying the result by 100. CSF samples from human immunodeficiency virus (HIV)-positive patients with clinical TBM were not included, because the overall low optical density readings at 450 nm (OD<sub>450</sub>) obtained with those specimens were not amenable to this type of analysis.

tibody in the CSF have the potential to surpass the performance of culture-based methods. However, the need for culture-based diagnostic methods, particularly for use in antibiotic-susceptibility testing, remains paramount in clinical practice.

## Discussion

The findings of our study indicate that TBM is associated with the presence of detectable levels of antibodies in the CSF of most patients. Two observations merit comment. One is that the proportion of antibody-positive CSF specimens decreased from 87% in the clinical TBM group to 72% in the culture-positive TBM group and to 65% in the autopsy-proven TBM group. Because clinical TBM may often be taken to represent an early stage of CNS involvement, this downward trend might reflect a decrease in immune responses that occurs during the progression of disease. However, we cannot exclude the possibility that the lower proportion of antibody-positive CSF samples among patients with autopsy-proven TBM, in comparison with the other groups, may be a consequence, at least to some extent, of some protein decay in autopsy samples of tissues. Second, none of 102 CSF samples from control patients reacted with any of the 6 *M. tuberculosis* antigens that we used. Because the estimated prevalence of latent tuberculosis in the adult population of India ranges from 40% [1] to 80% [12], this result strongly indicates that only active tuberculosis is associated with detection of anti-*M. tuberculosis* antibodies in CSF.

The unimpeded spread of HIV infection to areas of the world in which tuberculosis is endemic makes it necessary to investigate the effects of HIV coinfection on the immune responses elicited during tuberculosis. The present analysis suggests that HIV coinfection correlated with a decrease in the proportion of antibody-positive CSF specimens among patients with clinical TBM. However, the proportion of patients who had an-

tibody reactions in the HIV-positive, clinical TBM group (70%) was similar to that in the groups of patients with advanced TBM (72% for patients with culture-positive TBM and 65% for patients with autopsy-proven TBM). Thus, coinfection with HIV appears to reduce the possibility that antibody will be detected in the CSF of TBM patients significantly but not dramatically.

Some aspects of the antigen-recognition patterns delineated in the present study merit comment. First, most antibody-positive CSF specimens (55%–75%, depending on the patient group) reacted with 5 or 6 antigens. These findings contrast with the results of our previous studies on serum antibodies [8]. In those studies, most positive serum samples (~75%) reacted with 1–3 antigens, and only a small proportion reacted with  $\geq 5$  antigens. It is possible that the very different protein composition of serum and CSF may account for differences in signal-to-noise ratios and, therefore, in the analytical sensitivity of ELISA in testing these body fluids. However, we cannot exclude the possibility that there might be a real difference in antigen recognition by antibodies in CSF and serum. Parallel analyses of serum and CSF samples from patients with both TBM and pulmonary tuberculosis will be required to address this question. A second aspect of the antigen-recognition analysis concerns preferential recognition of some antigens in the different groups of TBM patients. For example, strong responses against the 14-kDa antigen were more frequently associated with culture-positive TBM than with clinical (i.e., culture-negative) TBM, which suggests that antibody recognition of this antigen may correlate with bacillary load in CSF. In another example, antibodies against MPT64 were lowest in CSF samples from patients with terminal (autopsy-proven) TBM. We speculate that the antibody response to MPT64 may be short-lived in patients with TBM. Thus, as has been proposed elsewhere [8, 13], different stages of TBM may correlate with preferential recognition of different antigens, and antigen availability and/or antibody turnover may change as disease progresses.

Does the presence in CSF of anti-*M. tuberculosis* antibody always indicate TBM? There is ample evidence that TBM is accompanied by intrathecal production of antibody [14–16]. However, antibody may leak into the CSF from the bloodstream, particularly when the blood-brain barrier is damaged

**Table 3.** Results of a comparison of antibody detection with culture methods for diagnosis of tuberculous meningitis (TBM) using cerebrospinal fluid samples from 370 patients who had received a clinical diagnosis of TBM.

<i>Mycobacterium tuberculosis</i> culture results	Antibody detection results, no. of samples		
	Positive	Negative	Total
Positive	50	19	69
Negative	254	47	301
Total	304	66	370

by an inflammatory process [17, 18]. The effect of such an event on the accuracy of antibody-based diagnosis of TBM is of concern in areas where tuberculosis is highly endemic (in countries where TBM is not endemic, no anti-*M. tuberculosis* antibody was detected in the CSF of control patients who had nontuberculous meningitis) [5] (A.C., unpublished observations). Thus, antibody-based diagnosis of meningeal disease in settings where the prevalence of tuberculosis is high may require parallel testing of anti-*M. tuberculosis* antibody levels in CSF and serum and evaluation of the permeability of the blood-brain barrier by measurement of the ratio of CSF albumin to serum albumin (an example is provided in [18]).

This article delineates the diagnostic usefulness of an early diagnostic assay for TBM that is based on detection of specific antibodies in CSF. Of all patients with TBM (both HIV positive and HIV negative) in whom the disease was diagnosed by clinical criteria, 82% tested positive for antibody, regardless of the results of culture for *M. tuberculosis*. Of patients with TBM (both HIV positive and HIV negative) in whom the disease was diagnosed by clinical criteria only, 84% tested positive for antibody. Even among HIV-positive patients, who are affected by the immunosuppression associated with HIV infection, the proportion with antibody-positive CSF remained as high as 70%. These results warrant future efforts toward the development and validation of antibody-based diagnostic assays for TBM. In particular, there will be a need to evaluate diagnostic accuracy, using CSF samples obtained from control patients with infectious meningitis of non-tuberculous etiology, and to evaluate the performance of antibody-based methods versus molecular diagnostic methods (e.g., methods based on amplification of mycobacterial nucleic acids), which tend to find an application in developed countries.

Future and ongoing research will address yet-unanswered questions. In particular, there is a need to define the correlation of antibody levels in CSF with outcome of antituberculosis treatment, with different stages of TBM (such as those described by the British Medical Research Council [19]), and with other clinical forms of CNS tuberculosis. Addressing these issues will, in turn, provide further validation of future antibody-based tests for the diagnosis of TBM.

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