Ann Natl Acad Med Sci (India), **41(1&2)**: 1-21, 2005

Diagnosis of tuberculous meningitis: newer approaches - suited to developing countries

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SUMMARY

Tuberculous meningitis (TBM) is a potentially curable infectious disease of the central nervous system. A complete neurological recovery in patients with TBM will depend upon an early confirmative laboratory diagnosis as well as institution of appropriate anti-tuberculous chemotherapy. Demonstration of *Mycobacterium tuberculosis* by the conventional bacteriological methods is not only less sensitive but also yield false negative results. Hence development of alternates parameters for the laboratory diagnosis of TBM not only relevant but also becomes essential. In this communication we describe simple, sensitive, specific, reproducible and newer immunoassays for the early laboratory diagnosis of TBM. Operational advantages of these immunoassays for laboratories in developing countries are also emphasized in this communication.

Key words: Tuberculous meningitis (TBM); *Mycobacterium tuberculosis*; Bacteriological methods; Immunoassays; laboratory diagnosis.

INTRODUCTION

Human tuberculosis still remains one of the major public health problems in most of the developing countries. Despite a worldwide tuberculosis control and eradication programme, as well as emergence of newer diagnostic and therapeutic modalities, human tuberculosis continues to carry high mortality and morbidity rates. During the past two decades, the incidence and prevalence of human tuberculosis showed a global increase and this has been attributed to the emergence of human immunodeficiency virus (HIV) infection. Patients with HIV infection are twenty- times more predisposed to acquire tuberculosis than patients without HIV infection. Every year, an estimated eight million new cases of tuberculosis are added and approximately two million deaths occur due to this infectious disease (1).

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Dr. Pran Nath Chuttani oration delivered at the Annual meeting of NAMS at Jaipur-2004.

CENTRAL NERVOUS SYSTEM (CNS) TUBERCULOSIS:

In general, the incidence of CNS tuberculosis in a geographic zone is directly proportional to the prevalence of tuberculosis. Among the extra- pulmonary sites, 15% of tuberculous infection occurs in CNS (2). The CNS involvement in tuberculous infection may clinically manifest in four distinct forms *viz* a) tuberculous meningitis, b) tuberculoma, as intracranial space occupying lesion c) cerebrovascular lesions in the form of stroke and d) encephalopathy. CNS tuberculosis constitutes approximately 45% of all forms of tuberculosis in pediatric age group. This assumes greater significance when one considers the fact that there is very high incidence of tuberculosis in the pediatric age group.

TUBERCULOUS MENINGITIS (TBM):

TBM is the most common clinical and neuropatholgical manifestations of tuberculosis of the central nervous system. TBM is usually caused by *M tuberculosis*. However, *M avium, M intracellulare, M kansasii* and *M fortuitum* have also been isolated in patients with TBM (3). Cranial form of TBM is, by far the most common clinical manifestation of neurotuberculosis and constitutes 80% of neurotuberculosis (4). TBM can manifest at any age but it is uncommon under the age of six months. The incidence of TBM is highest during the first five years of life. In the pediatric age group, TBM is usually a complication of primary complex. In adults, disease mainly occurs either as reactivated form of an old lesion or as an isolated form or in association with pulmonary tuberculosis. The latter is more often seen in patients with HIV infection.

LABORATORY DIAGNOSIS OF TBM:

The laboratory diagnosis of TBM can be made from

a) cytological and biochemical parameters in CSF
b) bacteriological methods
c) biochemical methods
d) immunodiagnostic approaches, and
e) molecular biologic approaches.

a) Cytological and biochemical parameters in CSF:

The usual cytological picture in the CSF of patients with TBM is lymphocytosis. However, cytological features in CSF specimens can vary from patient to patient of TBM. In some patients with TBM, the total leukocyte- cell count in CSF specimen may be normal. *Polymorphonuclearcytosis* can occur in the initial stages of the disease or can also manifest as a reaction to the release of tuberculoprotein into the subarachnoid space (5,6). *Pleocytosis*: Composed of lymphocytes, plasmacytoid lymphocytes and macrophages. Occasionally, several macrophages or monocytes may aggregate and they are referred to as "wandering tubercles".

The biochemical parameters in CSF include elevated protein. The protein concentration can vary from 50-1000 mg % (7). Proteins are made up of albumin and gamma globulins. Some of these gamma globulins contain specific antibodies against antigens of *M tuberculosis*. CSF glucose level is grossly reduced (usually less than 40 mg %). Chloride concentration is often reduced.

b) Bacteriological methods

The 'Gold Standard' for establishing the laboratory diagnosis of TBM is the demon-

stration of acid-fast bacilli *M tuberculosis* in CSF by Ziehl Neelsen smear or by culture method (8).

Acid-fast bacilli in CSF smear

To demonstrate the acid-fast bacilli in CSF smears, utility of an equipment - Cytospin is valuable, as it promotes the localization of the acid- fast bacilli within the inflammatory cells. It also promotes the preservation of cyto-morphological characteristics of the acid-fast bacilli and the inflammatory cells. Acid-fast bacilli can be stained using basic fuchsin dyes such as Ziehl-Neelsen and Kinyoun stains. *M tuberculosis* bacilli can also be demonstrated using fluorescent dyes such as auramine and rhodamine (9).

CSF culture for M tuberculosis

In contrast to the other types of bacteria, mycobacteria have a longer generation time of 18-24 h. Conventional culture method using Lowenstein-Jensen medium (LJ) usually require 8-10 weeks to grow *M tuberculosis*. This is not only cumbersome but also less sensitive and may lead to false negative results. As an alternative to LJ medium, several short-term culture methods are recently in use for the isolation of *M tuberculosis* (10).

(i) BACTEC- 460 systems: - In this method, the clinical specimens are inoculated into the BACTEC 7H12 medium. If the clinical specimen contains optimum numbers of viable *M tuberculosis*, then ^{14}C will be released from radiolabelled palmitic acid substrate into the medium and this can be measured. Positive results can be obtained within 7-11 days.

(ii) Biphasic culture (MB-Check, Septicheck):- Consists of a biphasic medium containing Middle Brooke's 7H9 broth and an agar slant. The liquid phase consists of modified MB 7H9 broth with a supplement containing oleic acid, albumin, glucose, catalase, glycerol, pyridoxal hydrochloride, polyoxyethylene-40-stearate, polymyxin B, Amphotericin B, Naladixic acid, trimethoprin and azlocillin in a screw-capped glass bottle. The agar slant consists of Middle Brooke's 7H11 agar on one side and the opposite side has two portions. One portion contains MB 7H11 media with p- nitro a- acetylamino-3-hydroxy propiophenone (NAP) and the other portion contains chocolate agar. By inverting the system during incubation with the clinical specimen, this broth culture gets flooded over the slope. Mycobacteria growing in the broth will form visible colonies on the Middle brook agar whereas the microbial agent in clinical specimens does not belong to the tuberculosis complex, they will also grow on the Middle brook agar containing NAP. Bacteria other than mycobacteria will grow on chocolate agar.

(iii) Mycobacterial growth indicator tube: - A non- radiometric broth method consists of MB 7H9 broth and a silicon rubber impregnated with a ruthenium metal complex as fluorescence quenching based oxygen sensor. The growth of mycobacteria is accompanied by the consumption of oxygen, which allows the indicator to fluoresce under 365nm UV light.

(iv) MB Redox medium: - Consists of 4 ml-modified serum supplemented Kirchner medium with a colorless tetrazolium salt as a growth indicator.

During growth, this tetrazolium salt gets reduced to a pink, red or violet colored formazan insoluble material and is secreted to the cell surface in a granular form. Media contains vitamin for the acceleration of growth and formation of formazan.

c) Biochemical methods

Sophisticated biochemical parameters have been described in the literature for the laboratory diagnosis of TBM. These include measurement of metabolic products of *M tuberculosis* bacilli. These are as follows:

i. Tuberculostearic acid (11) - It is a component of the cell wall. This can be measured by mass-spectroscopy and electron gas liquid chromatography.

ii. 3,2 ketohexyl indoline (12) - Substance of unknown origin, related to seratonin. It is measured using mass spectroscopy and electron gas liquid chromatography.

iii. Radiolabelled Bromide partition test (13) - Measures the ratio of radioactive bromide in serum and CSF. Values less than 1.6 are usually characteristic and diagnostic for TBM.

iv. Adenosine Deaminase (ADA) activity (14) - It is an enzyme secreted by T lymphocytes. ADA has been used as a biochemical marker and values more than 6-8 U are diagnostic to TBM.

d) Immunodiagnostic approach

The CSF in patients with TBM contain breakdown products of *M tuberculosis* i.e. mycobacterial antigens. Elevated immunoglobulins in CSF are antibodies intrathecally synthesized by *M tuberculosis*. Therefore, the immunodiagnostic approaches in TBM are based upon the detection of antibodies to *M tuberculosis* or mycobacterial antigens in CSF specimens. Immunodiagnostic methods described for the laboratory diagnosis of TBM are:

- o Fluorescent Immunoassay
- o Radio Immunoassay
- o Enzyme Linked Immunosorbent Assay
- o Immunoblot assay
- o Dot Immunobinding Assay

e) Molecular biologic approach

The CSF in patients with TBM contains genomic DNA of *M tuberculosis*. The mycobacterial DNA can be amplified with the help of primers. The different molecular biologic methods used to detect the mycobacterial DNA in the CSF include

- o Polymerase chain reaction (PCR)
- o Mycobacterium direct test (MDTB)
- o Ligase chain reaction (LCR)

PROBLEMS IN THE LABORATORY DIAGNOSIS OF TUBERCULOUS MENINGITIS IN 'DAY TO DAY' CLINICAL PRACTICE:

Cytological:

The cytological features in CSF of patients with TBM often show marked variation from patient to patient with TBM.

a) The leukocyte count may be normal.

b) Lymphocytosis and Pleocytosis can be seen in meningitis due to non-tuberculous aetiology such as partially treated pyogenic meningitis, fungal meningitis and granulomatous meningitis like sarcoidosis. Cytological appearances are more or less similar in these diseases.

c) Anti-tuberculosis chemotherapy administration prior to CSF analysis of-

ten significantly alters the cytopathological picture.

d) "Wandering" tubercles are seldom seen in the CSF samples in patients with TBM.

Due to the above, the cytological parameter cannot be relied upon, for the laboratory diagnosis of TBM

Biochemical

Elevated proteins and reduced glucose concentrations in CSF can be observed in other neurological diseases like chronic meningitis, demyelinating disorders and viral meningoencephalitis. Therefore, routine biochemical parameters in CSF are non-specific and seldom contribute to the laboratory diagnosis of TBM.

Bacteriological methods

Demonstration of *M tuberculosis* from CSF by bacteriological methods i.e. smear as well as culture still remains the "Gold standard" for the laboratory diagnosis of TBM. Inspite of several advances in bacteriological methods, successful demonstration of *M tuberculosis* in CSF in patients with TBM is infrequent (15). Those laboratories which employ conventional media such as Lowenstein Jensen media may require 8-10 weeks to culture *M tuberculosis* in CSF specimens. Short-term culture methods consume less time but they are less sensitive, particularly in CSF specimens (16). Demonstration of acid-fast bacilli in Ziehl-Neelsen stained smears is often infrequent and carry low sensitivity. Kennedy et al. 1979 has reported that minimum 100 mycobacteria per milliliter of CSF are required for the successful demonstration of AFB in CSF (17). Most CSF in TBM does not contain sufficient number of ***M tuberculosis.*** Thus, the bacteriological methods are often ineffective and not useful for the early laboratory diagnosis of TBM.

There are several reasons for the infrequent isolation of *M tuberculosis* in the CSF of patients with TBM.

a) CSF samples from most patients with TBM are obtained from the lumbar route. The lumbar CSF contains lower concentration of *M tuberculosis* bacilli than ventricular and cisternal CSF (18).

b) *M tuberculosis* bacilli become embedded in the dense exudates in the base of the brain and these exudates form a barrier for *M tuberculosis* to circulate in the lumbar CSF.

c) more importantly, most of the patients with TBM would have received a course of anti-tuberculosis chemotherapy before they are referred to specialized centers for neurological diseases. Lumbar CSF from partially treated patients with TBM will seldom contain *M tuberculosis;* hence the culture is invariably negative. For these reasons, bacteriological methods are seldom useful in so far as early laboratory diagnosis of TBM.

Biochemical methods

The sophisticated biochemical methods such as estimation of Tuberculostearic acid, 3,2 ketohexyl indoline assay, radiolabelled bromide partition test and adenosine deaminase activity hold definite promise for the laboratory diagnosis of TBM. However, these are not feasible for routine application in laboratories of developing countries because of the sophisticated instrumentation as well as technical expertise needed to perform these biochemical parameters.

Immunodiagnostic approaches

The CSF in patients with TBM usually contains a) antibodies to *M tuberculosis* and b) circulating mycobacterial antigens. Any attempt to demonstrate either circulating mycobacterial antigen or antibody by an immunological technique will be useful as an adjunct in the diagnosis of TBM. Several types of mycobacterial antigens have been isolated from the cultures of *M tuberculosis* and these have been used for the immunodiagnosis of TBM. Demonstration of mycobacterial antigens in CSF of patients with TBM has immense diagnostic value. Several sophisticated immunoassays like Inhibition ELISA, Competitive ELISA, Sandwich ELISA and Dot-Iba have been established in the past three decades for the laboratory diagnosis of TBM. However, the following problems are often encountered in these immunoassays 1) requirement of specific antibodies, which will react only with *M tuberculosis* antigen and not with other microbial agents 2) requirement of technical expertise 3) these immunoassays may yield false negative results in a patient who has already received partial treatment with anti-tuberculosis chemotherapy (ATT) for more than 4 weeks. In tertiary referral hospitals, most of the patients would already have already received ATT for more than 4 weeks and hence in such patients, the immunoassays for mycobacterial antigens may yield false negative results in CSF specimens.

Though detection of antibody to *M tuberculosis* in CSF carries diagnostic significance, it has several disadvantages. 1) Detection of antibodies cannot distinguish the active disease from the inactive stage. 2) Wherever there is disruption in blood-brain-barrier, serum antibodies to *M tuberculosis* will mix with the CSF, which will yield erroneous and false positive results. This phenomenon can also occur in patients with partially treated pyogenic meningitis and thus the detection of antibodies to mycobacterial antigen may give false positive results. 3) There are several non-specific mycobacterial antigens, which may cross-react with the antibodies in CSF specimens from patients with non-tuberculous meningitis and thus false positive results can occur in the immunoassays.

Molecular biologic methods

Although considerable data are now available on their use with respiratory specimens for the diagnosis of pulmonary tuberculosis, the precise role of PCR for the routine diagnosis of TBM is still not defined. The low sensitivity of PCR for the diagnosis of TBM could be due to the following reasons:

a) Pausibacillary status of CSF in patients with TBM and particularly, the lumbar CSF contains less ***M tuberculosis*** than cisternal or ventricular CSF (18).

b) The presence of host factors in CSF, which may inhibit the PCR.

c) The methodology used for the isolation of DNA requires breaking the tough cell- wall of *M tuberculosis*, and this in turn could result in sub-optimal yield of amplifiable mycobacterial DNA and low PCR sensitivity.

d) In a tertiary referral hospital, many patients with TBM are referred from peripheral hospitals and majority of these patients would already have received partial treatment with anti tuberculosis chemotherapy (ATT). ATT treatment may also have an effect on the bacterial load, which in turn would re-

sult in sub-optimal yield of DNA. Bonignton et al (16) have suggested that increasing the volume of CSF can enhance the sensitivity of PCR and they have emphasized the importance of obtaining CSF before the commencement of ATT.

e) Above all, requirement of clean laboratory area, sophisticated instrumentation, expensive chemicals and reagents and an unforgiving precaution in reagent and sample handling render PCR inappropriate for routine laboratory application in the developing world.

Development of newer parameters for the early laboratory diagnosis of TBM: their need and relevance to laboratories in the developing world:

Complete neurological recovery in a patient with TBM depends upon early diagnosis and institution of appropriate anti-tuberculosis chemotherapy. Any delay in diagnosis and institution of anti-tuberculosis treatment will invariably lead to the development of neurological complications and irreversible sequalae of the disease. Since TBM is a potentially curable disease and conventional bacteriological techniques are seldom useful in making a confirmative diagnosis of TBM, there is a need to devise alternate laboratory methods for the early laboratory diagnosis of TBM. However, any newly proposed laboratory parameter prior to its application in patients must fulfil the following criteria:

(a) It should carry a high degree of specificity so that there is no potential danger of false positivity

(b) It should carry a sensitivity higher than conventional bacteriological methods

(c) It should be rapid, cost-effective, and reproducible

(d) It must possess operational advantages over conventional bacteriological methods, and

It must be feasible for application in the laboratories in developing countries where there are constraints in laboratory resources and technical expertise.

Broad outlines of this study:

Tuberculosis of the central nervous system is one of the common infectious diseases among patients admitted into the neurological services at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, which is a major tertiary referral hospital for neurological diseases in Kerala. Patients from several hospitals in Kerala and adjoining states are referred to this hospital. A clinical diagnosis of TBM was made in all these patients, based on the relevant clinical features and this was supported by (a) compatible biochemical and cytological parameters in the cerebrospinal fluid specimens (b) neuro imaging features in Magnetic imaging resonance (MRI) scans. CSF specimens from all these patients were routinely sent bacteriology laboratory for the isolation of Mycobacterium tuberculosis. In addition to these investigations, in some selected patients, CSF specimens were also subjected to PCR studies.

At my department, we initiated a study on 'immunodiagnostic approaches in TBM' in early 1988 and over the past one and half decades, my colleagues and I developed several immunoassays for the diagnosis of TBM. This includes immunocytochemical method in the cytospin smears of CSF specimens, dot-immunobinding assay, ELISA

and immunoblot methods. The results of these methods have provided additional dimensions to the immunodiagnosis of TBM.Several methods developed at our laboratory were unique and original. I wish to discuss these newer methods for the laboratory diagnosis of TBM in detail.

(A) Direct Immunocytochemical method to demonstrate mycobacterial antigen in the CSF cytospin smears (reference#19)

Principle of the assay: The cerebrospinal fluid (CSF) in patients with TBM during active stages of the disease contains monocytoid cells (macrophages) and lymphocytes. The function of these monocytoid cells is to phagocytose the acid-fast bacilli and process the antigenic component of the *M tuberculosis* bacilli. Thus, the cytoplasm of the monocytoid cells in the CSF during the active stages of the disease contains mycobacterial antigen. The presence of mycobacterial antigen in these monocytoid cells has been demonstrated by a direct immunocytochemical method.

Materials: CSF specimens (2-3 ml) from 22 patients with TBM and diseased controls were collected under aseptic conditions and immediately centrifuged by a cytospin. Three smears each of CSF specimen from TBM and diseased controls were made and two CSF smears were immediately fixed in acetone and stored at +4°C.The third CSF cytospin smear was stained with hematoxylin and eosin and was examined under the microscope for the presence of monocytoid cells. Those CSF cytospin smears containing more than 5 monocytoid cells per high power field were selected for the immunocytochemical assay.

Immunocytochemical method for the demonstration of mycobacterial antigens: The CSF 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 smears were washed several times with 0.05 M Tris-buffered saline with Tween 20 (pH 7.6) (PBS-T). CSF-cytospin smears were then treated with 3% hydrogen peroxide for 5 minutes and washed thrice in 0.05 M TBS-T. Smears were then incubated with primary antibody (20mg of polyvalent rabbit IgG to *M tuberculosis* for one hour at 37°C. Subsequently, the smears were incubated with the anti-rabbit IgG-biotin conjugate and streptavidin - horseradish peroxidase (Dako LSAB2 system) for 45 minutes each at room temperature. Following that, the smears were thoroughly washed with TBS-T and they were incubated with a substrate-diaminobenzidine tetrachloride (10 mg dissolved in 5ml of 0.05 M Tris- phosphate buffer and 5ml 3% hydrogen peroxide) for 10 minutes. Finally the smears were counterstained with Harris' hematoxylin, dehydrated, cleared in xylene, mounted in permount and visualized under a microscope.

Results: Of the 22 CSF-cytospin smears from TBM patients, 16 showed a mixture of lymphocytes and monocytoid cells. Approximately, 15% monocytoid cells and lymphocytes showed degenerative changes in their cytoplasm. All the well-preserved monocytoid cells in the cytospin smear showed positive immunostaining for mycobacterial antigen in the form of brownish red granules in the cytoplasm. About 70 to 80% of monocytoid cells in the smears showed positive immunostaining for mycobacterial

antigens. Besides this, aggregates of immunostained extra-cellular brownish material was also seen in the smears of TBM patients. In six CSF smears of TBM patients, the immunostaining was negative because in these cases smears showed only few lymphocytes. Positive immunostaining in the monocytoid cells was also seen in the three TBM CSF samples in which *M tuberculosis* was isolated by bacteriological culture. In order to evaluate the reproducibility of the assay, immunostaining was repeated on the third CSF- cytospin smear of the same patient. There was no variation in the immunostaining pattern. All the 22 patients received anti-tuberculosis chemotherapy based on the immunocytochemical staining. A total of 16 patients had optimal neurological recovery and in 6 patients, the neurological recovery was sub optimal. None of these CSF-cytospin smears from the patients in the disease control group showed positive immunostaining, indicating that the nonspecific immunostaining did not occur by this technique.

Discussion: Earlier immunoassays described in the literature for the demonstration of mycobacterial antigen in the CSF of patients with TBM include the latex agglutination test with anti-plasma membrane antibody (Krambovitis et.al 1984), a sandwich enzyme-linked immunosorbent assay (ELISA) with anti- BCG antibody (Sada et.al 1983), an inhibition ELISA with polyvalent antibody against *M tuberculosis* (Bal et.al 1984), and a dot-immunobinding assay (Mastroianni et al (1991). The goal of these earlier studies was to detect the circulating mycobacterial antigens in the CSF of patients with TBM. In this study, however, I have demonstrated the presence of mycobacterial antigens in the monocytoid cells instead of CSF by a direct immunocytochemical method. This method carries a sensitivity of 72.5% and a specificity of 100%. The technical aspects of this assay are simpler than the methodology described in the earlier studies. The result of this assay can be easily visualized under the microscope and can be obtained within 5 hours of the receipt of CSF samples in the laboratory. The presence of an adequate number of monocytoid cells (> 5/high power field) in the CSF is essential for immunostaining and this should be ascertained in the initial hematoxylin and eosin stained smear. A positive result obtained by this immunocytogical method has potential diagnostic application in patients with TBM. **Hitherto, a similar study has not been described in the literature.** This newer diagnostic approach may have potential application in the early diagnosis of TBM, particularly in cases where bacteriological methods did not confirm the diagnosis. More importantly, this particular assay does not require any elaborate instrumentation and, therefore is, best suited to laboratories in developing countries where there are constraints in laboratory resources.

(B) Isolation of 14-kDa mycobacterial antigen from the culture filtrates of *M tuberculosis* by immunoabsorbent affinity chromatography and its potential application for the rapid diagnosis of TBM:(reference#20,21)

Principle: In this study, an attempt was made to isolate a specific mycobacterial antigen from the unheated culture filtrates of *M tuberculosis* by an immunoabsorbent affinity chromatography. Immunoglobulin G antibody specific for *M tuberculosis* in a CSF specimen from a patient with culture-proven TBM was isolated and was coupled

with activated Cynogen Bromide - Sepharose 4B. By immunoabsorbent affinity chromatography, a 14-kDa antigen was isolated from the culture filtrate of *M tuberculosis*. The immunochemical property of the affinity column-purified mycobacterial antigen was characterized. An antibody against the 14-kDa antigen was used to standardize a simple dot-immunobinding assay (Dot-Iba) to measure the mycobacterial antigen in the CSF of patients with TBM

Methodology: **Isolation of human IgG to *M tuberculosis* in CSF:** A total of 10 -15 ml of cisternal CSF from a patient with culture-proven TBM as a positive control and 10 ml of cisternal CSF from a patient with rheumatic heart disease as a negative control were collected at autopsy. The immunoglobulin G (IgG) fraction in positive and negative control CSF specimens was eluted by passing the CSF through Protein A-Sepharose 4B columns. The elute was repeatedly dialyzed and concentrated with an ultrafiltration unit. The protein content was estimated, and the elute was reconstituted to 3 mg/ml and stored in aliquots at –20°C.

Immunoabsorbent affinity chromatography for the isolation of 14-kDa antigen from culture filtrates *M tuberculosis*: 1 gram of cynogen bromide-Sepharose 4B was reconstituted to 3.5 ml in distilled water and washed with large volumes (20 times the original gel volume) of cold 0.1 m sodium bicarbonate buffer (pH 9) This was resuspended as a slurry of 50% (wt/vol) by the addition of 0.1 M sodium bicarbonate buffer. Human CSF IgG (3mg/ml) to *M tuberculosis* was added 1:1 by volume to the activated cynogen bromide-Sepharose 4B and the immunoabsorbent was incubated for 16 hours at 4°C. The immunoabsorbent was washed five times with large volumes of 0.1 M sodium borate buffer (pH 9) alternating with 0.1 M sodium acetate buffer (pH 5), suspended in 0.1 M phosphate buffered saline (PBS), poured into a glass–chromatographic column (diameter 1cm) and equliberated with 0.15 M PBS.The column was washed three times with 4 m urea in 0.15 M sodium bicarbonate buffer (pH 9) alternating with 0.15 M PBS to minimize the leaching out of IgG from the immunoabsorbent column. One milliliter (5mg/ml) of culure filtrates of *M tuberculosis* was added, and the column was run with 0.15 M PBS. Every 10 minutes, a 1 ml fraction was collected until a blank reading at 280 nm was obtained. The specific mycobacterial antigen that was bound to immunoabsorbent column was eluted with 4 M urea in 0.15 M sodium bicarbonate buffer, and the absorbances of fractions at 280 nm were recorded. Fractions with absorbances of >0.05 were pooled and dialyzed against PBS.The protein content of the dialyzed material was estimated by a Lowry's method before it was stored in aliquots (100mg/ml) at –20°C. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the elute gave a single band, and the molecular mass was found to be 14-kDa. The IgG from the control CSF did not bind to the 14-kDa antigen. Antibody to 14-kDa mycobacterial antigen was raised in rabbits by the conventional immunization schedule. The IgG fraction in the immune rabbit serum was recovered by the Protein A-Sepharose 4B column chromatography, dialyzed, concentrated and stored in aliquots (1 mg/ml) at –20°C.

Standardization of Dot-Immunobinding Assay (Dot-Iba): Prior to patient sampling, Dot-Iba was standardized with different concentrations (5-500 ng/ml)

of 14-kDa mycobacterial antigen in a nitrocellulose membrane (NCM). Circular NCM discs (diameter,1 cm) were placed in each well of a flat-bottom microtiter plate. Five microliters of the 14-kDa mycobacterial antigen at each concentration was spotted onto the NCM discs, and the plates were incubated at +4°C for 12 hours, after which the NCM discs were washed repeatedly with PBS-Tween 20 (PBS-T). The unbound sites in the NCM discs were quenched with 3% bovine serum albumin-PBS-T. Subsequently, the NCM discs were treated with rabbit IgG to the 14- kDa antigen (1:1000 dilution) and were washed three times with PBS-T. The NCM discs were subsequently incubated with (i) anti-rabbit IgG-biotin conjugate (1: 4000 dilution) and (ii) Extr-avidin alkaline phosphatase (1:900 dilution) for one hour each and were washed repeatedly with PBS-T. The NCM discs were then immersed in a substrate containing ortho-dianisidine tetrazolid (0.25 mg/ml), and magnesium sulfate (6 mg/ml) in 0.6 M sodium borate buffer (pH 9.7) for 10 minutes. The reaction was stopped by decanting the substrate, followed by thorough washing in PBS-T. The NCM discs were fixed in a solution containing methanol, acetic acid and distilled water in a ratio of 5:1:5. a positive reaction was indicated by the development of insoluble purple colour in the NCM discs. The standardized Dot-Iba gave positive reaction for those NCM discs that contained 100 ng of the 14-kDa antigen per ml and above. The assay gave negative results for NCM discs that contained the 14-kDa antigen at <100 ng/ml.

CSF samples from patients with culture-proven cases of TBM and diseased control groups were assayed for the mycobacterial antigen as described above for the standardized Dot-Iba. All CSF samples were assayed on two different occasions to evaluate the reproducibility as well as the reliability of the assay. The assay was performed with batches of 10 CSF specimens along with a positive antigen standard (100 ng/ml in PBS) and a negative control containing of PBS only).

Results and discussion: In contrast to conventional bacteriological methods, the Dot-Iba required only 6 hours to perform in the laboratory. The NCM discs containing 14-kDa antigen standard can be routinely stored at +4°C for at least six months. Dot-Iba gave positive results in all the five culture-positive patients with TBM. Ten of the thirty-five CSF specimens from culture-negative patients with TBM were positive for by the Dot-Iba. For 25 of 35 culture- negative patients with TBM, the Dot-Iba consistently gave negative results. In order to confirm the diagnosis of these 25 culture- negative patients with TBM, PCR was performed, and the PCR results were again negative for tuberculous aetiology. Serological and PCR for pneumococci and fungi were also negative. Hence a microbiological diagnosis for these 25 patients with probable TBM could not be ascertained. For the forty patients CSF samples from with nontuberculous neurological diseases, the Dot-Iba yielded consistently negative reactions. Thus the specificity of Dot-Iba was 100%. The results of Dot-Iba for patients in TBM and control groups were reproducible when the assay was performed on two different occasions and there was no variation in the results for any one the CSF samples from patients in the TBM and control groups.

In the earlier immunoassays described in the literature, the antibodies used in the assay to detect mycobacterial antigen either

were commercial products or were induced in other species. However, in this study, I have used specific human CSF IgG to *M tuberculosis* to isolate a mycobacterial antigen from the culture filtrates of *M tuberculosis*. Immunoabsorbent affinity chromatography was applied to isolate a specific mycobacterial antigen, and this antigen showed a single band in SDS-PAGE and had a molecular weight of 14-kDa. All TBM patients with a positive Dot-Iba received anti-tuberculosis chemotherapy (ATT) during their hospital stay and they showed positive neurological response to ATT within 3 weeks after the commencement of chemotherapy. Thus, a positive Dot-Iba result may have definite diagnostic value. The Dot-Iba established in this laboratory is rapid and specific for the detection of mycobacterial antigen in CSF. More importantly, it can be readily performed in a routine clinical laboratory and does not require sophisticated equipment and the results can easily be interpreted by visual examination of the NCM strips. Further, the staff of a single laboratory can easily handle large numbers of CSF specimens. The reagents used in the assay have shelf lives of more than six months. Because of these operational advantages, I consider that this diagnostic approach is not only unique but is also appropriate to laboratories in developing countries.

(C) Diagnostic utility of Immunohistochemical (IHC) method for the demonstration of mycobacterial antigens in intracranial tuberculoma. (Reference #22)

Background information: Intracranial tuberculoma clinically manifests either as a single or multiple space-occupying lesion. The advent of neuroimaging techniques such as magnetic resonance imaging (MRI) has definitely enhanced the pre-operative diagnosis of intracranial tuberculoma. However, there are still few clinical instances where the clinical diagnosis of intracranial tuberculoma remained uncertain and the neuroradiological features in MRI scan could not distinguish tuberculoma from solitary enhancing lesion, i.e.-abscess or metastasis. Confirmatory laboratory diagnosis of tuberculous etiology viz the **'gold standard'** in the surgical specimens of intracranial tuberculoma depends upon the demonstration of *M tuberculosis* either by bacterial culture or demonstration of acid-fast bacilli in Ziehl-Neelsen–stained smears. However, these conventional bacteriological methods are not only cumbersome but also less sensitive Thus, bacteriological confirmation of tuberculosis is often negative in a vast majority of specimens of intracranial tuberculoma. Histopathological features of caseating granulomatous lesions though suggestive of mycobacterial infection, but similar caseating granulomatous lesions can be caused by other microbial agents such as *Aspergillus* fungi. At times, pathologists are faced with the problem of distinguishing tuberculosis from fungal etiology in a surgical specimen of intracranial tuberculoma. This distinction becomes relevant and assumes greater clinical significance because the treatment modalities differ in tuberculosis and fungal infections of the Central Nervous System. Thus institution of appropriate chemotherapy in patients with intracranial granuloma depends upon an accurate laboratory diagnosis. With this objective, we have standardized two methods in the laboratory i.e. PCR and IHC methods to establish tuberculous etiology in surgical specimens of intracranial tuberculous granulomatous lesions as well as ten

surgical specimens of intracranial granulomatous lesions due to fungal infections.

Methodology: In this study, ten surgical specimens from patients with intracranial tuberculomas were collected. The pre-operative diagnosis of intracranial tuberculoma was made by the clinical features and supported by neuroimaging techniques-CT and MRI scans as well as compatible operative findings at surgery. Representative tissues collected at surgery were subjected to bacteriological and histopathological studies simultaneously. Under aseptic conditions, tissue homogenates of surgical specimens were inoculated into Lowenstein-Jensen (LJ) medium for culturing *M tuberculosis* and the smears from homogenates simultaneously stained with Ziehl-Neelsen stain for the demonstration of acid-fast bacilli. For histopathological studies, two representative blocks of tissue were sampled from every formaline fixed specimen and they were routinely processed. Five- micron thick paraffin sections under hematoxylin and eosin showed classical caseating epitheliod granulomatous lesions in eight specimens and were suggestive of tuberculous aetiology. Two specimens showed extreme degree of caseous necrosis and also showed minimal inflammatory reaction around the caseous necrosis. Ziehl-Neelsen staining for acid-fast bacilli was also undertaken in all these ten specimens. Paraffin sections from ten cases of intracranial fungal granulomas (Aspergillus granuloma 8, cryptococcal granuloma 1,phaeohyphomycosis 1) were selected as disease control for this study.

Immunohistochemical method to demonstrate mycobacterial antigen in tuberculoma: 5 micron- thick paraffin sections from tuberculous and non-tuberculous intracranial granulomatous lesions were subjected to a series of three preliminary incubations with (a) 3% hydrogen peroxide and methanol (1:5) for 30 minutes (b) concentrated hydrochloric acid and methanol (1:500) for 10 minutes and (c) 10% normal rabbit serum diluted in Tris-buffered saline (pH 7.4). Sections were then incubated with rabbit immunoglobulin G to *M tuberculosis* (20mg/ml) for 12 hours at +4°C. Following that the sections, were incubated for 20 minutes with (a) anti-rabbit IgG-biotin conjugate (1:100 dilution) and (b) streptavidin-peroxidase for 20 minutes each.. Subsequently the sections were washed thoroughly with Tris-buffered- saline containing 1% normal rabbit serum. Following this, sections were stained for 20 minutes with 2 mg of diaminobenzidine tetrachloride (5mg dissolved in 4 ml of 0.05 M Tris-buffer and 50 micro liter of hydrogen peroxide. Finally the sections were counterstained with hematoxylin, dehydrated and mounted in permount. The sections were examined under the microscope and mycobacterial antigens were characteristically located within the cytoplasm of several macrophages and Langhan's giant cells in the granuloma. They appeared as diffusely stained granular brownish-pink material. In order to evaluate the specificity of the immunostaining, normal rabbit serum was substituted instead of rabbit serum to *M tuberculosis* and this substitution gave negative results in all the specimens of intracranial tuberculoma. This would suggest that localization of mycobacterial antigen in the granulomatous lesions was brought by the addition of rabbit IgG to *M tuberculosis.*

Extraction of mycobacterial DNA from formaline fixed paraffin embedded tissues: Three 20 mm thick paraffin sections from each par-

affin block of tuberculoma and fungal granuloma were cut with a rotary microtome. In order to prevent carry-over contaminating DNA, a fresh a microtome disposable blade was used for each test and control specimens. After processing each specimen, the microtome was cleaned with xylene and 100% ethanol. The paraffin sections from intracranial tuberculous and fungal granulomas were collected in 1.5 ml microcentrifuge tubes and deparaffinized using xylene. Rest of the procedures of DNA extraction, PCR amplification of 123 bp regions from the IS6110 and analysis of amplified PCR product were performed using the conventional protocol in the laboratory.

Results and discussion: The results of bacteriological cultures were available 6-8 weeks after the inoculation of the specimen onto Lowenstein-Jensen medium and *M tuberculosis* was isolated in one out of ten specimens of tuberculoma while acid-fast bacilli were demonstrated by Ziehl-Neelsen stain in 2 out of ten specimens of tuberculoma. Immunohistochemical method yielded positive results in 8 out of 10 cases of intracranial tuberculoma. In none of these eight cases, was the characteristic morphological appearance of acid-fast bacilli identified. This may be as a result of phagocytosis and subsequent disintegration of the *M tuberculosis* bacilli within the granulomatous lesions. In two cases of intracranial tuberculoma, mycobacterial antigens were not demonstrated, because there was extensive caseation and paucity of macrophages and giant cells within the lesions. Thus the sensitivity of the immunohistochemical method depends upon the presence of macrophages and giant cells within the granuloma. In none of the paraffin sections of ten intracranial fungal granuloma, was the characteristic intra-cytoplasmic localization of mycobacterial antigens demonstrated. In other words, the rabbit IgG to *M tuberculosis* did not cross- react with fungal antigen present within the macrophages and giant-cells in fungal granuloma. Thus the specificity of immunohistochemical method was 100% in this study. Hitherto, reports on larger series highlighting the utility of immunohistochemical method as an adjunct for the diagnosis of intracranial tuberculoma have not been published in the literature.

Six out of 10 intracranial tuberculoma gave positive results by PCR method. All positive cases depicted single band that corresponded to mycobacterial DNA standard, used as positive control in PCR.Hence PCR is less sensitive than immunohistochemical method. It requires more than 72 hours to perform the test while the immunohistochemical method required 12-16 hours to perform the assay. Immunohistochemical method could be undertaken in routine laboratories and requires only a small laboratory space while PCR requires a well-equipped laboratory and also requires individual stations for isolation, preparation of amplification as well as gel documentation system. Immunohistochemical method is cost-effective whereas PCR requires elaborate instrumentation and expensive chemicals. While Immunohistochemical method can be performed in a large number of specimens at a time, PCR can only be done in small numbers and requires technical expertise. Thus, based on the results of this study, I consider that immunohistochemical method for the demonstration of mycobacterial antigen in a tuberculous granuloma possesses several operational advantages over PCR.

(D) Correlation between culture of *Mycobacterium tuberculosis* and antibody concentrations to *Mycobacterium tuberculosis* antigen-5 in lumbar, ventricular and cisternal cerebrospinal fluid specimens in patients with tuberculous meningitis. (Reference#23)

Background information: The CSF specimens in vast majority of patients with TBM are collected from the lumbar route for the routine bacteriological studies and isolation of *M tuberculosis* in lumbar CSF samples are often less sensitive. There are several factors that may account for the low isolation rates of *M tuberculosis* in lumbar CSF samples in patients with TBM. (i) *M tuberculosis* bacilli, are embedded in the dense exudates in the basal cisterns (cisterna magna and interpeduncular cisterns) and basal leptomeninges of the brain. These exudates form a barrier for *M tuberculosis* bacilli to circulate in lumbar CSF. (ii) Perhaps more importantly, most of the patients with TBM have received a course of antituberculosis chemotherapy before being referred to tertiary referral centers for neurological diseases. Lumbar CSF samples from partially treated patients with TBM contain less numbers of viable *M tuberculosis* and hence the smears and cultures are invariably negative. (iii) The cisternal and ventricular CSF contains more numbers of *M tuberculosis* than lumbar CSF.

Methodology: In an attempt to extrapolate the above, I undertook a prospective study in 10 patients with TBM. CSF during the hospitalization was collected through the lumbar route and they were subjected to bacteriological studies. Acid-fast bacilli were not demonstrated in any one of the 10 lumbar CSF specimens. *M tuberculosis* was isolated by cultures in two out of ten patients. All these ten patients were treated with anti-tuberculosis chemotherapy during their hospitalization. Because of the development of neurological complications and sequalae of the disease like –hemiplegia, seizures ans cerebral edema, these patients did not recover and expired during their hospital stay. Autopsy studies were available only in eight patients. At the time of autopsy, CSF samples (2-3 ml) were collected from the lateral ventricle and basal cisterns in two separate sterile glass centrifuge tubes. The deposits were subjected to bacteriological studies. The supernatant CSF samples were stored in vial and preserved at-70°C. CSF samples were also collected from ventricles and basal cisterns from 8 patients with chronic meningitis at autopsy and were used as controls. An indirect ELISA assayed the supernatant ventricular, cisternal CSF samples and also lumbar CSF samples for IgG antibody to *M tuberculosis* antigen-5. In brief, the wells in the polystyrene microtiter plates were coated with *M tuberculosis* antigen-5 (2mg/well). CSF samples collected from cisternal, ventricular and lumbar routes from patients with TBM and chronic meningitis were serially diluted (through the range 1:40-1:320), before they were added to the respective well in duplicate. IgG antibody to *M tuberculosis* antigen-5 present in the CSF samples was measured with an antihuman IgG-alkaline phosphatase as an enzyme conjugate and p-nitrophenyl phosphate as a substrate. The end-point antibody titer in the CSF samples was recorded. Subsequently, the absorbances at each dilution end-point were recorded with an automated ELISA reader. The arithmetic mean IgG antibody titer in lumbar, cisternal and ventricular CSF samples was calculated in patients with

TBM and in patients with chronic pyogenic meningitis.

Results and discussion: The lumbar CSF samples in all the ten TBM patients did not demonstrate acid-fast bacilli and positive cultures for *M tuberculosis* was recorded only in 2 patients. In contrast, cisternal CSF samples were positive for acid-fast bacilli in 6 patients and positive cultures were obtained in 7out of 8 patients. In ventricular CSF samples, positive results for acid-fast bacilli and cultures were obtained in 4 and 6 patients respectively. The CSF- I gG antibody titer in lumbar CSF samples of patients with TBM ranged between 1:80-1:320 with a mean antibody titer of 1:170. The mean IgG antibody titers in cisternal and ventricular CSF samples of patients with TBM were 210 and 177.5 respectively. The results of this study highlights (a) low culture sensitivity in lumbar CSF is due to the low density of circulating *M tuberculosis* in lumbar CSF samples than cisternal or ventricular CSF samples in patients with TBM (b) IgG antibody concentrations in lumbar, ventricular and cisternal CSF samples circulate in significant titers and are not significantly different from one another(c) since the specimens of CSF can not be obtained from cisternal or ventricular routes for the routine bacteriological investigations in patients with TBM, detection of IgG antibody to *M tuberculosis* antigen-5 in lumbar CSF by an indirect ELISA should be considered as an aid for the diagnosis of TBM ,particularly when repeated CSF cultures are negative for *M tuberculosis* . Hitherto such a study has not been published in the literature earlier.

(E) Demonstration of specific antibody to 35-kDa mycobacterial antigen in the CSF specimens by an immunoblot method. (Reference #24)

In order to define the mycobacterial antigens that may be specifically associated with human tuberculosis infection, the cerebrospinal fluid (CSF) specimens of thirty patients with tuberculous meningitis and of an equal number of patients with non-tuberculous meningitis were compared by means of an immunoblot method for detecting antibody to *Mycobacterium tuberculosis.*

Methodology: Culture filtrate antigen (CFA) of *M tuberculosis* was subjected to the conventional discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The various protein components of CFA in the gels were stained with 1% coomassie brilliant blue dye. The CFA showed multiple bands, the molecular weight of which ranged between 10-kDa and 89-kDa. The components of CFA were then transferred to Nitrocellulose membrane (NCM) by means of a horizontal LKB Novoblot apparatus. After the transfer, the NCMs containing the components of CFA were thoroughly washed in 0.15 M phosphate buffered saline (PBS-pH 7.4) and then quenched in 2.5% skimmed milk for one hour at 40°C. Subsequently, the NCM strips were incubated with 1 ml CSF (1 in 10 dilution) from each of the patients with TBM and each of the controls for 2 hours. The NCMs strips were then washed several times with PBS before being incubated with 1 ml (1: 1000) anti-human IgG-alkaline phosphatase conjugate for 2 hours. The NCM strips were again washed several times with PBS. The immunostaining was performed by the addition of a substrate containing O-dianisidine tetrazolid (0.25 g/L), â-naphthyl acid phosphate (0.25g/L) and magnesium sulphate (1.2g/L) in 0.006 m borate buffer. After 15 minutes, the reaction was terminated by dipping the NCM strips in a fixa-

tive-containing methanol, acetic acid and distilled water (5:1:5). A positive reaction was indicated by the development of a purple pink band in the NCM. The immunoblot technique was performed with batches of 10 samples of CSF at a time. The intensity of immunostaining was not affected by the storage of either the NCM strips at +4°C.

Results and discussion: The CSF samples specimens from most of the patients in control group either did not contain any demonstrable antibody to CFA or when present, it was found to react with 27,30,45-kDa antigens in the CFA of *M tuberculosis*. In patients with TBM, the numbers as well as intensities of the immunostained bands were greater than those of the controls. The most striking observation in the immunoblots however, was the presence of a antibody in the CSF samples of patients with TBM which had specifically reacted with a 35-kDa antigen of CFA.This specific antibody was present in all the culture-positive patients as well as in 19 out of 25 culture-negative patients with TBM.In the CSF samples of the control group, specific antibody was not found to react with 35-kDa mycobacterial antigen. Thus we consider that this unique observation allows patients with TBM to be differentiated from patients with partially treated pyogenic meningitis (PM).Such distinction has important clinical implications because a differential diagnosis between TBM and partially treated pyogenic meningitis at times poses considerable difficulties, particularly in the absence of bacteriological confirmation of the nature of the disease.

Thus, I consider that the immunoblot method established in this study can meet the needs of most laboratories in developing countries. The NCM strip containing the CFA can be stored in +4°C for at least 2 years and can be readily transported to all the laboratories located in outlying hospitals. The technical procedures of the immunoblot methods are very simple. It is necessary only to incubate the NCM strip and compare the result with that of a positive control CSF. Neither elaborate instrumentation nor exceptional technical expertise is required. Many samples of CSF can be handled at a time and the results can be made available within 8 hours after the receipt of the CSF specimens in the laboratory.

(F) Detection of Mycobacterium *tuberculosis* antigen 5 in CSF specimens by inhibition enzyme-linked immunosorbent assay (ELISA) and its diagnostic potential in patients with TBM (reference #25)

In an attempt to establish a specific diagnosis of TBM, we standardized an inhibition ELISA to quantitate circulating *M tuberculosis* antigen 5 in the cerebrospinal fluid specimens of 40 patients with TBM and in equal numbers of patients with meningitis due to non-tuberculous aetiology.

Methodology: Prior to application in clinical specimens, the inhibition ELISA was initially standardized by incubating different concentrations of *M tuberculosis* antigen-5 (1-500 ng/ml) in 0.15 M phosphate-buffered saline (PBS-pH 7.4) with an equal volume of 100-mg/ml gamma globulin to *M tuberculosis* antigen-5. After 12 hours incubation at+4°C, the antigen-antibody complex was centrifuged at 5000rpm for 10 minutes and 200ml of supernatant was transferred to each well of microtiter ELISA plates. The microtiter plates were presensitised with *M tuberculosis* antigen-5

(2mg/well in carbonate-bicarbonate buffer pH9.6) and subsequently quenched with 1% bovine serum albumin. After 12 hours incubation, the micro titer plates were washed three times with 0.05% Tween 20 in 0.15 M PBS. Next, 200ml of a 1:40,000 dilution of anti-rabbit-biotin conjugate was added to each well, and plates were incubated for 2 hours at room temperature. Following thorough washing with PBS-T, 200ml of Avidin-alkaline phosphatase was added to each well, and plates were incubated for 2 hours. The color reaction was developed by the addition of 200ml of Para nitrophenyl phosphate. The reaction was stopped after 30 minutes by the addition of 3 N sodium hydroxide. The control well in the standardization procedure contained antigen-5, antibody to antigen-5, anti-rabbit IgG –biotin conjugate, alkaline phosphatase and para nitrophenyl phosphate. The assay was repeated at six different concentrations of the antigens. The absorbances in test and control wells were recorded at 405nm in an automated ELISA reader. Differences in absorbance between test and control wells were plotted against the logarithmic value of the antigen used in the assay. A linear relationship, reproducible even at a low concentration of antigen (5ng/ml) was obtained. CSF specimens from tuberculous and control groups were assayed for *M tuberculosis* antigen-5 concentration in an identical fashion as described in the standardization inhibition ELISA and antigen concentration in each CSF specimen was directly measured from the standard linear graph.

Results and discussion: For the 40 patients in the control group, the mean antigen concentration was 1.45 ng/ml. An assay was regarded as positive if the antigen concentration was more than 5.2 ng/ml (mean+3SD). Antigen-5 concentrations in 10 culture-positive patients with TBM were 10.0-73.5 ng/ml (mean 48.5 ng/ml). Of the 30 culture-negative patients with TBM, the assay was positive in 21 patients with the antigen concentrations of 9-82 ng/ml (mean 45.5 ng/ml). The antigen-5 concentrations in the other 9 culture-negative patients with TBM were 1.05-4.6 ng/ml. Because no control CSF specimen had antigen concentration more than 5.2 ng/ml, the specificity of the assay was considered to be 100%. We also correlated the antigen-5 concentrations in CSF specimens and neurological recovery during anti-tuberculosis chemotherapy in 8 patients. The antigen concentration showed a gradual decrease during weeks 2-4 of therapy in 5 out of 8 patients following the chemotherapy and also showed positive correlation with neurological recovery.

The results of this study would highlight

(a) Inhibition ELISA is one of the specific immunoassays for the early diagnosis of TBM particularly in culture- negative patients with TBM, and (b) besides its diagnostic potential, this assay can also be used to monitor the therapeutic response to anti-tuberculosis chemotherapy in patients with TBM.

(G) Demonstration of heat stable 82 kDa mycobacterial antigens in CSF specimens by immunoblot method (reference#26)

In this prospective study, an immunoblot was standardized for measuring circulating mycobacterial antigen in the CSF of patients with TBM. The heat- inacti-

vated CSF specimens from tuberculous and non-tuberculous patients were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and they were subsequently transferred onto a nitrocellulose membrane (NCM). Using rabbit IgG to *M tuberculosis*, a heat stable 82-kDa mycobacterial antigen was demonstrated in the CSF samples of patients by this immunoblot method. The antigen was conspicuous by its absence in the CSF samples of non-tuberculous subjects. Due to heat inactivation of CSF specimens, there is minimal risk of handling of infectious material to the laboratory personnel processing the CSF specimens. This has assumed greater significance, because of high incidence and prevalence of tuberculosis in patients with HIV infection. Besides its diagnostic potential, this newer approach is simple and can be readily applied in any routine clinical laboratory and it is particularly suited to the laboratories in developing countries.

Conclusions: We have developed two newer immunoassays for the early diagnosis of TBM (a) direct immunocytochemical method for the demonstration of mycobacterial antigen in the cytospin smears of CSF specimens (b) a dot-immunobinding assay for the demonstration of specific mycobacterial antigens in CSF specimens. Both these immunoassays are simple and best suited to laboratories in developing countries where there are constraints in the resources as well as technical expertise. We also wish to propose that these immunodiagnostic systems may be made available to all the medical college hospitals in our country so that patients with TBM are diagnosed and treated in their early stages.

ACKNOWLEDGEMENTS:

We dedicate this work to all those patients who gave their valuable clinical specimens for this study. We are indebted to Department of Science and Technology, New Delhi for the financial assistance to undertake the study. We wish to express our sincere thanks to the Director of this institute for providing all the facilities for this research. We also take this occasion to convey our sincere thanks to all the colleagues in the departments of Pathology, Neurology and Microbiology for their unflinching support and co-operation during the past fifteen years.

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