IMMUNODIAGNOSIS OF TUBERCULOUS MENINGITIS

A THESIS PRESENTED

BY

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CERTIFICATE

I, Annamma Mathai, hereby certify that I had personally carried out the work depicted in the thesis entitled ***IMMUNODIAGNOSIS OF TUBERCULOUS MENINGITIS****, except where external help sought are acknowledged.*

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DECLARATION

This is to certify that Smt. Annamma Mathai in the Division of Pathology of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, has fulfilled the requirements of the regulations, relating to the nature and prescribed period of research for the Ph.D. degree of that Institute. The work relating to her thesis entitled **IMMUNODIAGNOSIS OF TUBERCULOUS MENINGITIS** was carried out under our joint supervision.

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The thesis
entitled

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Submitted
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ANNAMMA MATHAI

for
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OF
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TO

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SYNOPSIS

1. Purpose of the study

The diagnosis of Tuberculous meningitis (TBM) still remains a challenge for clinicians because the clinical and cerebrospinal fluid (CSF) findings of this infection often overlap with other bacterial infections of central nervous system (CNS), particularly with partially treated pyogenic meningitis. Conventional microbiological methods to demonstrate **Mycobacterium tuberculosis (M tuberculosis)** in CSF do not yield positive result in large proportion of patients with TBM[1]. A number of other laboratory tests like latex agglutination, reverse passive haemagglutination, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA)[2] have been developed in recent years as an aid in the diagnosis of TBM. However in majority of these studies, sensitivity and specificity of these assays are not found to be high because the antigens used in the tests are not specific for the diagnosis of TBM. In 1987 when the present study was undertaken, a purified mycobacterial antigen - **M tuberculosis** antigen 5 of molecular weight 35 kDa was used in ELISA. There are only few reports about the application

of this antigen in the immunodiagnosis of pulmonary tuberculosis[3] and TBM[4].

II. Basic Procedure

A. Preparation of mycobacterial antigen and antibody

(i) **Antigen:-** H_{37} Ra **M tuberculosis** was used for the preparation of mycobacterial antigens. Two mycobacterial antigens namely culture filtrate antigen[5] and **M tuberculosis** antigen 5 were isolated[6] from H_{37}Ra **M tuberculosis** in this laboratory.

(ii) **Antigen:-** Antibodies to culture filtrate antigen and **M tuberculosis** antigen 5 were raised in rabbits[7].

B. Collection and testing of clinical specimens

(i) **Patients with TBM:-** Sera and lumbar CSF were collected from 75 patients admitted in the neurology unit of this hospital between March 1987 and February 1991. 16 of them were classified as "definite" cases of TBM because **M tuberculosis** was isolated from their CSFs. In the remaining 59 patients, the diagnosis was based on the clinical features and they showed compatible cytological and biochemical parameters in the CSFs. However CSF cultures were negative for **M tuberculosis** and other microbial agents. These patients were therefore classified as "Probable" cases of TBM.

(ii) **Control group:-** Sera and lumbar CSF from 75 patients with non-tuberculous neurological disorders were collected. (Pyogenic meningitis n=45; viral encephalitis n=8; cryptococcal meningitis n=3, non-infectious neurological disorders n=19).

C. Techniques

(i) Estimation of mycobacterial antigens in CSF was carried out by an inhibition ELISA[8].

(ii) Detection of antimycobacterial antibody to **M tuberculosis** antigen 5 in CSF was carried out by an indirect ELISA[9].

(iii) Demonstration of mycobacterial antigen and antibody in CSF by an immunoblot method[10].

(iv) CSF-IgG index was calculated using the formula, CSF IgG index = CSF IgG: Serum IgG/CSF albumin: serum albumin.

III. Main Findings

(i) The data for the quantitation of mycobacterial antigen in CSFs of patients with TBM and patients in the control group are shown in Table I. A test was considered positive for tuberculous aetiology when the antigen concentration in CSF was more than

5.35 ng/ml (mean antigen concentration in the control group + 3SD).

(ii) The data for the antimycobacterial antibody in CSFs of patients with TBM and control group are shown in Table II. None of the patients in the control group gave positive ELISA at 1/80 dilution and therefore 1/80 was selected as a "cut off" point for scoring a test CSF positive for the presence of antimycobacterial antibody. The test was also considered positive, if the absorbance in a test sample at 1/80 dilution is more than 0.56 (mean absorbance in control + 2SD).

(iii) CSFs of patients with TBM contained two mycobacterial antigens of 52 kDa and 27 kDa while these antigens were absent in the CSFs of the control group. IgG antibody present in CSF of patients with TBM specifically reacted with 35 kDa present in the culture filtrate of **M tuberculosis**. The IgG present in CSFs of patients in control group did not react with the 35 kDa antigen.

(iv) Mean CSF-IgG index in 'definite' cases TBM was 0.92 ± 0.11;. In 'probable' cases of TBM the CSF IgG index was 0.89 ± 0.18, and in control group the IgG index was 0.31 ± 0.12.

(v) Sensitivity and specificity of the inhibition ELISA and indirect ELISA are shown in Table III. Inhibition ELISA is more sensitive than indirect ELISA. However with both ELISAs no false negative results were recorded in "definite" cases of TBM. All the "definite" cases of TBM showed the presence of mycobacterial antigen and antimycobacterial antibody in their CSFs. Thus there was a good correlation between the results of ELISA and results of bacteriological methods in "definite" cases of TBM.

IV. Principal Observations

1. There is a positive correlation between the results of bacteriological method for the isolation of **M tuberculosis** in CSFs and the results of ELISA in "definite" cases of TBM. The results of cultures were known only after 6 to 8 weeks while the results of ELISA required only 48 hours to predict a tuberculous aetiology. Positive ELISA has diagnostic value in "probable" cases of TBM and these patients should get the benefit of antituberculous chemotherapy.

2. Sensitivity of inhibition ELISA is 76% and sensitivity of indirect ELISA is 69% in this study. These sensitivities are greater than 21% sensitivity obtained

with the bacteriological methods in our study. The efficiency of prediction for tuberculous aetiology with both ELISA methods ranged between 84-88%.

3. An immunoblot analysis revealed the presence of two mycobacterial antigens 52kDa and 27kDa and a specific antimycobacterial antibody to 35kDa antigen was also present in the CSFs of patients with TBM.

4. ELISA method should be therefore considered as an adjunct in the rapid diagnosis of "probable" cases of TBM. ELISA method should also be considered as one of the laboratory investigations for establishing a tuberculous aetiology in a patient with meningitis of unknown aetiology.

TABLE - I

Mycobacterial antigen concentration in CSF

Patients	No. of patients with antigen concentration < 5 ng/ml	No. of patients with antigen concentration > 5 ng/ml	Range ng/ml	Mean ng/ml
Definite TBM n = 16	0	16	14.5-290	158.8
Probable TBM n = 59	16	43	15.5-280	151.6
Control group n = 75	75	0	1.15-3.77	1.45

TABLE - II

Antimycobacterial antibody to M tuberculosis antigen 5 in CSF

Parameters	Definite TBM n = 16	Probable TBM n = 59	Control Group n = 75
End-point titre			
Less than 1:40	0	23	75
Between 1:80-1:640	16	36	0
Absorbance at 1/80 dilution			
Range	0.7-1.3	0.65-1.2	0.1-0.38
Mean	0.66±0.21	0.78±0.19	0.25±0.15

TABLE - III

Data on Inhibition ELISA Vs Indirect ELISA

	Inhibition ELISA	Indirect ELISA
Sensitivity (%)	76	69
Specificity (%)	100	100
False negative in 'definite' TBM (%)	0	0
Efficiency of prediction (%)	88	84
Inter-assay variation	5-8	3-6

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INTRODUCTION

CHAPTER I

Human tuberculosis still remains a major public health problem in many parts of the world including India. The current global prevalence of tuberculosis is estimated to be 15-20 million cases with an annual incidence of about 10 million new cases and at least 3 million deaths each year. In developing countries the incidence, mortality and morbidity rates in patients with tuberculosis have not shown any decline during the past decade. In India, 500,000 people die from this infectious disease every year (97). In developed countries the incidence of tuberculosis was low during the past decade. It is now on the increase partly because of the emergence of AIDS (20).

Tuberculosis is caused by a bacillus M tuberculosis. Robert Koch in 1882 isolated a rod-shaped bacilli from a lesion and he inoculated the bacilli into 94 guinea pigs, 70 rabbits, 9 cats and 14 mice. All the animals developed tuberculosis. Subsequently Robert Koch in 1891 reported an enhanced immune response when the tubercle bacilli were reinoculated into the same host, in whom the tubercle bacilli were inoculated earlier. Robert Koch's observations were the forerunner of what we recognise today as tuberculin immunity and hypersensitivity. Despite the fact that more

than one hundred years have passed since Robert Koch isolated the tubercle bacillus, the precise immune responses in patients with tuberculosis are not well understood and considerable lacunae still exists in our knowledge regarding the host immune mechanisms in tuberculosis.

1.1 Pathogenesis of mycobacterial infection

Human tuberculosis is caused by **M tuberculosis** and **M bovis.** Less frequently atypical mycobacteria like **M intracellulare, M kansasii** can also induce lesions which are similar to **M tuberculosis** (54). The sequence of events involved in the pathogenesis of mycobacterial infection is represented in Fig. I.I. Primary infection due to **M tuberculosis** commonly occurs in the lungs and this is usually acquired by the inhalation of the infected droplets, which pass down the bronchial tree into the bronchioles or alveoli. Phagocytosis may occur at this stage but the mycobacteria may remain viable and can replicate within this intercellular environment. The tubercle bacilli then enters the blood as well as the regional lymphatic channels and are carried to regional and distant organs. Tubercle bacilli are usually deposited in the upper lung zones and less frequently in the renal parenchyma, cerebral cortex and regional lymph nodes. This allows bacterial multiplication before immunity develops in the host. The primary site of infection usually gets healed as the immunity develops in

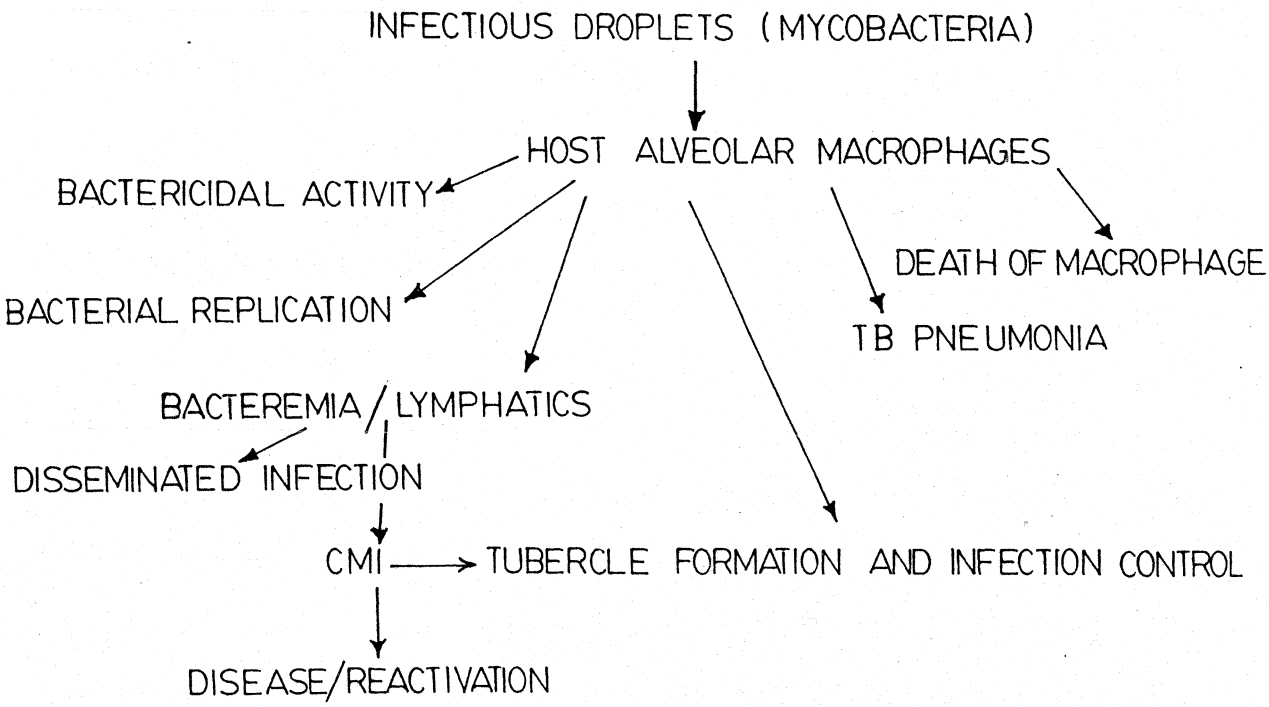


FIG. I.I SHOWS THE SCHEMATIC REPRESENTATION OF THE EVENTS IN THE PATHOGENESIS OF TUBERCULOSIS

(Adapted from Edwards and Kirkpatrick; Am Rev Respir Dis 1986).

the host. A significant proportion of individuals who develop immunity after successful recovery from the primary infection, do not eliminate the tubercle bacillus and remains at the risk of reactivation. The risk factors for the reactivation of dormant tuberculosis are not well understood, but it may include age, socio-economic factors, malnutrition and treatment with immunosuppressive drugs. In general, the risk of developing tuberculosis is about 8% during the first two years following the primary infection.

15% of tuberculous infection occur in the extrapulmonary sites. CNS involvement by tuberculous infection is one of the common neurological diseases among hospitalised patients. In general the incidence of CNS tuberculosis in a given geographic zone is directly proportional to the prevalence of the tuberculous infection. The CNS involvement by the tuberculous infection may clinically manifest in three forms - TBM, tuberculoma and cerebro-vascular accidents. CNS tuberculosis constitutes nearly 45% among all forms of tuberculosis in the paediatric age group. This fact assumes greater significance when one considers that this very high incidence persists despite the advent of potent ATT during the past 15 years. Cranial form of TBM is by far the commonest form of neurotuberculosis. TBM can occur at any age but is uncommon under the age of 6 months and rare before the age of 3 months. The highest

incidence of TBM occurs in the first five years of life. In the paediatric age group, TBM is usually a complication of primary infection. In adults, the disease may occur as an isolated form of tuberculosis or in association with pulmonary or miliary tuberculosis. The tubercle bacilli reaches the CNS by the way of haematogenous dissemination from the primary site which is most frequently from the lungs. The majority of cases of TBM are caused by **M tuberculosis**. Meningitis caused by atypical mycobacteria is extremely uncommon although there are reports in whom **M kansasii** and **M scrofulaceum** were isolated from the CSF (54).

Until the decisive study of Rich and McCordock (105) it was believed that TBM was a direct and immediate result of haematogenous infection of the meninges. These investigators have proposed that (a) Many cases of TBM occur in the absence of miliary tuberculosis. (b) Meningitis may be absent in most extreme cases of miliary tuberculosis in which every susceptible organ is riddlled with tubercles. (c) When miliary tuberculosis and TBM occur together, the age and character of the miliary tuberculosis do not correspond with the stage of meningitis. (d) Introduction of large number of tubercle bacilli into the blood stream of a susceptible animal invariably produce miliary tubercles but fail to cause meningitis eventhough the inoculum has been

made directly into the carotid artery. (e) Typical diffuse leptomeningitis can be induced when tubercle bacilli are introduced directly into the subarachnoid space of susceptible animals.

On the basis of these observations, Rich and McCordock postulated that TBM occurs in two stages. Initially tuberculous lesions are formed in the brain or in the meninges by the haematogenous dissemination of the tubercle bacilli from the primary site of infection. The meningitis develops from the discharge of the tubercle bacilli from a cortical caseous focus directly into the subarchnoid surface. This phenomenon may occur immediately after the lesion is formed. Although many host factors such as malnutrition, alcoholism, diabetes and impairment of CMI are associated with a high incidence of tuberculosis, no data exist to prove that any of the factors play a role in the development of meningitis (89).

1.2 Immune response in tuberculosis

It is relevant to highlight the immune response in tuberculosis in brief because it will promote the understanding of the pathogenesis and pathology of the tuberculosis infection. The basic expression of tuberculo-immunity is shown in Fig.I.II.

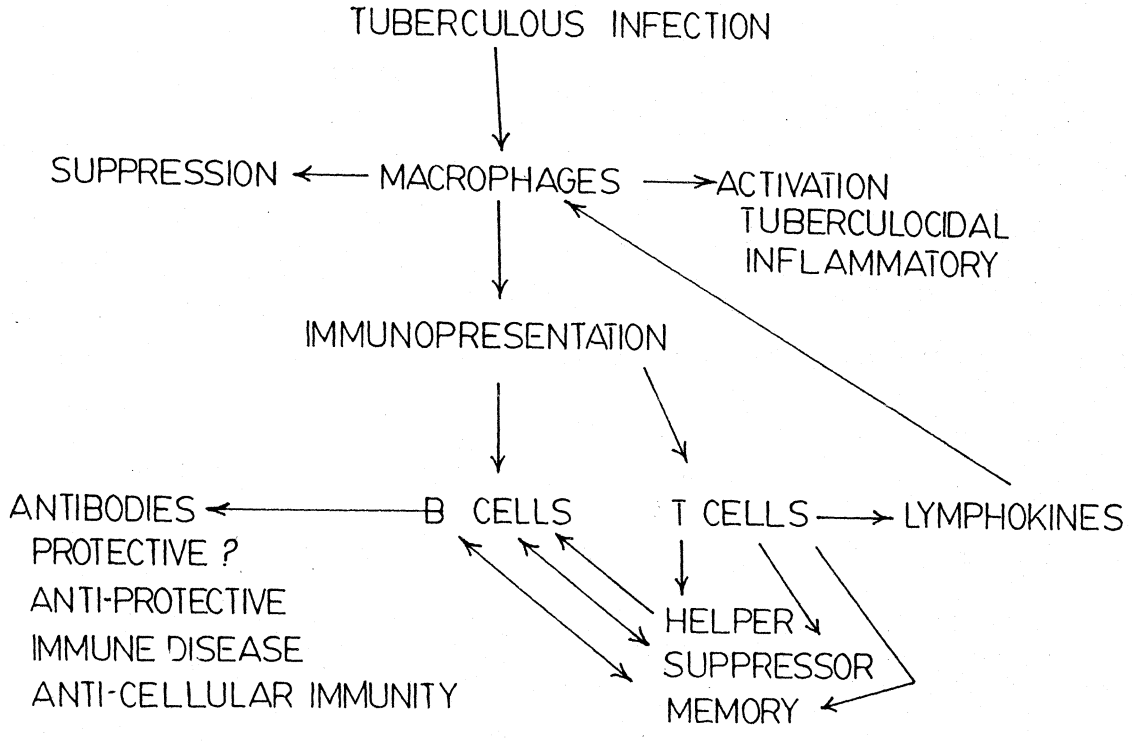


Fig I. II SHOWS THE SCHEMATIC REPRESENTATION OF THE BASIC IMMUNOLOGICAL EXPRESSION IN TUBERCULOSIS

(Adapted from Chapras; Bull WHO 1982)

In primary tuberculosis, neutrophils constitute the first line of defence and this is soon followed by macrophages which play a crucial role in generating an immune response. Besides providing an effective natural defence mechanism, the macrophages also initiate the afferent limb of the immune response. The macrophages process the mycobacterial antigens and present them to T-lymphocytes. The macrophages once activated, exhibit profound morphological changes in them. These are characterised by an increase in the number of mitochondria, lysosomes and enhanced rates of phagocytosis as well as tuberculocidal activity. Phagocytosis is an orderly process whereby mycobacteria are engulfed into the cytoplasm of the macrophages and thus a phagosome is formed. The sequence of events of phagocytosis of tubercle bacilli by the macrophages are illustrated in Fig. I.III. Within the cytoplasm of macrophages, enzyme containing lysosomes contacts and fuses with the phagosome and thus a phagolysosome is formed. Enzymes released by the lysosomes then exert digestive activities on the ingested bacilli and cause the break-down of the bacilli. Fig. I.IV summarises the processes involved in the immune response to the tubercle bacilli or any antigen. They are (a) antigen presentation by the macrophages and activation of helper T-lymphocytes (T_H cell) (b) secretion of lymphokines and

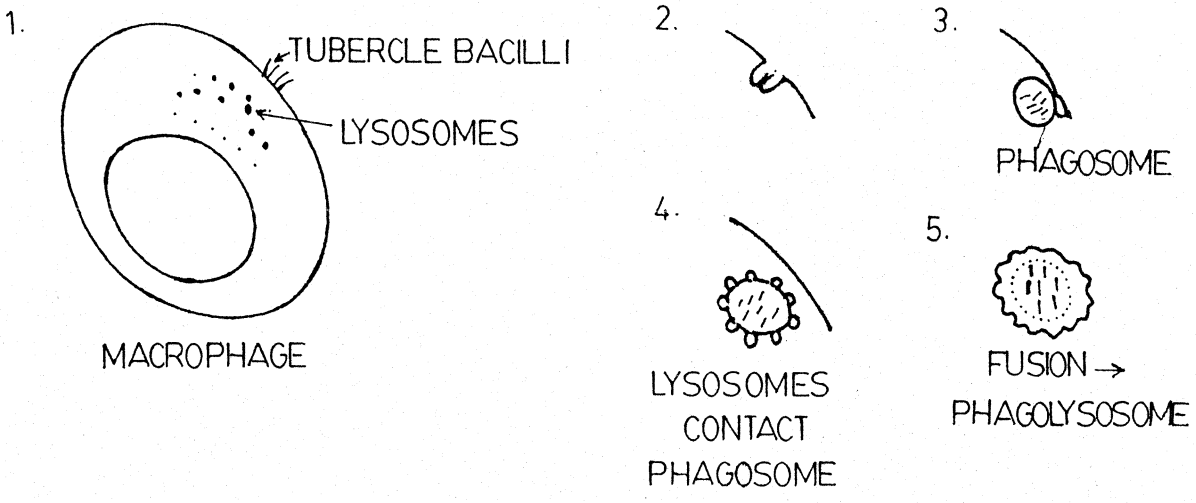


Fig. I. III. SHOWS THE DIAGRAMMATIC REPRESENTATION OF THE SEQUENCE OF EVENTS IN THE PHAGOCYTOSIS OF TUBERCLE BACILLI

(Adapted from Chapras; Bull WHO 1982)

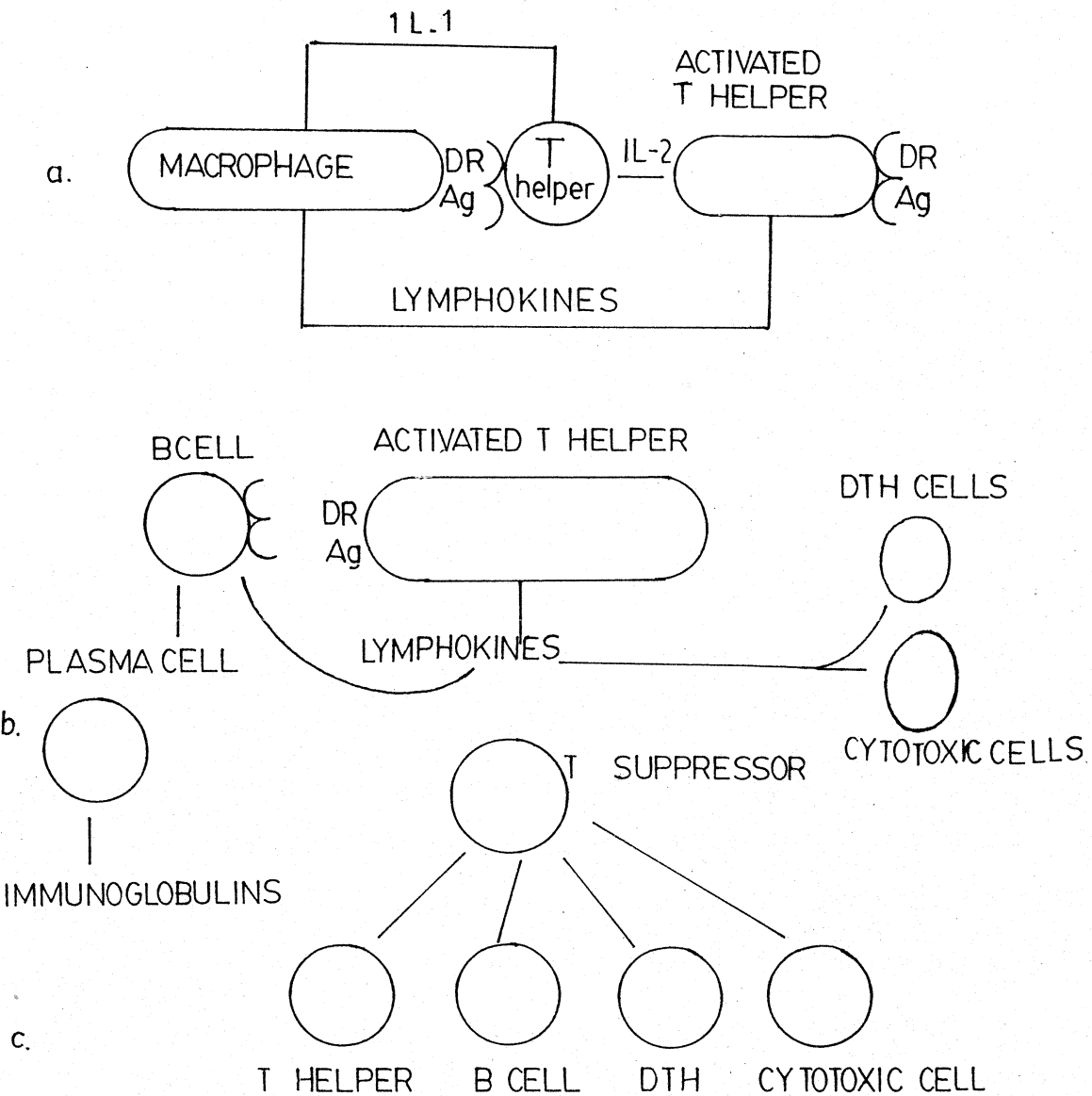


Fig.1 IV SHOWS THE SCHEMATIC REPRESENTATION TO HIGHLIGHT THE INTERACTION BETWEEN MACROPHAGES AND LYMPHOCYTES

(Adapted from Edwards and Kirkpatrick; Am Rev Respir Dis 1986).

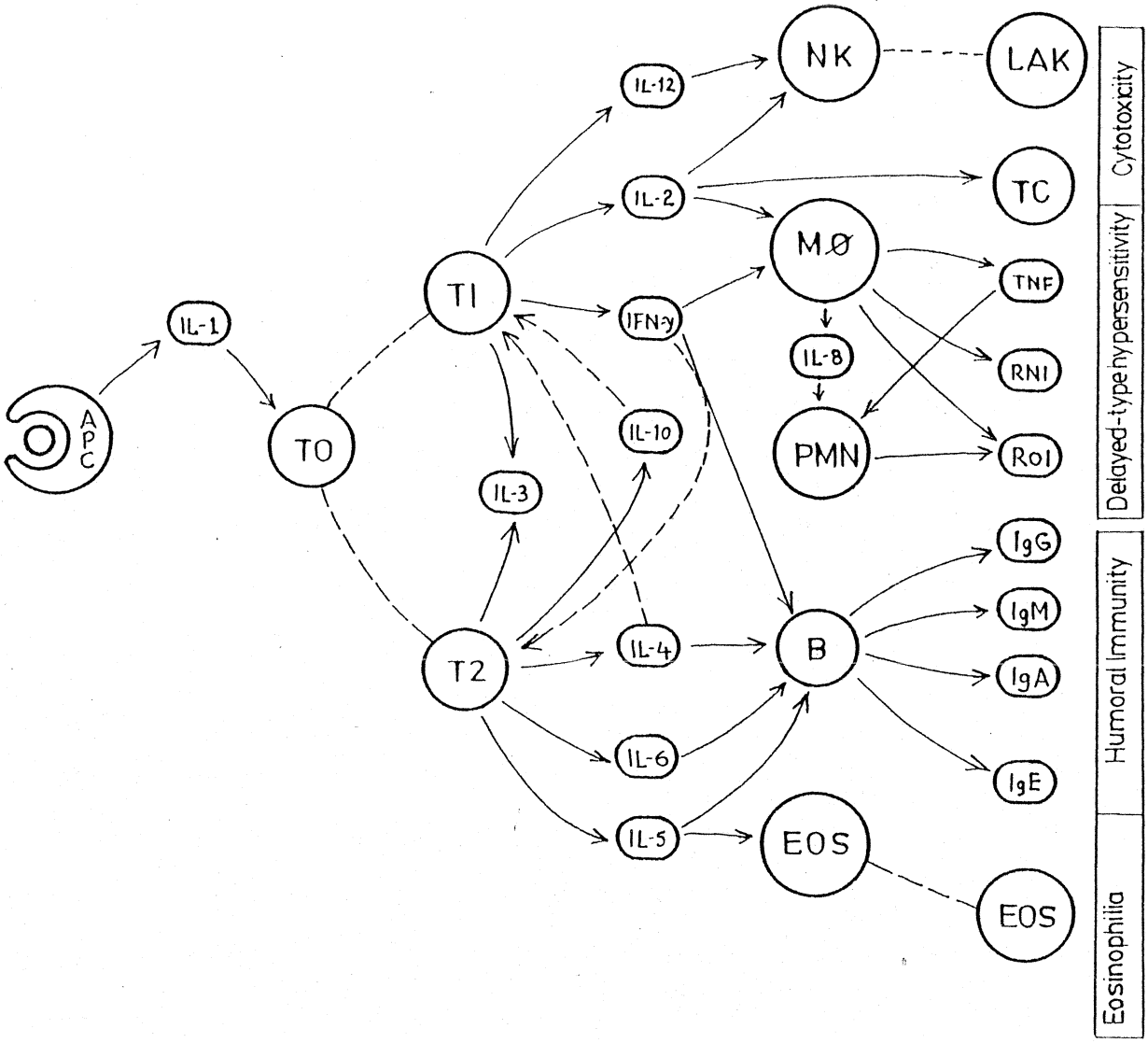
activation of effector cells (c) inhibition of effector cells by suppressor T-cells (T_S cell). Activation of macrophages is dependent largely on the activities of T-lymphocytes. The interaction of immune T_H cells with tubercles bacillus and its antigen results in the release of a number of mediators (lymphokines) with different activities. The list of lymphokines released is shown in Table-I.1

Mediators which mobilise and activate macrophages, play a significant role in the destruction of tubercle bacilli. Movement of phagocytic mononuclear cells to the site of tubercle bacilli is prompted by the release of lymphocyte and macrophage chemotactic factors. Macrophages so attracted are encouraged to remain at the site by MIF. Increased enzymic activity is induced by MAF which is responsible for the increased tuberculocidal activity. The release of SRF leads to inflammation and increased vascular permeability. The T-lymphocyte activation requires synthesis and release of IL-1, a soluble cytokine from the macrophages. IL-1 appears to stimulate T-cells to produce IL-2. IL-2 in return promotes T-cell proliferation. An intricate network of cytokine is involved in the generation of humoral and CMI as depicted in Fig. 1.V. There are also indications that T_H-cells are further classified into T_HI

TABLE - 1.I

The lymphokines released by activated lymphocytes

(A)	**Inhibitors of growth**
	proliferation inhibitory factors (PIF)
	clonal inhibitory factors
	cytotoxic factors (CF)
(B)	**Promoters of growth**
	blastogenic factors (BF)
(C)	**Inhibitors of motility**
	migration inhibitory factors (MIF)
	leukocyte (Polymorphonuclear) inhibitory factors (LIF)
(D)	**Promoters of motility**
	Chemotaxis
(E)	**Activators and inflammation inducers**
	macrophage activating factors (MAF)
	skin reactive factors (SRF)
(F)	**Cytokines**
(G)	**Interferon**
(H)	**Antibody**



Abbreviations:

Cells: *B, B cells, T1, $T_H1.CD4^+$ and $CD8^+$ T cells; T2, T_H2 $CD4^+$ and $CD8^+$ T cells; TC, cytotoxic $CD8^-$ T cell; TO uncommitted T lymphocytes; APC antigen presenting cell; NK, natural killer cell; LAK, lymphokine activated cell; PMN, polymorphonuclear cell or neutrophil, EOS, eosinophil, MO, macrophage.*

Cytokines and effector molecules. *IL interleukin; IFN- , interferon - gamma. TNF, tumor necrosis factor , ROI, reactive oxygen intermediates RNI, reactive nitrogen intermediates.*

Fig.1.V *Showing the T-cell subset and the cytokines released in infections.*

(Adapted from Cox and Liew; Immunology Today 1992).

and T_H2 lymphocytes, the former participating in the generation of gamma interferon, IL-2 and delayed type of hypersensitivity (Fig. 1.VI).

The cell type most associated with protective immunity in tuberculosis is the helper T_H-cell. The positive effects of T_H cells may be countered by T_S cells. In tuberculosis, both types of T-cells may be produced at the same time. Initially T_H cells activity is more prominent but in the advanced stages of the disease as well as in the presence of a large bactericidal load there appears to be a predominant activity of T_S cells. Activity of T_S cells are demonstrable in advanced stages of tuberculosis and they are presumed to function by the release of soluble suppressor substances and they also modify the production of B cells, as well as T_H cells. In advanced tuberculosis, an enormous bacterial load perpetuates the T_S cell activity and this in turn promotes the unrestricted growth of the bacilli. Soluble factors inhibitory to CMI have been found in the sera of such patients. Therefore exposure to large levels of antigen results in the suppression of T-cell functions. This could be due to either clonal depletion or blockage of specific receptor sites by antigen-antibody complexes.

The role and function of NK-lymphocytes in tuberculosis is not well understood. Interferon is known to potentiate NK-cell differentiation and its activity but the

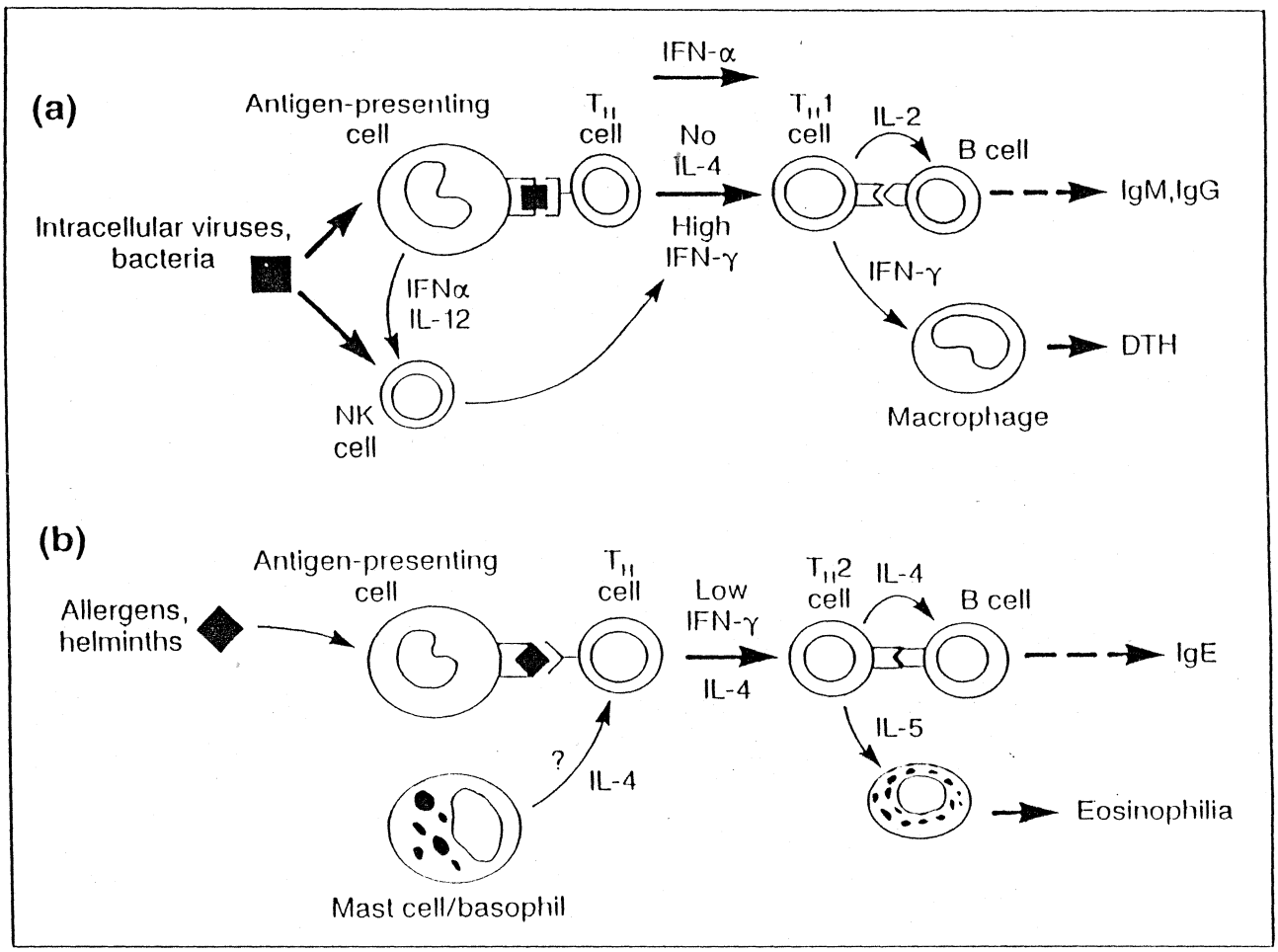


Fig. 1.VI *Showing the role of T_HI and TH_22 lymphocytes in infection and allergic conditions*

(Adapted from Romagnani; Immunology Today 1992)

role of interferon in increasing the NK-cell activity in tuberculosis remains speculative.

The precise role of antibodies in the immunity to tuberculosis is not clear. However, patients with more severe form of the disease demonstrated polyclonal hypergammaglobulinemia in their sera. Lenzini (75) described a spectrum of tuberculosis ranging from patients with fully active CMI to anergy in which the serum immunoglobulins progressively increased as the CMI decreased. Their study also showed high serum titers of antibodies to PPD antigen. The studies conducted by Kalpan et al (63) showed an increase in the antibody titers to mycobacterial antigens during the treatment and that the antibody titres correlated with the clinical improvement in patients with tuberculosis. Thus serum immunoglobulin estimation may be useful for the clinical staging of the disease but their role in immunity in tuberculosis still remains unclear.

1.3 Role of immune complexes

Total hemolytic complement in serum is increased in patients with tuberculosis (106). Many investigators (18,59,86,110) found CICs in the sera from patients with tuberculosis. The immune complexes were composed of IgG, IgA and IgM and mycobacterial antigen. This immunological response correlated with a poor prognosis because a rise in

CICs might suggest an increase in the virulence of **M tuberculosis.** Though the significance of CICs in patients with tuberculosis remain unclear, they offer a modality by which one can assess a patient's clinical response to ATT and estimation of CICs may also be considered as one of the markers in the prognosis (86,110).

1.4 Defects in host phagocytic activity

Multiple defects in the macrophage functions have been reported in patients with tuberculosis. Cruchaud (27) observed reduced phagocytic and bactericidal functions in patients with severe pulmonary tuberculosis. Reduced bactericidal activity may be related to the inhibition of phagolysosomal fusion (3). In addition to the defective bactericidal effect, macrophages from patients with tuberculosis may also show defective chemotaxis (19). However, chemotactic defects, reduced phagocytosis and bactericidal defects alone cannot explain the anergy observed in some patients with active tuberculosis.

1.5 Salient pathological features of TBM

The pathologic picture of TBM depends upon the age of the patient, the severity of the infection, state of immunity or hypersensitivity, the duration of illness as well as the type of treatment received. Clinically and pathologically it would be more appropriate to designate the

disease as tuberculous meningoencephalitis rather than TBM (98).

1.5.1 Meninges :

A thick exudate usually fills the interpeduncular and pontine cisterns (40). The exudate may extend laterally to involve the lateral sulci and posteriorly to the cisterna magna and anteriorly to the chiasmatic cistern. The convexities of the brain are relatively unaffected. The exudate surrounds the cranial nerves and the major blood vessels at the base of the brain. The choroid plexus involvement within the ventricles is almost always present. Vasculitis, mainly involving vessels in the base of the brain constitute an important feature of TBM both from the clinical and pathological stand point. The vascular changes are diffuse and involve large, medium and small arteries as well as the veins. Partial or complete occlusion especially of the arteries may be seen. The histopathological examination of the involved arteries reveal inflammatory, proliferative and degenerative changes. The adventitial involvement represents an extension of a tuberculous process from the subarachnoid space and consists of inflammatory cellular infiltration with or without tubercle formation and caseation necrosis. The media of the artery may show no lesion. A mild cellular infiltration or occasional fibrinoid change may be seen in the media. The intimal lesions may

include subendothelial inflammatory cellular infiltration, intimal proliferation, degeneration and fibrinoid changes. Owing to the characteristic distribution of the basal exudate, the middle and anterior cerebral arteries and their branches are most commonly involved. The meningeal veins traversing the inflammatory exdudate show varying degree of phlebitis which may lead to thrombosis with partial or complete occlusion of their lumen. The mechanisms of vasculitis in TBM is not entirely clear although mononuclear cell - infiltration and the fibrinoid changes of the vessel walls suggests a delayed hypersensitivity reaction.

1.5.2 Brain

The degree and extent of brain involvement is variable. The usual parenchymatous lesions are (A) border zone reaction (B) infarcts and ischaemic lesions (C) hydrocephalus (120).

(A) Border zone reaction

The neural parenchyma immediately underlying the tuberculous exudate show a variable degree of oedema, perivascular inflammatory cellular infiltration and microglial reaction. Like the submeningeal region, the subependymal region may constitute a border zone and often depict identical changes as seen in the sub-meningeal region.

(B) Infarcts and ischaemic lesions

The vascular changes described earlier may result in the ischaemia or infarct. The majority of infarcts are seen in the territory of the middle cerebral artery. They may be superficial but often include the basal ganglia and hypothalamus. Owing to the involvement of smaller blood vessels, minute and even microscopic infarcts have also been observed in the brain stem.

(C) Hydrocephalus:

At autopsy, some degree of hydrocephalus is nearly always present in patients with TBM. The most frequent type is a communicating hydrocephalus resulting from the blockage of the basal cisterns by the tuberculous exudate or by the arachnoid granulations clogged by the inflammatory exudates. Less frequently obstructive hydrocephalus is caused by occlusion of the aqueduct of Sylvius or blockage of the outlet foramina of the fourth ventricle at the ponto-cerebellar angle (77). The narrowing of the aqueduct of Sylvius is usually due to the oedema of the midbrain or compression of the brain stem by the surrounding meningeal exudate.

1.6 Clinical manifestations of TBM

The clinical manifestation of TBM may be classical but during the past two decades the clinical presentation of

the disease has become increasingly varied and atypical manifestations are now much more common (45,70). The factors responsible for the changing pattern in patients with TBM are multiple and probably attributed to the dominant pathological process in an individual patient. These are (a) a thick exudate in the base of the brain may manifest as cranial nerve palsies and hydrocephalus (b) vasculitis and vascular occlusion may clinically present as focal neurological deficits (c) cerebral oedema results in impaired consciousness, seizures and raised intracranial pressure and (e) the presence of tuberculoma can present as a space occupying lesion.

Most cases of TBM progress through three stages as defined by the British Medical Council (88) (a) stage-I prodromal phase with no definite neurological symptom. (b) stage-II - meningeal irritation with mild or no clouding of consciousness, cranial nerve palsies, or no neurological deficit (c) stage-III clouding of consciousness, stupor or coma, convulsions, hemiplegia and involuntary movements.

In most patients with TBM there is a history of vague ill-health lasting for two weeks or longer prior to the onset of meningeal irritation. The symptoms are nonspecific except in patients in whom association of tuberculosis of another organ is known. In children apathy, irritability,

restlessness, minor headache, loss of appetite, nausea, vomiting and abdominal pain are the usual presenting symptoms. Intermittent low grade fever may also be present. In adults lassitude, depression, confusion or behavioural changes are the usual presenting features. With the onset of meningeal irritation, headache and vomiting becomes the major symptoms. In children below the age of three years vomiting is more frequent while in adults headache is more common. Fever of varying degree is usually present although 5% of paediatric and 10% of adult patients do not have fever at the time of admission. Neck stiffness, Kernig's and Brudzinski's signs are present in most patients. Cranial nerve palsies occur in 20-30% of patients with TBM and sometimes this may be the only presenting clinical feature (76). The sixth cranial nerve is most commonly involved followed by the third and then the fourth cranial nerve. Cranial nerve palsies may be unilateral initially but may become bilateral. The facial nerve is less commonly involved. The other cranial nerves are rarely affected, although involvement of the optic nerve may produce optic atrophy and blindness. Convulsions may be encountered at all stages of the illness. Focal seizures are more common in adults, whereas generalised seizures are more common in children. Convulsions may be either presenting or early symptoms in 10-15% patients in paediatric age group.

Elevated intracranial pressure usually does not manifest in the initial stages of illness, but occasionally signs and symptoms of raised intracranial pressure may precede the classical signs of meningeal irritation (107). It is not therefore uncommon in areas of high prevalence to discover that a child with hydrocephalus and an adult with suspected brain tumor are found to have TBM. Gradually the signs and symptoms of increased intracranial pressure become more prominent. Enlargement of head and a tense fontanelle in infants and papilloedema in adults are often present in significant percentage of cases. As the disease progresses, defective CSF circulation may result in hydrocephalus. At this stage the signs and symptoms of hydrocephalus (headache, papilloedema, diplopia, visual blurring) may dominate the clinical picture.

The clinical course may be punctuated with sudden onset of focal neurological deficits. Hemiplegia may occur at the onset of the disease or at later stages and usually correlates with the ischaemia or infarction in the territory of middle cerebral artery (126). Less commonly it may be due to the severe brain oedema and quadriplegia secondary to bilateral infarction. Severe cerebral oedema is a less common feature and occurs only in the advanced cases. Monoplegia is uncommon and is usually due to the small vascular lesions occurring at an early stage of the disease.

Paraplegia of cerebral origin has also been reported. In a small percentage of cases, usually in later stages of the disease, abnormal movements may dominate the clinical picture. Choreiform or hemiballistic movements, generalised tremors, have been observed more commonly in children (96).

During the illness, spread of inflammation to spinal meninges may result in symptoms and signs of radiculomyelopathy which may be due to progressive organisation of the inflammatory exudate. Following the development of spinal block, progressively increasing paraparesis and less commonly tetraparesis may develop in some patients. In some cases vascular lesions, similar to those seen in the intracranial lesion resulting in acute transverse myelitis may also be seen (25,39,133). In rare instances, the clinical picture is dominated by the evidence of spinal involvement from the very beginning. A thick tenacious exudate develops around the spinal cord resulting in complete or partial spinal block. Paraplegia occurs early and headache, confusion, neck stiffness appear later, and terminally coma sets in (70). This type of presentation is seen more frequent in adults than in children.

As the disease progresses, increasing evidences of cerebral disfunctions set in. Apathy and irritability tend to evolve into lethargy, confusion, stupor and coma. Signs of meningeal irritation may disappear in some but fever

continues to persist. The terminal illness is characterised by the deep coma, decerebrate or decorticate posturing, extensor rigidity and spasm. The pupils become dilated, fixed and the respiration becomes irregular. The clinical picture of brain-stem disfunction, though commonly due to the tentorial herniation may result in brain-stem infarcts secondary to vascular lesions or compression by dense organising exudate in the base of the brain.

Residual neurological defects have been reported in 10-30% cases of TBM (123). The incidence of neurological sequelae is higher in infants and children (135). Delay in the diagnosis and institution of therapy is responsible for an increase in the incidence of sequelae in patients with TBM. Visual and otological sequelae are most common. Blindness and impaired vision is usually due to the compressive ischaemic damage to the optic nerve or chiasma. Occasionally this may be secondary to a raised intracranial pressure and hydrocephalus. Deafness or decreased hearing is due to the eighth cranial nerve damage by the basilar exudate or drug toxicity. The incidence of major deficits has been reported to vary between 10-25%. These sequelae are more common in children especially those admitted in an advanced stage of illness. Mental retardation occurs in these children who developed meningitis under the age of three years. Mental retardation is characterised by the

intellectual subnormality and some degree of abnormal behavioural manifestations (41). Endocrinal disfunctions due to hypothalamic involvement can give rise to hypogonadism, diabetes insipidus and growth retardation. Intracranial calcification is seen in 20-40% of patients with TBM. Calcification can be detected two to three years after the onset of the disease (78). Intracranial calcification is usually seen in the sellar and basal regions. Hydrocephalus has been an important sequelae and usually occurs in most of the patients. This is due to obstruction and blockage of foramina of Luschka in the pontocerebellar angle.

1.7 Problems in the diagnosis of TBM

The "**gold-standard**" for establishing the laboratory diagnosis of TBM is the demonstration of either AFB in the Z-N stained CSF smears or by successful isolation of **M tuberculosis** in the CSF specimens by the culture method. However the demonstration of the **M tuberculosis** in CSF specimens by bacteriological methods are often infrequent. Despite several advances, the bacteriological confirmation in patients with TBM is available in only 10-20% of cases (120). Besides the low isolation rates, the bacteriological methods are cumbersome, time-consuming and requires at least 6-8 weeks. Since the clinical recovery is

dependent on an early diagnosis and the institution of appropriate ATT, the clinicians usually make the diagnosis of TBM on the relevant clinical manifestation in a patient and ATT is usually started on empirical basis. However, atypical clinical manifestation of the disease will pose considerable diagnostic difficulties. Secondly meningitis due to other aetiological causes such as partially treated pyogenic meningitis often mimic the clinical feature of TBM so much so that a distinction between these two infectious diseases becomes difficult on clinical grounds.

A positive intradermal tuberculin test in patients with TBM are helpful but cannot be considered as a tool in the diagnosis of TBM. The tuberculin intradermal test has been reported to be negative in 5-50% of cases of TBM (46). Moreover a negative intradermal skin test has been ascribed to a state of anergy observed in malnourished individuals or those patients on corticosteroid therapy. Thus tuberculin test cannot offer a definite diagnosis.

The CSF specimen from patients with TBM are usually collected from the lumbar route. In a classical case of TBM, the CSF is clear, colourless and may show a pellicle or a cobweb on standing. There is a moderate degree of pleocytosis usually not exceeding 500 cells/mm^3. A CSF with leukocyte cell-count greater than 1200/mm^3 is extremely rare

in TBM. The majority of leukocytes are lymphocytes. In the early stages of the disease and sometimes during the course of the disease, a polymorphonuclear reaction may be observed (117). At other times, the onset of meningitis is acute and the CSF leukocyte cell-count may show 1000 cell/mm^3, with a predominance of polymorphonuclear leukocytes. This atypical cytological features may be due to sudden release of tuberculoprotein into the CSF (117). The CSF protein level in TBM usually ranges between 100-500mg%. The protein concentration increases gradually in CSF as the disease progresses. In advanced stages when a spinal block develops, the CSF may become xanthochromic with a protein concentration of 1000 to 1500 mg% may even be recorded. The CSF glucose concentration is usually below 40 mg% and it tends to decrease in the untreated patients with TBM.

These cytological and biochemical features in 'classical' cases of TBM may not uniformly be seen in all patients with TBM. Wide variations are seen in the CSF leukocyte cell-count and proteins. In occasional cases, the CSF leukocyte cell-count may be normal or show a mild increase (20-40 cells). It needs to be emphasised that these CSF cytological and biochemical features may be seen in other forms of meningitis particularly in patients with partially treated pyogenic meningitis. Therefore CSF

cytological and biochemical parameters cannot be considered as a marker for making the diagnosis of TBM.

Microscopic examination of CSF for AFB is a useful method in the laboratory diagnosis of TBM. The frequency with which tubercle bacilli are seen varies widely depending both on the quality of examination as well as number of specimens examined. In Western countries the AFB positivity has been shown to the tune of 50-90% of CSF specimens. Steward (118) was able to demonstrate the tubercle bacilli in 91 out of 100 patients with TBM. Her method consisted of centrifuging 10-20ml of CSF at 2500 rpm for 30 min and preparing a thick smear from the pellicle. The smear was examined for 30-90 min. Illingworth (56) applied the same method in 14 patients with TBM and they were able to demonstrate AFB in 85% cases. But from the developing countries where the incidence of TBM is very high, the AFB have been demonstrated only in 0-10% cases (100)

There may be several reasons for the infrequent isolation of **M tuberculosis** from the CSF of patients with TBM. CSF from these patients are usually obtained through the lumbar route and the lumbar CSF contains a low concentration of **M tuberculosis** than the ventricular or cisternal CSF. Secondly, tubercle bacilli become embedded in the dense exudate in the basal cisterns or basal

leptomeninges and the exudate forms a barrier for **M tuberculosis** to circulate into the lumbar CSF. Thirdly and perhaps more importantly, most patients with TBM received a course of ATT before being referred to the specialised centre for neurological diseases. CSF in the partially treated patients with TBM is seldom found to contain **M tuberculosis** and hence culture is invariably negative.

In the absence of a definite laboratory diagnostic criterion, alternate methods are essential for establishing the diagnosis of TBM. This is particularly relevant because effective and specific ATT is available in patients with TBM. Secondly, an early laboratory diagnosis and an effective ATT will minimise the mortality and morbidity rates in patients with TBM.

During the past decade several alternate methods have been introduced in the laboratory diagnosis of TBM. These include biochemical, immunoassays and more recently molecular biological techniques. The biochemical parameters include estimation of tuberculostearic acid (84), 3- (2') ketohexyl indoline assay (17), and radioactive bromide partition test (83). All these biochemical parameters hold definite promise in the laboratory diagnosis of TBM. However, they are not feasible for a routine application

because of the need for elaborate instrumentation and expertise, which are generally not available in most of the clinical laboratories in the developing countries. Therefore, any of the alternative methods if they are to be used in the developing countries, must be economical and should be performable by the staff trained in these settings. In addition an alternate method must offer operational advantages over direct microscopy and that the sensitivity of the newer technique should be at least as good as those of direct microscopy. Most importantly the test should be rapid and should also carry high degree of specificity.

Identification of the antigenic components of the causative agent in several infectious diseases has been widely used as a tool in making the diagnosis. The first report of the serodiagnosis in tuberculosis was that of Arloing (2) which was published in 1898, sixteen years after Robert Koch's identification of tubercle bacilli. Arloing, developed an agglutination test and reported that sera from 57% of patients with pulmonary tuberculosis was positive for antibody. Serum from 11% of healthy control subjects and patients with other illness was also positive for antibody in this test. During the next ensuing eight decades many serologic techniques have been employed for the laboratory diagnosis of mycobacterial diseases. In 1972 Engvall and

Perlmann (44) described a highly sensitive and reproducible technique of ELISA. This ELISA technique is widely used for the serodiagnosis for several infectious diseases. ELISA measures the binding of antibody to the antigen which is fixed on to a solid phase absorbent. The use of ELISA with highly specific antigen offers greater precision in the diagnosis.

In this study we have developed ELISA as an immunodiagnostic tool and evaluated its application for the rapid laboratory diagnosis of TBM. The results of ELISA in CSF specimens were correlated with culture proven patients with TBM. Specificity of the ELISA was critically evaluated in the CSFs of patients with non-tuberculous neurological diseases. The application of ELISA in culture-negative patients with TBM as well as the clinical response to ATT in patients with TBM who were given the benefit of ATT on the basis of a positive ELISA were also critically evaluated in this study.

REVIEW OF LITERATURE

CHAPTER - II

2.1 Mycobacterial antigens: their isolation,characterisation, immunological properties and application in the immunodiagnosis of tuberculosis

The tubercle bacillus - **M tuberculosis** is a complex organism. Likewise the antigenic components of **M tuberculosis** are also complex. The antigenic components of **M tuberculosis** are derived from the cell wall as well as from the cytoplasm of the bacilli. Immunoreactive properties have also been demonstrated in lipid, polysaccharide and protein components of tubercle bacilli. Some of the antigenic components of **M tuberculosis** possess immunosuppressive properties while others are capable of inducing granuloma formation, macrophage activation as well as adjuvanticity. Some of the antigens derived from mycobacteria are species specific while others are known to be shared among many species and thereby contribute to the antigenic cross-reactivity (35).

In this review an attempt will be made to highlight the methods of isolation and evaluation of immunochemical properties of those mycobacterial antigens that are relevant for the immunodiagnosis of TBM.

It was not until the work of Janicki and his collaborators (31,32,57,58) in 1971 that a reference system for

the identification and nomenclature of individual mycobacterial antigen became available. Janicki and associates (31) prepared an unheated CFA from the H_{37} Rv strain of **M tuberculosis** and they raised a homologous polyvalent antiserum in goats. Subsequently additional reagents were prepared to provide a reference system for the cell-extracts as well as for the CFA of **M tuberculosis.** An IEP method was applied to evaluate the individual mycobacterial antigens against the polyvalent goat antiserum to **M tuberculosis.** Precipitin arcs obtained in IEP were numbered sequentially. Eleven major mycobacterial antigens were recognised in the IEP. Janicki and co-workers (58) emphasised the importance of the standardisation of IEP for the application of this reference system for mycobacterial antigens. Daniel et al (31), demonstrated different antigenic preparations of **M tuberculosis** demonstrated different patterns in IEP eventhough identical laboratory conditions were provided to duplicate the culture and immunisation schedule. Daniel et al however emphasised that this variation of patterns in IEP did not limit the usefulness of the reference system for mycobacterial antigens. Additional supplies of standard reagents including cell-sonicate antigens from the same strain of **M tuberculosis** as well as its homologous goat antiserum were prepared and made available for researchers who were

interested in the field of mycobacterial immunology (31). The reference reagents were designated as United-States-Japan Reagents because they were formed under the sponsorship of the United States-Japan Co-operative Medical Sciences Programme through the National Institute of Allergy and Infectious diseases, USA.

Chaparas and Hedrick (23) used the reference antiserum to demonstrate the antigenic similarity between **M bovis** and BCG strain of **M tuberculosis** and they showed no significant antigenic variation between these two strains of mycobacteria. Subsequently Chaparas (24) studied culture filtrates from twelve species of mycobacteria with the reference antiserum and observed that none of the antigens detected by the reference antiserum are unique to **M tuberculosis.**

Robert Koch in 1890 first reported an antigenic preparation of tubercle bacilli which he named as OT. It consisted of a concentrated sterile filtrate of autoclaved, heat-inactivated liquid cultures of **M tuberculosis.** Subsequently several investigators substituted synthetic media and improved the methods of preparation of the OT. Tuberculin skin testing, using such material was rapidly accepted in the clinical and epidemiological fields. However non-specific reactions to OT were soon encountered.

Later it was recognised that non-specific reactions could have resulted from the host environmental contact with other non-pathogenic strains of mycobacteria. These non-pathogenic mycobacteria contained cross-reacting antigens which shared with the antigens of virulent mammalian tubercle bacilli.

Seibert et al in 1932 first prepared a purified tuberculo-protein from the OT by using trichloroacetic acid precipitation method (111,112) and they designated the preparation as tuberculin PPD. PPD was subsequently prepared from the OT by repeated precipitation with ammonium sulphate at 50% saturation. Seibert et al (113) later used 8 weeks-old surface cultures of **M tuberculosis.** Filtrates were prepared from these cultures without prior heating and thus avoiding the heat denaturation incurred during the preparation of OT and PPD. Subsequently Seibert characterised four proteins as well as two polysaccharides, each of which was distinct in their physicochemical properties. Seibert et al designated these proteins as A,B,C,D and polysaccharides as I and II. Protein A was capable of eliciting tuberculin skin reactions in humans and was more potent than PPD (127). Protein A contained at least two components, one of which was thought to be protein and possesses a molecular weight of 35,000-42,000 daltons (115). Immunoelectrophoretic studies by Daniel and Affronti

(30) have shown that protein A contained antigens 1,2,5,6 and probably also 4. Protein B also was capable of eliciting tuberculin skin reactions in humans and was also more potent than PPD (127). Protein B was found to have a sedimentation constant of 2.0s and a molecular weight of approximately of 20,000 daltons (115). IEP showed protein B contained antigens 1,2,5,6,7 and found to be distinct from protein A by the virtue of containing much less antigen 6 and significantly higher quantities of antigen 7. Protein C was relatively inactive as a skin test antigen (127). On electrophoresis, protein C contained a rapidly moving component which Seibert designated as protein D (115). Protein D was not isolated or studied further. In IEP, protein C contained considerably less antigenic material than the other two proteins.

Polysaccharide I was further characterised by Birnbaum and Affronti as a heteropolysaccharide containing arabinose, galactose and mannose (14). It was initially described as being non-reactive in the sensitised guinea-pigs as well as in humans (87,115). Subsequently polysaccharide I was shown to elicit cellular hypersensitivity responses in-vivo as well as in-vitro. However in these instances its antigenicity has been attributed to the presence of the contaminating proteins (15,52). It did not react with the polyvalent goat

antiserum to **M tuberculosis** (15). Immunoelectrophoretic studies revealed polysaccharide I contained predominantly antigen 2, with smaller amounts of antigen I and also an unidentified anodal component (30).

Polysaccharide II was found to be a glucan with a molecular weight of 100,000 daltons (114,115). There was a variation in its concentration in different culture filtrate preparations. It did not elicit delayed skin test reactions. In IEP, polysaccharide II was shown to be antigen-3 (30).

The predominant source of polysaccharides is the cell wall of **M tuberculosis** (35). Arabinogalactans, arabinomannans, mannans and glucans have been isolated from the cell wall of mycobacteria. These polysaccharide did not elicit the delayed type skin reactions (5-7,90-93).

2.1.1 M tuberculosis antigen 5

Affinity chromatography procedures have been widely used for the isolation of mycobacterial antigens. Affinity chromatography is based on highly specific, dissociable interaction between the two macromolecules. It is among the most versatile and powerful purification methods available in the field of immunochemistry. Using an immunoabsorbent affinity chromatography, Daniel and Anderson (34,36)

isolated a dialysable antigen from the unheated culture filtrates of H_{37} Ra **M tuberculosis.** The immunoabsorbents were prepared by coupling cynogen bromide-sepharose 4B with goat immunoglobulin to **M tuberculosis** antigen 5. In this procedure, the culture filtrates of H_{37} Ra **M tuberculosis** was initially subjected to a concanavalin-A sepharose-4B chromatography and the elution of the antigen 5 from the culture filtrates of **M tuberculosis** was achieved with 1% dextrose. The elute was subjected to PAGE. Two millimeter segments of gel columns selected to contain antigen 5 region by the use of a parallel stained gel column, were cut from the unstained gel column. These gel segments were homogenised and injected subcutaneously on three consecutive days out of each week for 5 weeks. Subsequently two gel slices were emulsified with 1 ml **Bordetella pertussis** vaccine as well as with an equal volume of IFA. The material was injected into two intramuscular and two subcutaneous sites. The goat was bled and the immune sera was coupled with cynogen bromide sepharose-4B. The immunoabsorbents were thus prepared. The antigen 5 in the culture filtrate of H_{37} Ra **M tuberculosis** was eluted from the immunoabsorbents by the addition of 4**M** urea. This antigen was found to be a protein and the amino acid composition suggested that it is derived from the cytoplasm of the bacilli. This antigen had a sedimentation constant of 2.0S and a molecular weight of

35kDa. This antigen was termed as antigen 5 because it identified with antigen 5 in the numeric number of the nomenclature for mycobacterial antigens. Antigen 5 did not contain muramic acid and diaminopimelic acid which indicate that antigen 5 did not originate from the cell-wall of the mycobacteria. Daniel et al also found that antigen 5 is present in 4 strains of **M tuberculosis** and 6 strains of **M bovis** and absent in 30 other strains of mycobacteria (37). Benjamin and Daniel (12) used antigen 5 in an ELISA to detect antibodies against antigen 5 in the sera of patients from Bolivian and North American patients with tuberculosis as well as in several groups of control subjects. They found antigen 5 carried a higher specificity than other mycobacterial antigens in the ELISA. In a prospective study of ELISA with antigen 5, Balestrino and colleagues (9) found no serum from healthy control subjects was positive for antibodies to antigen 5 in the ELISA. Using identical technique, Ma and colleagues (81) found 73 of 84 patients with tuberculosis gave positive results for antibody to antigen 5 and none of the sera in 30 control subjects showed antibody to antigen 5 in the ELISA. In their study, an ELISA method clearly discriminated tuberculous and non-tuberculous subjects. There was also a positive correlation between antibody titer to antigen 5 and the extent of the pulmonary disease. Among various mycobacterial antigens, antigen 5 was

found to be more specific for the serodiagnosis of tuberculosis.

2.1.2 M tuberculosis antigen 6

M tuberculosis antigen 6 was originally purified from the culture filtrate of $H_{37}Ra$ **M tuberculosis** by Daniel (29) and was identified as protein A_2. The sedimentation constant of antigen 6 determined to be 2.5s and the molecular weight was estimated to be 45,000 daltons. Antigen 6 is probably one of the major cytoplasmic antigens of **M tuberculosis** and it is also the major constituent of Seibert's protein A (30).

Stroebel et al (119) purified antigen 6 from the culture filtrate of $H_{37}Ra$ **M tuberculosis** by an immunoabsorbent affinity chromatography and they studied IgG antibody to antigen 6 in the sera from 36 patients with bone and joint tuberculosis. 16 of these patients were in the active stage of the disease for which they received ATT for less than 9 months. 20 patients were in the inactive stages of the disease. 21 control sera were also obtained from patients with non-tuberculous bone and joint infections. Using a dilution end-point and 'cut-off' point 1:64, 15 of 16 sera from patients with active disease were positive and none of the sera from patients with inactive disease or from control subjects gave positive results in the ELISA.

Mycobacterial lipids are found in the cytoplasm of the bacilli but cell-wall of **M tuberculosis** is the predominent source for lipids. Mycolic acids, phospholipids, wax D, mycosides and other glycolipids are all present and isolated from the cell walls of **M tuberculosis** (49).

Virulent strains of **M tuberculosis** have the ability to form serpentine cords of bacilli (48). This virulence is attributed to glycolipids, cord factor and sulphatides which are present in **M tuberculosis** bacilli. Cord factor possesses lethal effects when injected intraperitoneally into the mice (16). Cord factor can also induce granuloma formation (11) which is probably secondary to the macrophage chemotaxis and stimulation (66,121). Cord factor can also activate the alternate complement pathway (101) and hence can provoke an acute inflammatory cellular reaction in a tuberculous lesion.

The sulfatides are lysosomotropic for the macrophages and they antagonise the fusion of lysosomes with the phagosomes. This in-turn prevents the degradation of the bacilli. sulphatides also increases the toxicity of cord factor(65). The combination of increased cord factor toxicity and antagonism of phagolysosome formation often leads to the progression of the disease by two mechanisms.

Firstly, the inhibition of phagolysosomal fusion would result in the persistence and further growth of the mycobacteria. Secondly as the bacterial population increases, more cord-factor and sulfatides will be produced and this will enhance the tissue destruction.

Phosphatidyl inositol monomannosides and oligomannosides have been described as the cementing substances for the cell-wall skeleton (71). Purified phosphatidyl inositol monomannosides are haptens and they can generate antibody responses when administered with IFA (64,68). The significance of formation of antibodies in tuberculosis is not known. However the generation of antibodies against phosphatidyl inositol monomannosides offers a serodiagnostic tool. ELISA studies have shown that 95% of cases of advanced tuberculosis contained an antibody against phosphoinositol antigen (104).

Wax D is a peptidoglycolipid present in the cell-wall of mycobacteria and it is composed of mycoloylarabinogalactan linked to the muramic acid by a phosphodiester and peptides (49). Wax D has predominently an adjuvant activity.

2.2 Immunoassays for the diagnosis of TBM

2.2.1 Immunoassays for the detection of antibody to M tuberculosis in CSF

Published reports for the detection of antibodies to **M tuberculosis** in the CSFs of patients with TBM are summarised and shown in Table 2.1.

Kuo (73) introduced a haemagglutination test for the immunodiagnosis of patients with TBM. In their study a haemagglutination test was standardised. They used either a Pasteur antigen (a polysaccharide antigen) or tuberculin PPD to sensitise the red blood cells. The antibodies to **M tuberculosis** in the CSFs of patients with TBM and control groups were tested by the haemagglutination method. The assay gave positive results in 33 out of 34 patients with TBM and thus carried a sensitivity of 97.1%. The assay gave negative results in 44 patients with other CNS infections. AFB were demonstrated by the Z-N method only in 55.9% and **M tuberculosis** was isolated by the culture in 70% of patients with TBM. Kuo also observed higher globulin content in the concentrated CSFs and therefore they emphasised the need to concentrate CSF specimens prior to their application in the test. The concentrated CSF specimen showed complete agglutination than the unconcentrated CSF specimens. Kuo emphasised that the

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TABLE 2.I

Data showing the published reports for the detection of antibody to M tuberculosis in CSF of patients with TBM

Sl.No.	Authors	Year	Antigen	Method used
1.	Kuo	(1969)	PPD	IHA
2.	Munoz et al	(1978)	PPD	IHA
3.	Kalish et al	(1983)	PPD	Indirect ELISA
4.	Samuel et al	(1983)	Sonicate antigen	RIA
5.	Hernandez et al	(1984)	BCG	Indirect ELISA
6.	Chandramuki et al	(1985)	MTSE	RIA
7.	Coovadia et al	(1986)	**M tuberculosis** antigen 5	Indirect ELISA
8.	Prabhakar et al	(1987)	Sonicate antigen, PPD, BCG	Indirect ELISA
9.	Ashtekar et al	(1987)	Sonicate antigen	RIA
10.	Watt et al	(1988)	BCG	Indirect ELISA
11.	Vanvooren et al	(1989)	P - 32 antigen	Dot-Iba
12.	Chandramuki et al	(1989)	Lipoarabinomananan	Indirect ELISA
13.	Sindic et al	(1990)	A-60 antigen, **M tuberculosis** cytoplasmic antigen	Immunoblot

concentration of the CSF specimens yielded more positive results and helped in establishing the diagnosis of TBM as well as increasing the sensitivity of the test.

Munoz et al (94) introduced an IHA using PPD antigen for the diagnosis and prognosis of patients with meningoencephalitis. The test gave 100% sensitivity in all the 22 patients with TBM. The test was negative in 53 patients with viral or pyogenic infections of the CNS. **M tuberculosis** was isolated only in 31.8% of patients with TBM while IHA gave positive results in all the 22 patients of TBM. The test according to these authors was specific because no false positive reaction was recorded in any of the 53 patients in the control group. Munoz et al observed no correlation between the antibody titer and severity of the disease. They also emphasised small volume of CSF was sufficient for the test and that the CSF samples following ammonium sulphate precipitation should be dialysed thoroughly prior to their application in the test because ammonium sulphate present in the CSF specimen can interfere with the haemagglutination reaction.

Kalish et al (62) for the first time introduced an indirect ELISA method for the immunodiagnosis of TBM. Kalish et al found high titers of IgG antibodies to PPD antigen in three culture proven patients with TBM. The CSFs

from 33 patients with chronic meningitis (control group) were negative for IgG antibody to PPD antigen. Serial CSF and serum specimens from one of their patients with TBM demonstrated greater amounts of IgG antibody to PPD in CSF than in the serum. The antibody levels in CSF showed positive correlation with the clinical course of the illness as well as with the leukocyte cell-count, protein as well as glucose concentration in the CSFs. Kalish et al emphasised, ELISA methods for measuring the IgG antibodies to PPD antigen should be evaluated as one of the means of early diagnosis in patients with TBM.

In order to demonstrate that IgG antibodies are formed in patients with TBM during the course of illness, Kalish et al measured IgG and albumin concentration in the serum and CSF and calculated the CSF-IgG index. An elevated CSF-IgG index in patients with TBM suggests that the IgG antibodies are synthesised within the CNS during the course of the disease, rather than be present due to the passive transfer of serum IgG across the blood brain barrier. In addition to the CSF-IgG index, Kalish et al also observed elevated anti-PPD index in their patients with TBM. This observation also suggests a local synthesis of antibody to **M tuberculosis** in patients with TBM.

Samuel et al (109) applied a RIA for the detection of mycobacterial antigen and antibody in CSF specimens of patients with TBM. They studied four groups of patients - controls, pyogenic meningitis, TBM and pulmonary tuberculosis without meningitis. In their study, detection of mycobacterial antigen in CSF specimen was not found to be useful for the diagnosis of TBM. However antibody titers to **M tuberculosis** in CSFs of patients with TBM were statistically higher than the other three groups of patients. Samuel et al also observed a decrease in the antibody titer between second week to 8 months after the commencement of ATT. They attributed the decrease in the antibody titer due to the steroids administration because steroids are known to suppress the antibody production. They also studied the effects of dilution of antibody titer with the development of hydrocephalus in their patients with TBM. Samuel et al considered false negative results in RIA may be due to ATT.

Hernandez et al (53) used BCG antigen as the solid phase absorbent in the ELISA. They evaluated the sensitivity of the ELISA in 20 bacteriologically proven cases of TBM and the specificity of ELISA was assessed in 31 patients with acute pyogenic meningitis, 20 patients with viral meningitis and 10 patients with non-infectious disease of CNS. Hernendez et al simultaneously measured both IgG

and IgM antibody titers in the ELISA. In their assay, simultaneous detection of IgG and IgM antibody against BCG gave a sensitivity of 100%. They also observed a marked difference in the antibody titer between the CSF samples of TBM and non-TBM group. Hernandez et al concluded, that on the basis of predictive value of the assay, the analysis of their data showed a 100% sensitivity and 100% specificity for the diagnosis of TBM.

In their study, Hernandez et al used a disk coated with BCG antigen as the solid phase absorbent instead of the conventional microtiter plates and they consider that this has made the test more economical. The plate method required expensive equipment like ELISA readers for the interpretation of the results. In contrast, disk method required less expensive equipment.

Chandramuki et al (21) standardised three different assays to detect antibodies in CSFs of their patients with TBM and pyogenic meningitis. (a) A solid-phase RIA in which the microtiter plates were coated with a soluble extract of mycobacterial antigen. The microtiter plates were incubated overnight at $4^{o}C$ with four serial doubling dilutions of CSF specimens. The plates were washed and incubated with ^{125}I labelled rabbit antihuman immunoglobulin. The presence of radioactivity in the CSF

specimen was expressed as the relative value. Antibody levels were expressed as the reciprocal dilutions of CSF that gave 50% binding (b) An antibody competition test for the detection of MAb like-epitope-specific antibodies in CSF. ^{125}I labelled probes were prepared using the following MAbs - ML 34, TB-72, ML-30, TB-23, TB-68, TB-78 and TB-71. Competitive inhibition of binding of each of these MAbs to MTSE coated microtiter plates in the presence of antibodies in the patients CSF samples were measured. The results were expressed as relative inhibition (%) of ^{125}I-MAb binding. (c) An immunoblot method to detect antimycobacterial antibodies in the CSF. The MTSE antigen was resolved by an electrophoresis in SDS-15% polyacrylamide gels. Subsequently the proteins were electrotransferred onto NCPs. The NCP were incubated with CSF samples and they were that treated with ^{125}I-RaHG. The radioactive blots were developed by an autoradiography. Analysis of the data showed that the assay carried 74% and 66% sensitivity in culture positive and culture negative patients with TBM. However the assay also showed the positive results in 37% of patients with pyogenic meningitis and 18% of patients with non-meningitic neurological diseases.

Coovadia *et al* (26) evaluated three parameters-ADA activity in the CSF, an indirect ELISA to detect antibody to **M tuberculosis** antigen 5 and a radioactive bromide partition

test in their patients with TBM. Most of the patients in their study were in the paediatric age-groups. Three groups of patients were studied. Group A - consisted of 38 children with TBM and this group was further subdivided into two subgroups. Group A-1 consisted of 13 children with a strong clinical suspicion of TBM. These patients showed typical CSF-cytological and biochemical findings and also positive Mantoux test. Radiogram of the chest was suggestive of pulmonary tuberculosis. CSF was positive for AFB either by direct Z-N stain or by culture. These children were assessed within 72h of admission or before the commencement of ATT. Group A2 comprised 25 children who are on ATT for varying periods. Group B was made up of 47 children presenting with clinical features which were suggestive of meningitis. This group included 16 patients with non-tuberculous bacterial meningitis (B2), 13 patients with aseptic meningitis (group B3) and 20 with normal CSF (group B). Group C included 14 children with pulmonary tuberculosis without any clinical evidences of meningitis. Clinically patients with TBM were classified into three stages depending upon the severity of meningitis. In stage-1 patients were fully conscious with neurological signs of meningeal irritation but they showed neither any focal neurological signs nor signs of hydrocephalus. In stage-2 patients were either in a confused state or showed

focal neurological deficits. In stage 3 - patients were mentally inaccessible, owing to the depth of stupor or delirium and they also showed complete hemiplegia or paraplegia. The results of the ELISA in their study showed that in patients group A, who were tested within the first month of admission, only 53% had antibody titer of 1:20 or more. Similarly, among all the CSF specimens tested in group A, only 42% (23/55) were found to be positive for antibody at this titer. Twelve of the children in group A had serial antibody assays performed during the course of their illness. Peak concentration of CSF antibodies was recorded as early as one week or as late as two and half months of the disease. Coovadia et al did not observe any correlation between antibody concentration and severity of the disease or the presence of neurological sequelae of the disease. With the exception of five children who had titers of more than or equal to 1:20, the remaining 31 children tested in group B had titers less than 1:20. All the children in group C had values less than 1:20. CSF specimens from the children in group A who were tested during the first month of admission gave a geometric mean titer 1:6 which was significantly higher than that obtained in the normal children and in patients with pulmonary tuberculosis. No significant difference was evident between antibody titer to **M tuberculosis** antigen 5 in the CSFs of

patients with either tuberculosis or bacterial meningitis. At CSF dilutions of more than or equal to 1:20, 1:40 and 1:80, the ELISA test showed sensitivities of 53% (8/15) 40% (6/15) and 27% (4/15). In the control groups B and C, a titer less than 1:20 was 90% (45/50) specific for the absence of TBM whereas titer less than 1:40 and 1:80 were 94% (47/50) and 100% (50/50) specific for the absence of TBM.

In an ELISA, Prabhakar and Oommen (99) used three antigens - commercial PPD, BCG and sonicate antigen of $H_{37}Rv$ **M tuberculosis** for the detection of antimycobacterial antibodies in the CSFs of patients with TBM. In a retrospective study, a total of 260 CSF samples from the patients admitted to the neurology unit were analysed. The CSF samples were classified into 6 groups on the basis on their clinical diagnosis. Group I was composed by patients with a clinical diagnosis of TBM and these patients had either positive CSF culture for **M tuberculosis** or showed definite clinical response to ATT. The patients in this group were regarded as being 'definite TBM'. Group II consisted of 'Probable' cases of TBM. Patients in this group had negative bacteriological culture for **M tuberculosis** and they also showed an equivocal clinical response to ATT. Group III consisted of patients who underwent spinal anaesthesia or lumbar puncture for

myelographic studies. Group IV consisted of patients with CNS infections due to non-tuberculous aetilogy viz., pyogenic meningitis, viral meningo-encephalitis and cerebral cysticercosis. Group V comprised of patients with post-infectious demyelinating disorders. Group VI consisted of patients with altered blood brain-barrier, strokes, brain tumors, recurrent seizures and benign intracranial hypertension. This particular group was selected to study whether the transfer of antimycobacterial antibodies occurs across the blood brain barrier. The CSFs from all the patient groups were used at different dilutions and tested in the ELISA. Sonicate antigen of $H_{37}RV$ **M tuberculosis** gave 70% sensitivity and 90% specificity in the ELISA. Lower sensitivity was observed with the BCG antigen although specificity was found to be 90%. Studies with PPD as antigen indicated a weakly positive reaction in five 'definite' TBM patients and hence PPD antigen was not used further in their study. Based on these results Prabhakar and Oommen concluded, sonicate antigen of **M tuberculosis** is the most appropriate antigen and that there is a good correlation between ELISA results and the cytological picture in the CSFs of patients with TBM. The ELISA test in their study differentiated tuberculous from non-tuberculous patients and that they emphasised the use of ELISA as an adjunct in the laboratory diagnosis of TBM.

Ashtekar et al (4) introduced a RIA to detect specific mycobacterial antigen and antimycobacterial antibody in CSF and serum of patients with TBM. They studied 84 patients on the basis of (a) clinical evidence of meningitis (b) history of contact (c) X-ray evidence of primary complex or progressive tuberculous disease (d) typical biochemical and cytological changes in CSF and (e) identification of **M tuberculosis** either in smears or by the culture of CSF specimens. 75% of the patients with TBM were positive for AFB either in the smears or in the cultures. They also selected 30 controls consisting of pyogenic meningitis, viral meningitis and patients with febrile convulsions. The results of their study revealed mycobacterial antigen levels and CICs in CSF specimens were significantly elevated in patients with TBM than in the controls. 54% of patients were categorized into TBM if both CICs as well as antimycobacterial antibody were estimated in the CSF specimens. If only antimycobacterial antibody was measured in the CICs, then 70% of patients could be classified into TBM. The sensitivity increased to 86% if either of the two parameters were measured. Repeat CSF examinations were done in 16 patients during the course of the treatment. In 14 patients antigen levels were elevated despite treatment. In 11 patients antimycobacterial antibody showed an increase while in 4 patients the antibody

titers were decreased. They also observed a positive correlation between the isolation of **M tuberculosis** by culture and estimation of mycobacterial antigen in the CSF specimen. Ashtekar et al concluded the detection of mycobacterial antigen and antibodies are more specific and therefore RIA should be carried out routinely in all the patients with TBM.

Watt et al (134) applied an indirect ELISA to detect antimycobacterial antibody in 29 bacteriologically confirmed patients with TBM and 83 patients with non-tuberculous infectious diseases of CNS. They compared the sensitivity of the assay with the direct staining method and also evaluated the specificity of the assay in the non-tuberculous subjects. ELISA was performed with BCG as the coating antigen. Watt et al recorded a positivity in only 7 out of 29 culture positive patients with TBM. However, the assay carried 98% specificity. The assay also showed false positive results in four patients with bacterial meningitis.

Van Vooren et al (128) established a dot-blot assay to detect antimycobacterial antibody in CSFs, using a purified BCG P-32 antigen. They studied five patients with pulmonary tuberculosis with suspected meningeal involvement. The dot-immunoblotting procedure is much simpler than the

conventional ELISA. Specific IgG class of antimycobacterial antibodies were found in all the five patients in the serum as well as in CSFs. However, a higher ratio of anti-p32 immunoglobulins and total immunoglobulin was observed in the CSFs of patients with TBM than in serum while a reverse situation was observed in non-tuberculous patients. Vooren et al concluded, specific antimycobacterial antibodies are synthesised intrathecally in patients with pulmonary tuberculosis with menigeal involvement.

Chandramuki et al (22) detected specific antibody to five mycobacterial antigens by an indirect ELISA in the CSFs of patients with TBM. These mycobacterial antigens are (a) soluble extract of $H_{37}Rv$ **M tuberculosis** (b) lipoarabinomannan (c) 14,19,38 kDa antigens from $H_{37}Rv$ **M tuberculosis** were isolated by an affinity chromatography (d) a recombinant 65 kDa antigen. They studied 74 patients with TBM, 26 culture positive, 26 patients with purulent meningitis, 68 patients with suspected TBM and 29 patients with other neurological diseases. Chandramuki et al observed antibody response to 14 kDa antigen as well as lipoarabinomannan. The assay gave 100% specificity and 74% sensitivity with these two antigens. Chandramuki et al emphasised, estimation of levels of antibody in CSFs to selected mycobacterial antigens should be considered as a valuable aid in the diagnosis of TBM.

Sindic et al (116) studied the CSFs and sera from six bacteriologically proven and two clinically suspected cases of TBM for the presence of antimycobacterial antibodies against an unfractionated cytoplasmic antigen of $H_{37}Ra$ **M tuberculosis.** They used an affinity mediated immunoblot technique. This technique is based on agarose gel isoelectric focussing of paired CSF and serum of patients with TBM as well as transfer of the specific IgG antibodies onto mycobacterial antigen loaded nitrocellulose sheets. An intrathecal synthesis of antimycobacterial oligoclonal IgG antibodies often superimposed on the diffuse polyclonal production and this was shown in all patients with TBM, but not in patients with tension headache or other neurological disorders. Similar results were obtained with another purified mycobacterial antigen (A_{60}) prepared from **M bovis.** This antigen was used for the coating the NCP sheets instead of whole mycobacterial homogenate. In their study, the number of antimycobacterial oligoclonal IgG bands increased with the time and persisted for years even in clinically cured patients. The authors concluded, the demonstration of such antimycobacterial antibodies in CSF could be an useful adjunct for the diagnosis of TBM especially in patients with negative CSF culture for **M tuberculosis.**

2.2.2 Immunoassays for the detection of mycobacterial antigen in CSF

The published studies on the detection of mycobacterial antigen in CSFs of patients with TBM are shown in Table 2.II

Sada et al (108) studied 16 patients with TBM and 12 patients with non-tuberculous infectious diseases and 11 patients without any neurological diseases. Sandwich ELISA was standardised in their study. The microtiter plates were filled up with (1:800) rabbit anti-BCG antibody. Following 12 h incubation, the CSF samples were added and incubated for 2 h. The amount of antigen present in the CSF was measured by the addition of anti-rabbit IgG-alkaline phosphatase conjugate. p-nitrophenyl phosphate was used as a substrate. The microtiter plates were read at 405nm. The assay showed a sensitivity of 81.25% and a specificity of 95% for tuberculous aetiology. False positive result was obtained in a patient with Cryptococcal meningitis. This false positive reaction may be due to the cross reactivity of the rabbit anti-BCG antibody with the polysaccharide capsular antigen of **Cryptococcus neoformans.** The authors concluded, serial determination of antigen concentration in the CSFs of patients with TBM during treatment may be useful in the follow-up of the patients as well as assessing the progress of the disease.

TABLE 2.II

Data showing the published reports for the detection of mycobacterial antigen in CSF of Patients with TBM

Sl.No.	Authors	Year	Antibody used	Assay
1.	Sada et al	(1983)	Anti-BCG	Sandwich ELISA
2.	Bal et al	(1983)	Antibody to sonicate antigen	Inhibition ELISA
3.	Krambovitis et al	(1984)	Antibody to plasma membrane antigen	Latex agglutination test
4.	Chandramuki et al	(1985)	Mouse MAb to MY_4 antigen	Reverse passive haemagglutination
5.	Kadival et al	(1986)	Anti-BCG	Sandwich ELISA
6.	Donald et al	(1987)	Anti-BCG	Sandwich ELISA
7.	Kadival et al	(1987)	Anti-**M tuberculosis**	Radioimmunoassay
8.	Watt et al	(1988)	Anti-BCG	Sandwich ELISA
9.	Ramkisson et al	(1988)	Anti-BCG	Competition ELISA
10.	Wu et al	(1989)	Anti-BCG	Sandwich ELISA
11.	Dhand et al	(1989)	Anti-BCG	Sandwich ELISA
12.	Mastroianni et al	(1991)	Anti-BCG	Dot-blot

Bal et al (8) estimated mycobacterial antigen in CSF specimens using an inhibition ELISA. They studied 41 CSFs from patients of whom only 9 were clinically diagnosed as TBM. In their assay, irradiated cells of $H_{37}Rv$ **M tuberculosis** was used as the solid-phase absorbent. A polyvalent antibody was raised in rabbits. In the standardisation ELISA, the globulin fraction of rabbit serum was conjugated with an alkaline phosphatase. The antibody-enzyme conjugate and the soluble fraction of mycobacterial antigen were incubated at 37°C for 1h. This antigen-antibody complex was added to a solid phase and incubated for 12 h at 4°C. The enzyme activity of the conjugate adherent to solid phase was estimated by the addition of p-nitrophenyl phosphate. The difference between the absorbence in the test and control sample was plotted against the logarithmic value of the antigen concentration. A linear relation was obtained. The CSF samples from patients were similarly tested. The antigen concentration of CSFs was calculated from the standard graph. Any reduction of absorbence of 0.15 or more was considered as significant. Besides the ELISA, the CSF specimens were also subjected to biochemical, cytological and bacteriological investigations. All the patients were followed-up either to confirm or to exclude the initial diagnosis of TBM.

Krambovitis et al (72) described a simple latex particle agglutination test for the rapid detection of mycobacterial antigen in the CSFs of 18 children with TBM and 134 controls with other neurological disorders. In 15 out of 18 children with TBM, AFB were demonstrated in the CSF smears. The test was carried out by the sensitization of latex particles with a purified rabbit immunoglobulin against the plasma membrane antigen of **M tuberculosis.** The control latex reagent was similarly prepared with the normal rabbit immunoglobulin. The CSFs were heat-inactivated at 56^{o}C to remove the non-specific substances. 40 ul of CSF was placed around two black circles in a glass test slide. 20 ul of latex reagent was added to the first circle and 20 ul of control latex reagent to the second circle. A positive result was indicated by the development of agglutination within 3 minutes. Those CSFs samples that did not contain any mycobacterial antigen showed no agglutination. The latex agglutination test was positive in 17 out of 18 CSFs and was negative in 130 out of 134 CSFs from the control group. A patient with **Haemophillus influenzae** meningitis gave a false positive result. The authors concluded, latex particle agglutination test has potential application in the laboratory diagnosis of TBM. The test is inexpensive and does not require elaborate instrumentation.

Chandramuki et al (21) applied a reverse passive haemagglutination test for the detection of soluble non-protein mycobacterial antigen using an IgM murine MAb. The murine IgM-MAb was prepared by immunising BALB/C mice with the MY4 antigen. The globulin fraction in the ascitic fluid of the mouse contained the IgM-MAb. A reverse passive haemagglutination test was performed by coupling IgM-MAbs to chymotrypsin treated sheep-red-blood-cells. (0.033% chromic chloride was added by drops to a mixture of 25ul of ML 34 globulin (400ng/ml). This suspension was incubated on a mixer for 1 h at 20°C. The cells were washed and made up to 1% suspension in PBS. Serial doubling dilutions of CSFs were made in the 'U' shaped microtitration plates. ML 34-coated red-blood-cells were added and the haemagglutination reaction was read after the incubation for 1 h at 20°C. Controls included neat CSF samples with uncoated SRBC and coated SRBC. The ML-34 antibody coated SRBC agglutinated by an MTSE was used as a positive control. Haemagglutination occurred in presence of 1.0 ug/ml MTSE. The test was applied to analyse the CSFs of 89 patients with TBM from India, 127 control subjects from UK and India. The mycobacterial antigen was demonstrated in 88% culture positive and 73% culture negative patients of TBM. However the test also detected false positive results in 21% of Indian patients with pyogenic meningitis.

Kadival et al (60) standardised a double antibody sandwich ELISA to detect mycobacterial antigen in the CSFs of treated and untreated patients with TBM. They used rabbit anti-BCG antibody as the primary antibody and antirabbit IgG-biotin conjugate as the secondary antibody. Avidin-alkaline phosphatase and p-nitrophenyl phosphate were used as enzyme and substrate respectively. Kadival et al studied a total of 90 patients. Group I consisted of 38 CSF samples from patients with TBM which was further subdivided into treated and untreated groups. Only 4 of these 38 patients with TBM had shown the presence of AFB in their CSF specimens. The diagnosis of TBM in the rest of 34 patients was based on the clinical presentation and biochemical abnormalities in the CSFs. Group II consisted of 22 control samples from patients with febrile convulsions, epilepsy, viral encephalitis and tetany. None of the patients in group II had evidence of pulmonary tuberculosis. Group III consisted of 30 CSF samples from patients with symptomatic aseptic non-mycobacterial meningitis. The CSF showed elevated proteins in most of these patients with culture proven bacterial meningitis. The sandwich ELISA was performed in the microtiter plates. The wells were coated with anti-**M tuberculosis** antibody (raised in burros) for overnight at 28°C. After the three washes the micotiter plates were incubated with the sonicates of **M tuberculosis.**

To study the cross-reactivity with other mycobacterial species, sonicate antigens of **M intracellulare, M avium, M kansasii, M vaccae, M fortuitum** were used. PBS with 1% BSA was used as the washing medium in between the phases of incubation. Anti-BCG rabbit serum was added and incubated for 2 h. This was followed by antirabbit IgG-alkaline phosphatase conjugate. The plates were incubated for another 2 h at 20°C. The absorbances were read after the addition of p-nitrophenyl phosphate. The biotin-avidin ELISA system was precisely used in this study to improve the sensitivity of the assay. With the avidin-biotin system, the assay detected the concentration of mycobacterial antigen as low as 3ng/ml while the sandwich ELISA method detected only upto 15ng/ml or more. The specificity of both types of ELISAs was evaluated with sonicate preparation from other mycobacteria ie **M intracellulare, M avium, M kansasii M fortuitum** and **M tuberculosis.** Sandwich ELISA showed a cross-reactivity of 5% with **M kansasii.** Only 2% cross-reactivity with other mycobacteria was observed in the ELISA with avidin-biotin system. The authors emphasised although the avidin-biotin system was five times more sensitive than the sandwich ELISA but it was less specific. Therefore sandwich ELISA was preferred for the detection of mycobacterial antigen in the CSF samples. The results in their study showed in 75% of the 24 untreated subjects with

TBM, the antigen concentration ranged between 0.3 to 10ng/ml. Among the 14 treated patients, only 5 patients showed the presence of antigen in the CSF. Among the patients with aseptic meningitis, non-tuberculous and non-bacterial meningitis, the antigen concentration in CSFs was present only in insignificant concentration and the ELISA gave no false positive results in the nontuberculous subjects. The authors have concluded that a good correlation was obtained between the demonstration of mycobacterial antigen in the CSFs and active stage of the disease. They recommended the application of ELISA technique for the diagnosis of patients with a clinical suspicion of TBM.

Donald et al (43) investigated 53 CSFs from patients with meningitis and detected the presence of mycobacterial antigen in CSF by a sandwich ELISA method. Prior to the application, the CSFs were heat-inactivated at 56^{o}C for 1 h to eliminate non-specific reactions. 22 out of 53 patients who presented with the clinical features of TBM, positive culture for **M tuberculosis** was obtained only in 9 patients. In 31 other patients with meningitis, the aetiology was due to the following organisms - **Haemophilus influenzae** (n=9) **Streptococcus pneumoniae** (n=8) **Neisseria meningitidis** (n=4) **Cryptococcus neoformans** (n=2) **Staphylococcus aureus** (n=1)

and **Klebsiella** pneumoniae (n=1), **Mumps virus** (n=2) **Coxsackie A virus** (n=1). The CSFs were obtained either before the start of the therapy or within a week after the therapy in patients with TBM while in non-tuberculous subjects the CSF was studied only with the initial CSF specimens from which the organisms were grown. The ELISA was standardised in the microtiter plates with varying concentration of BCG antigen. The plates were coated with rabbit anti-BCG antigen. The amount of antigen was determined using anti-BCG rabbit IgG-peroxidase as the enzyme conjugate and 0-phenylenediamine as the substrate. The absorbances were read at 494 nm. The assay gave 100% sensitivity and 81% specificity for tuberculous aetiology in their patients with TBM.

Kadival et al (61) applied a biotin-avidin RIA for the estimation of mycobacterial antigen in the CSFs of patients with TBM. The assay involved sandwiching mycobacterial antigen between the two antibodies to **M tuberculosis** raised in burros and rabbits. The reaction is amplified by the use of antirabbit IgG-biotin conjugate and ^{125}I labelled-avidin. The RIA detected antigen concentration up to 20ng/ml and showed less than 5% cross reactivity with six other strains of mycobacteria. The authors investigated 17 treated and 19 untreated patients

with TBM, 26 patients with non-bacterial meningitis and 30 patients with bacterial meningitis. The results showed the antigen concentrations in 15 out of 19 untreated patients in the TBM ranged between 20-10,000 ng/ml. However in the treated group only 2 out of 17 CSFs were positive for the presence of antigen. The authors concluded that RIA promises to be a rapid adjunct for the early diagnosis of TBM.

Watt et al (134) evaluated the use of sandwich ELISA to detect mycobacterial antigen in the CSFs of 29 culture proven patients with TBM and 83 patients due to non-tuberculous CNS infections. The ELISA was performed with anti-BCG antibody as the coating antibody. The CSFs from tuberculous and non-tuberculous subjects were added in their respective wells. BCG protein standards were used as the control. Following this, antirabbit IgG-HRP was added as the enzyme conjugate and O-phenylenediamine was added as the substrate. Watt et al compared the sensitivity of the assay with the direct demonstration of AFB and specificity of the assay was critically evaluated in the non-tuberculous subjects. The authors have observed that the ELISA detected antigen in 11 out of 28 patients with TBM while AFB was present in only 2 out of the 28 patients. Thus the ELISA was more sensitive than the direct smear examination in patients with TBM. The assay showed a specificity of 98% in

non-tuberculous subjects. False positive results occurred in 4 patients with bacterial meningitis. The authors also estimated predictive values for two extremes of prevalence. They observed a positive predictive value of 93% in their high prevalence setting which means that positive ELISA is very helpful whereas a negative test is much less useful. They have also emphasised that in area of low prevalence a negative test is very useful in excluding the diagnosis of TBM.

Ramkisson et al (102) described a competition ELISA for the detection of mycobacterial antigen in the tuberculous exudates. They studied 59 CSFs, 25 ascitic and pleural fluids of patients with tuberculosis. The technical procedure of the ELISA consisted of incubation of equal volumes of CSF specimen and the rabbit IgG anti-BCG antibody in an unsensitised wells of a flat-bottomed polysytrene microtiter plate. Following overnight incubation, 100 ul of CSF-rabbit anti-BCG complex was transferred onto each well in a microtiter plate. The wells were presensitised with 1 µg of BCG antigen. Following the incubation, goat antirabbit IgG-alkaline phosphatase conjugate was added into each well. p-nitrophenyl phosphate was used as the substrate. The absorbance was recorded. The results showed significant differences between the absorbance in the CSFs of patients with TBM and control groups. The authors have

suggested that the competition ELISA seems to possess a clinical application the diagnosis of TBM.

Wu et al (137) used commercial antibody against **M bovis** in a sandwich ELISA and detected mycobacterial antigen in the CSFs of five patients with TBM. The ELISA was capable of detecting antigen as low as 4ng/ml of antigen. Absorbance for five patients with confirmed TBM ranged between 0.150-0.600 (0.27±0.19). For 134 patients with non-meningitic neurological diseases and 6 treated patients with probable TBM the absorbances were 0.032±0.009 and 0.029±0.10 respectively. Specificity of the ELISA was evaluated by the negative results (0.028±0.066) obtained with the bacterial and cryptococcal antigens. The authors have emphasised that the ELISA will be useful for monitoring the effectiveness of anti-tuberculosis treatment in patients with TBM. The authors also observed the chemotherapy may be responsible for the rapid decline of antigen levels in CSFs of their patients with TBM. The authors concluded that the detection of antigen is more useful than the detection of antibody because detection of antibody will not differentiate patients with active TBM from patients with an inactive TBM.

Dhand et al (42) developed a sandwich ELISA for the detection of mycobacterial antigen in the CSFs of 20

patients with TBM, 9 patients with pyogenic meningitis and 15 others with a normal CSF. Double antibody sandwich method was employed in their study. Anti-BCG antibody was raised in rabbits by immunising the rabbit with 100 ug **M bovis** mixed with Freund's adjuvant. The second antibody was prepared from pooled immune sera from tuberculous subjects. The immunoglobulin were separated from the immune sera and were further purified by an affinity chromatography. The results showed 100% sensitivity in 7 culture-positive patients with TBM. Among the 13 culture negative patients, the assay was positive in 6 patients. The assay was negative in all the 9 patients with pyogenic meningitis but false positive results were seen in one case each of viral meningitis, neurocysticercosis, and enteric encephalopathy.

Mastroianni et al (85) standardised a dot-blot enzyme immuno-assay for the detection of mycobacterial antigen in the CSFs of patients with TBM. A total of 38 CSF specimens were collected for the assay. 11 patients were diagnosed as having TBM. In 8 patients, TBM was proved bacteriologically by culturing the bacilli from the CSF. In 3 patients, the diagnosis was made on the basis of clinical features together with the characteristic changes in the CSF (lymphocytosis, increased amount of protein and a

reduced concentration of glucose) as well as response to antituberculosis treatment. 27 CSFs from patients with bacterial, fungal and viral meningitis were also examined. The dot-blot assay was performed in the NCP sheets. The CSFs were spotted onto the NCP. After washing rabbit anti BCG-HRP conjugate was added and incubated for 2h. After washing, the substrate was added. Positive reaction appeared as a well-defined blue dot against the NCP in a white background.

In order to estimate the sensitivity of the dot-blot assay, sonicate antigen prepared between **M tuberculosis** was tested in concentrations ranging between 5 and 500ng/ml. The amount of antigen in CSFs detected by the dot-blot technique was 100ng/ml and above. Mycobacterial antigen was demonstrated in all the samples of CSF (11/11) from patients with TBM. 2 out of 25 CSF samples from patients with non-tuberculous meningitis also gave a positive reaction. Serial CSFs in patients with TBM following therapy showed a progressive decline in the concentration of antigen. Mastroiani et al concluded that dot-blot assay is simple, sensitive and rapid for the early diagnosis of TBM. It can be easily performed in clinical laboratories without the use of expensive apparatus and best suited to developing countries where the prevalence of TBM is still high.

2.2.3. Immunohistochemical technique for the demonstration of mycobacterial antigen in intracranial tuberculoma

Demonstration of mycobacterial antigen in intracranial granulomatous lesion will be useful for establishing a diagnosis of tuberculosis. This is particularly relevant because granulomatous lesion due to a fungal aetiology often mimic with the histopathological features of a tuberculous granuloma.

Humphrey et al (55) applied an immunohistochemical method to demonstrate mycobacterial antigen in the caseating pulmonary tuberculomas of 59 surgical specimens. Immunohistochemical technique was performed in the formalin fixed paraffin sections. The primary antibody to culture filtrates of **M tuberculosis** was raised in rabbits. Antibody present in the rabbit immune sera was further purified by the bioaffinity immunoabsorbent chromatography. Paraffin sections were incubated with the primary rabbit antibody for 4-5 h at $+4^{\circ}C$. Subsequently the sections were treated with antirabbit IgG and rabbit peroxidase-antiperoxidase complex. The enzyme reaction was developed by the addition of diaminobenzidine tetrachloride. Specificity of the staining reaction was evaluated by substituting the preimmune sera of the rabbit in an another paraffin section of a tuberculous lesion. This substitution gave negative results in all the

specimens of tuberculosis. Distribution of mycobacterial antigen in the lesion consisted of brown to pink granular particles within the cytoplasm of Langhan's giant cells and macrophages. This intracytoplasmic material was conspicuous by its absence, in cases of fungal granuloma. The second type of distribution was seen in the caseous zones where clusters of mycobacterial antigen staining masses were seen and were clearly demarcated from the amorphous caseous necrosis. The authors concluded, detection of mycobacterial antigen may be useful for establishing mycobacterial aetiology in cases of caseating pulmonary granulomas.

Barbolini et al (10) used four MAbs against **M tuberculosis.** MAb 60.25 recognised a broad range of mycobacterial antigens localised within cytoplasm of phagocytic cells. MAb 61.3 reacted with 35 kDa protein **M tuberculosis, M africanum** and **M bovis.** The specificity of MAb 61.3 was confirmed by the negative stanining of positive lymphnode specimen obtained from a patient who was infected with **M kansasaii.** MAb 105.10 and 2.16 reacted with 65 kDa heat-shock protein and they appeared as dark clumps of bacilli within the cytoplasm of phagocytic cells. On the basis of this study Barbolini et al emphasised that detection of mycobacterial antigen in a tuberculous granuloma by immunohistochemical method is useful for establishing the mycobacterial aetiology in a caseating granuloma.

MATERIALS AND METHODS

CHAPTER III

3.1 Specimen collection

3.1.1 Collection of serum:

5 ml of venous blood was collected in a sterile glass container and incubated at 37°C for 2 h. Followiing the clot retraction, the blood samples were centrifuged at 2000 rpm for 15 min. The clear serum was collected and dispensed into 0.5 ml sterile aliquots for the immunoassays. The sera collected from several groups the patients were coded and stored at - 20°C.

3.1.2 Collection of CSF

2 ml of lumbar CSF was collected under aseptic conditions from all the patients undergoing lumbar puncture in this hospital. In the laboratory, the CSF specimens were subjected to (a) smear examination for AFB under a Z-N preparation (b) The remainder of the deposits was reconstituted in 0.2 ml sterile normal saline and inoculated into L-J slope for culturing **M tuberculosis.** The supernatant CSF was dispensed in four aliquots and stored at - 20°C.

3.1.3 Collection of CSF and tissues at autopsy from patients with meningitis

Ventricular and cisternal CSFs were collected during autopsy from patients with TBM who died during their hospital stay . The CSFs were centrifuged at 3000 rpm for 10 min. The deposits were subjected to bacteriological studies. The supernatant CSFs were stored at -20°C for immunoassays. The brain was processed for the routine neuropathological studies as well as immunohistochemical techniques for the demonstration of mycobacterial antigen in the tuberculous lesions.

3.1.4 Collection of tuberculoma (tissues from neurosurgical material)

Surgical specimens from ten cases of intracranial tuberculoma were collected and they were used for the demonstration of mycobacterial antigen by an immunohistochemical method. Ten cases of fungal granuloma were also collected and used as controls.

3.2 Study population

Patients selected for this study are classified into the following groups.

3.2.1 Group I : Patients with TBM

Serum and CSF specimens were collected from 75 in-patients admitted to the Neurology services of this institution with a clinical diagnosis of TBM. This hospital is a tertiary referral centre for neurological diseases and all the patients included in this study were referred from several outlying hospitals in and around Kerala and Tamil Nadu states.

A possibility of TBM in these patients was considered on the basis of clinical manifestations of the disease - history of fever, headache, vomiting, presence of neck stiffness, Kernig's sign and compatiable features of pleocytosis, lymphocytosis, elevated protein (> 40mg%) reduced sugar (< 40mg%) in the CSFs as well as definite clinical recovery following a course of ATT. A proforma was prepared in every patient for recording the relevant clinical data as well as laboratory investigations. Patients with a clinical diagnosis of TBM were further subdivided into (a) Definite TBM (b) Probable TBM. **'Definite cases' of TBM** - are the patients in whom **M tuberculosis** was demonstrated either in the ZN stained smears or by the culture. **'Probable cases' of TBM** are the patients in whom the clinical and CSF findings were suggestive of TBM and these patients showed improvement following a course of

ATT. However in none of the patients with 'Probable' TBM, **M tuberculosis** was isolated in their CSF.

TABLE 3.I

Data showing the age and sex of patients in group I (TBM)

Sl No.	Age in years	Males	Females
1	0-10	2	3
2	11-20	8	8
3	21-30	19	8
4	31-40	4	7
5	41-50	2	2
6	51-60	4	2
7	61-70	4	2

3.2.2 Group II : Patients with Non-TBM

Sera and CSFs were collected from 75 in-patients admitted with a clinical diagnosis of pyogenic meningitis, viral encephalitis and fungal meningitis. These patients were grouped as Non-TBM.

a. **Pyogenic meningitis** (n = 55). In 10 patients, the CSFs showed positive cultures (Hemophilus influenzae n = 1, Hemophilus species n = 1, Streptococcal pneumoniae n = 3,

Pseudomonas aeruginosa n = 1, Acinetobacter calcoaceticus n = 3 and Nisseria meningitides n = 1). In 45 patients with pyogenic meningitis, the CSF cultures were negative for bacteria including anaerobes and **M tuberculosis** on more than one occasion. b. **Viral encephalitis** n = 17, c. **Fungal meningitis** n = 3 (Cryptococcus neoformans n = 2 : Aspergillus fumigatus n = 1.

TABLE 3.II

Data showing the age and sex of patients in group II (Non-TBM)

Sl. No.	Age in years	Males	Females
1	0-10	8	12
2	11-20	9	12
3	21-30	15	3
4	31-40	6	3
5	41-50	2	1
6	51-60	2	2

3.2.3 Group III. Patients with non-meningitic neurological disorders

Sera and CSFs were collected from 75 patients with non-meningitic neurological diseases. These include Benign intracranial hypertension n = 20; stroke n = 12;

polyradiculitis n = 10; myeloradiculitis n = 8; seizures n = 8; CNS vasculitis n = 6; demyelination n = 4; CSF collected at myelography, n = 7.

TABLE 3.III

Data showing the age and sex of patients in Group III (non-meningitic neurological disorders)

Sl. No.	Age in years	Males	Females
1	0-10	3	5
2	11-20	5	11
3	21-30	10	7
4	31-40	7	5
5	41-50	7	4
6	51-60	3	2
7	61-70	3	1
8	71-80	1	1

3.2.4 Group IV Patients with Pulmonary tuberculosis, Leprosy and Healthy controls.

5 ml sera were collected from 50 patients (sputum AFB positive) with pulmonary tuberculosis. Sera from 50 patients of leprosy and sera from 50 voluntary healthy blood donors were also collected and they were used as controls.

3.3 Preparation of mycobacterial antigens

Three mycobacterial antigens were isolated from **M tuberculosis.** The methodology for the preparation of each of these antigens are described below.

3.3.1 Preparation of CFA

CFA was prepared (29) from the cell-free culture filtrate of H_{37} Ra strain of **M tuberculosis.** This strain was supplied by Tuberculosis Research Centre, Madras. Pellicle cultures from the 10-14 day old "seed" cultures were grown on Proskauer-Beck medium (Hi-media, Bombay) for 8-10 weeks. At the optimum growth, the cultures were initially clarified by filtration through the whatman paper-IV and finally through the 0.45 μm millipore membrane. The cell-free culture filtrate were then dialysed several times against distilled water and concentrated to ten-folds using an ultrafiltration unit (Amicon-GMPH; Witten; Germany). The protein content of the culture filtrate was determined (79) dispensed in aliquots and preserved at -20°C. Sodium merthiolate was added (1:10,000) as a preservative.

3.3.2 Preparation of tuberculin PPD

PPD antigen was prepared (80) from H_{37}Ra **M tuberculosis.** Pellicle cultures from the 10-14 day old "seed culture" were grown on Proskauer-Beck medium for 8-10

weeks. At the optimum growth, the cultures were filtered through Whatman filter paper IV and 0.45 μm pore-sized millipore membrane. The culture filtrate was autoclaved at 120°C for 30 min and ammonium sulphate was added (297 gms/litre of culture filtrate) to bring the saturation at 50%. The precipitate was recovered by repeated centrifugation and reconstituted to 10 ml in PBS. The reconstituted material was dialysed against PBS for 48 h at +4°C. The protein content was measured, dispensed in aliquots and stored at - 20°C. This material was designated as tuberculin PPD.

3.3.3 Preparation of M tuberculosis antigen 5

This antigen was isolated from the unheated cell-free, culture filtrate of $H_{37}Ra$ strain of **M tuberculosis** by an immunoabsorbent affinity chromatography (33). The reference **M tuberculosis** antigen 5 and monospecific goat antiserum to this antigen were made available through the kind courtesy of Dr. T.M. Daniel, Cleveland, Ohio, USA. Gammaglobulin in the reference antiserum was recovered by the precipitation with ammonium sulphate at 50% saturation. The precipitate was reconstituted in normal saline and dialysed against 0.1 M sodium bicarbonate buffer (pH 9.0) to which 0.5 M sodium chloride was added. The protein content of the dialysed

gammaglobulin was estimated, dispensed in aliquots and stored at -20°C.

Immunoabsorbents were prepared with activated cynogen bromide-sepharose 4B (Sigma Chemicals, St. Louis). One gram of cynogen bromide-sepharose 4B was allowed to swell to 3.5 ml in double distilled water, washed with large volumes (20 times the original gel volume) of cold 0.1 M sodium bicarbonate buffer (pH 9.0) and finally resuspended as a slurry of 50 percent (w/v) by adding 0.1 M sodium bicarbonate buffer. Gammaglobulin (8 mg/ml) to **M tuberculosis** antigen 5 was added in an equal volume to the activated cynogen bromide sepharose 4B and the immunoabsorbents were coupled for 16 h at 4°C. Excess of gammaglobulin was washed with larger volume (50 ml) of 0.1 M sodium borate buffer (pH 9.0) alternating with 0.1 M sodium acetate buffer (pH 5.0) for five cycles, starting and finishing with sodium borate buffer. The immunoabsorbents were finally suspended in PBS and poured into a chromatography (1.0 cm in diameter) column and equilibrated in the column with PBS. The column was washed three times with 4 M urea in 0.15 M sodium bicarbonate buffer to minimise the 'leaching-out' of the gammaglobulin from the immunoabsorbents. One ml of cell-free culture filtrate of $H_{37}Ra$. **M tuberculosis** (5 mg/ml) was added to the column and

the column was run with PBS. One ml fractions were collected every 10 min till the absorbance at 280 nm showed a blank reading. Elution of antigen 5 was then carried out by the addition of 4 M urea in 0.15 M sodium bicarbonate buffer and the absorbance of fractions at 280 nm were recorded. Those fractions with an absorbance of more than 0.05 were pooled, dialysed and concentrated to 1 ml. The protein content of the elute was quantitated.

3.4 Characterisation of mycobacterial antigens by SDS-PAGE

The CFA and **M tuberculosis** antigen 5 were subjected to SDS-PAGE (74).

The electrophoresis and separation of proteins were performed in gel-slabs. The dimensions of the slab gels were 1.5 x 180 x 160 mm). The stacking gel contained 4% acrylamide and 0.1% SDS (Sigma Chemicals, St. Louis) in 0.125 M Tris-hydrochloride buffer (pH 6.8). The separation gel contained 12% acrylamide and 0.1% SDS in 0.375 Tris-hydrochloride buffer (pH 8.8). The cross linker was N,N, methylene-bisacrylamide 2.6%. 140 ugs of CFA, and 80 ug of **M tuberculosis** antigen 5 were diluted 1:1 in the sample buffer containing 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.02% bromophenol-blue in 0.06 M Tris-hydrochloride buffer (pH 6.8). The samples were boiled in a water bath for 5 min and they were applied to the gels.

The electrophoretic tank contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS(pH 8.3). The standards used as molecular weight markers were BSA (66 kDa), OVA (45 kDa), glyceraldehyde (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa). Electrophoresis was carried out at a constant current of 10 mA for 16 h in a vertical slab-gel LKB electrophoretic unit.

3.4.1 Staining with coomassie-brilliant blue R-250

After the electrophoresis, the gels were stained with coomassie brilliant blue (R-250 dye) for the identification of various protein components (47). The gel was fixed in 40 % methanol and 10% trichloroacetic acid for one h before it was immersed in the staining solution. The gel was then stained in 0.1% coomassie brilliant blue dissolved in 40% methanol, 10% acetic acid and then destained with a solution containing equal volume of 40% methanol and 10% acetic acid. The destaining solution was changed several times, until the background became clear.

3.4.2 Results in SDS-PAGE

The electrophoretic patterns of CFA and Antigen 5 in SDS-PAGE are shown in Figure 3.I. CFA demonstrated 22 stainable bands when stained with a coomassie stain. The molecular weights of these proteins ranged between 10 kDa to

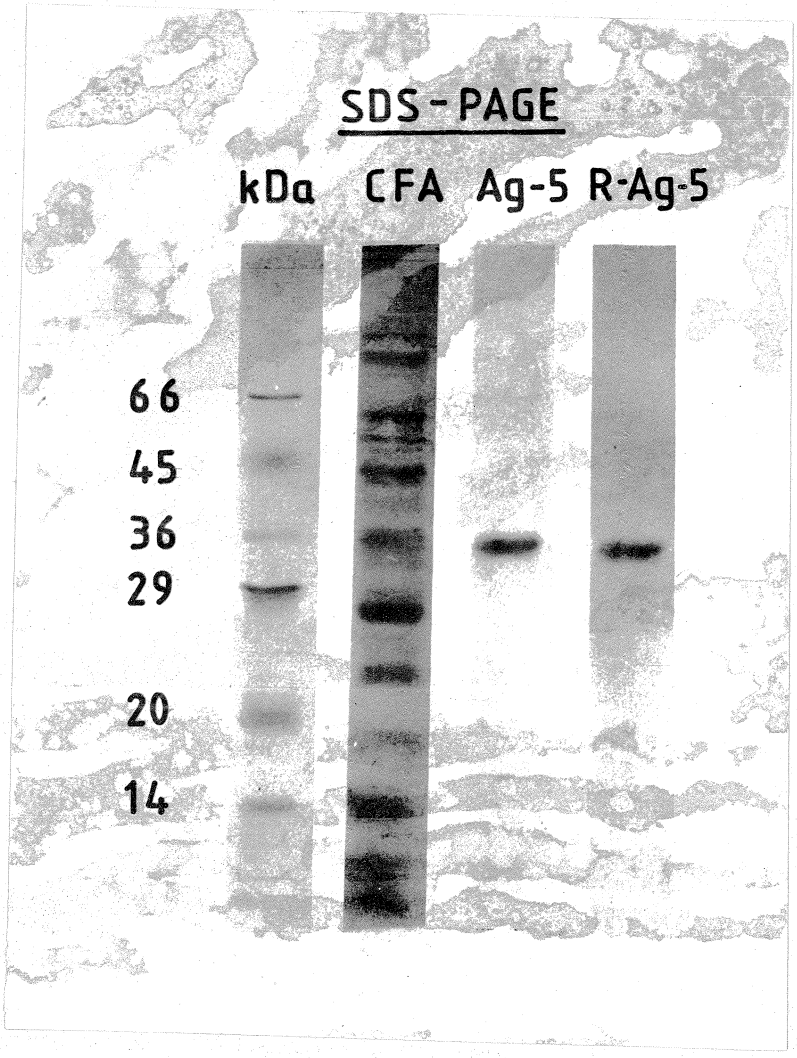


Fig. 3.I Showing the appearances of CFA and antigen 5 in SDS-PAGE. The gels are stained by coomassie brilliant blue. Note the similarity between antigen 5 and reference antigen 5.

89 kDa. **M tuberculosis** antigen 5 appeared as a single stainable band and was found to possess a molecular weight of 35 kDa. Antigen 5 prepared in this laboratory appeared identical with the reference antigen 5, in SDS-PAGE.

3.5 Immunisation schedule for raising antisera against mycobacterial antigens

3.5.1 Raising polyvalent antiserum to CFA (31)

Three adult male rabbits served as a source of antiserum. In the primary immunisation, each rabbit received 2 ml of water-in-oil emulsion that contained 2 mg of CFA, 2 mg of dried autoclaved bacillary sediment mixed thoroughly with IFA (Sigma Chemicals, St. Louis). The material was injected in divided doses into two gluteal intramuscular sites and into 2 flank subcutaneous sites. The immunisation schedule was repeated on 14th, 21st, 28th, 35th days following the primary immunisation. The antibody titer was sequentially assessed during the immunisation schedule by an IEP. On the 35th day following the primary immunisation, 20 ml of blood was drawn by an intracardiac puncture. Serum was separated and stored in aliquots at $-20^{o}C$. Sodium merthiolate (1:10,000) was added as preservative.

3.5.2 Raising of monospecific antiserum to M tuberculosis antigen 5 (34)

Two adult male rabbits served as the source of antiserum. For the primary immunisation, each rabbit was immunised with 3 ml of water in-oil emulsion that contained 1 ml of **M tuberculosis** antigen 5 (100 µg), 1 ml of IFA and 1 ml of **Bordetella pertussis** vaccine. The material was injected intramuscularly and subcutaneously at multiple sites. The secondary immunisation was repeated on the 14th day following the primary immunisation. Booster doses were given at weekly interval. High antibody titer was demonstrated on IEP following the third booster dose. 20 ml of blood was collected through an intracardiac puncture and serum was separated. Sodium merthiolate (1:10,000) was added as a preservative and the sera were stored in aliquots at $-20^{o}C$.

3.6 Evaluation of antibodies raised in rabbits to CFA and M tuberculosis antigen 5

3.6.1 Evaluation of antibodies by IEP

IEP was performed (57) on the LKB horizontal electrophoretic apparatus. The buffer used in the electrophoretic tank contained 1.38 g of barbital, 8.76 g of sodium barbital, 0.38 g of calcium lactate dissolved in 1000 ml of distilled water (pH 8.6). The buffer used for the

preparation of agar contained 0.55 g of barbital, 3.5 g sodium barbital, 0.51 g of calcium lactate and 10 g of agarose in 1000 ml of distilled water (pH 8.6). 15 ml of buffer-agar solution was spread uniformly on a (94 x 84 mm) clean glass slide. Wells and troughs were made in the agar with the LKB template. The diameter of each well was 4 mm and the trough measured 2.5 mm by 57 mm. Space between the well and trough was 4 mm. 10 µl each of CFA (A), **M tuberculosis** antigen 5(B) and reference **M tuberculosis** antigen 5(C) were placed in the respective wells and the antigens were separated electrophoretically for 90 min using a constant current of 5 mA per slide. After the electorphoresis, 150 µl each of rabbit polyvalent antiserum against **M tuberculosis** (Ab-I), monospecific rabbit antiserum against antigen 5 (Ab-2) and reference monospecific goat antiserum to antigen 5 (Ab-3) were added in the respective troughs. Following IEP, the slides were incubated in a humid chamber + 4^{o}C and examined daily for a period between 4-7 days. The patterns exhibited by CFA and antigen 5 in an IEP are shown in Figure 3.II

Interpretation of the results of IEP

1. Multiple precipitin arcs were seen with CFA and rabbit polyvalent antibody to **M tuberculosis.**

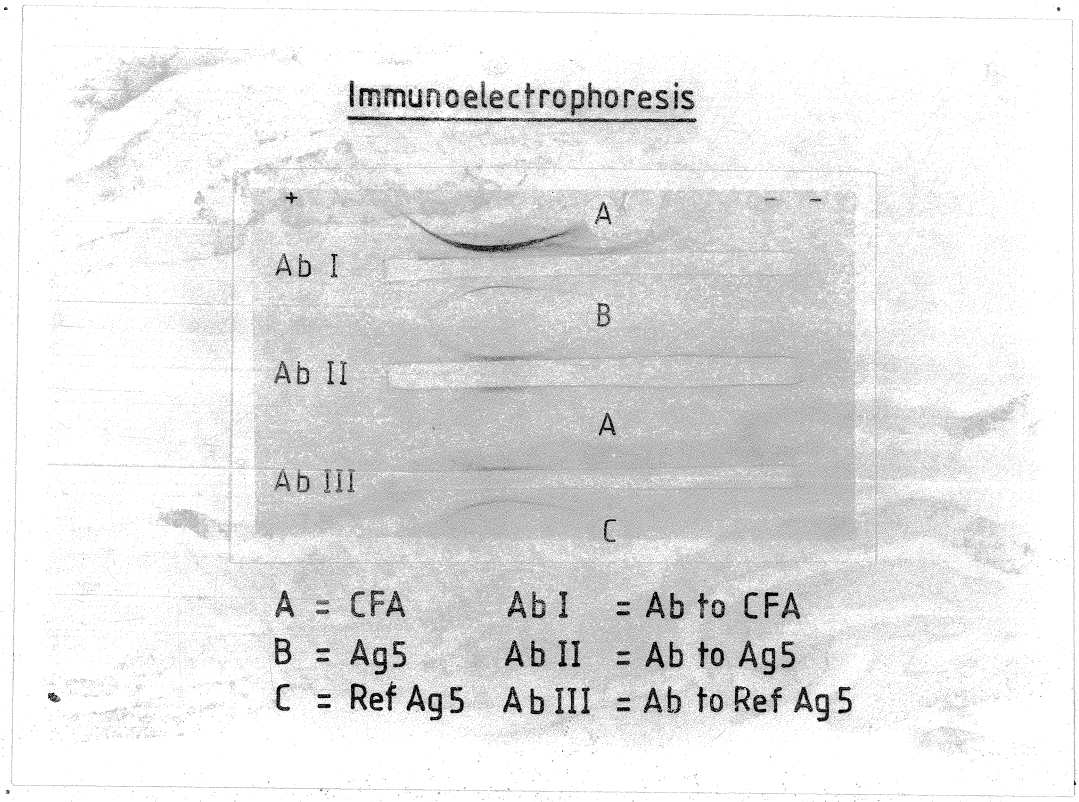


Fig. 3.II IEP showing the appearances of CFA, antigen 5 and reference antigen 5 against their antibodies.

2. Single precipitin arc was seen with **M tuberculosis** antigen 5 and polyvalent antibody to **M tubrculosis.**

3. Single precipitin arc was seen with CFA and rabbit antibody to antigen 5.

4. Single precipitin arc was seen with antigen-5 and rabbit antibody to antigen-5.

5. Single precipitin arc was seen when CFA was treated against reference goat antibody to antigen 5.

6. Single precipitin arc was demonstrated with reference antigen 5 with reference antibody to **M tuberculosis** antigen 5.

3.6.1.1 Evaluation of antibodies by modified Osserman IEP (95)

A modified Osserman IEP was performed. IEP was carried out with CFA as the antigen and rabbit polyvalent antiserum to **M tuberculosis** as the antibody. At the completion of electrophoresis, another well was cut and it was filled with antigen 5 (isolated from the CFA in this laboratory). Immunodiffusion was allowed for 48 h (Figure 3.III).A single precipitin arc developed between the antigen 5 and the rabbit polyvalent antiserum to **M tuberculosis.** Thus modified Osserman's IEP has confirmed that, antigen 5 is one of the immunodominant antigens of **M tuberculosis.**

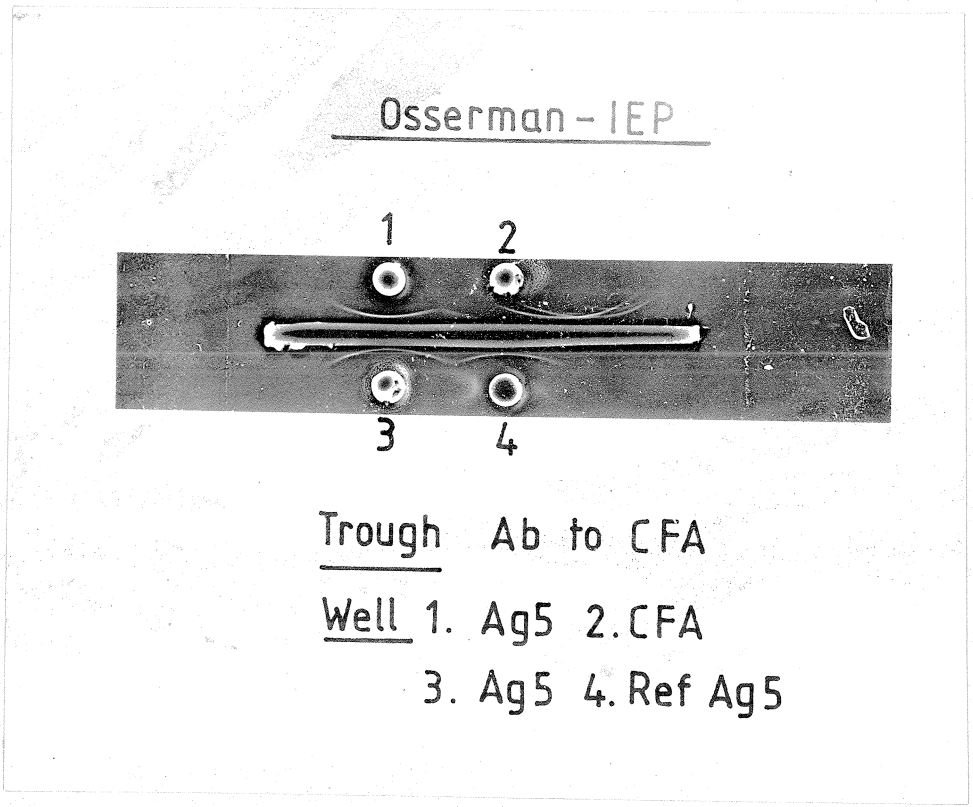


Fig. 3.III A modified Osserman IEP showing the identical appearances of antigen 5 and reference antigen 5 against polyvalent rabbit antibody.

3.6.2 Evaluation of antibodies by an indirect ELISA (44)

CFA and **M tuberculosis** antigen 5 were serially diluted through the range of 5-80 µg/ml in 0.1 M carbonate-bicarbonate buffer (pH 9.6). 100 µl of either antigens were added in the respective wells of a polyvinyl-chloride microtiter ELISA plates (Dynatech laboratories, Alexandria, Va; USA) and incubated at room temperature for 2 h. The plates were washed with PBS in 0.05% Tween 20 (PBS-T) for 3 to 4 times. The unreacted sites in the wells of the microtiter plates were blocked with 1% BSA (Sigma Chemicals, St. Louis, USA) in PBS for 1 h. Rabbit antibodies to CFA and **M tuberculosis** antigen 5 were diluted serially (1:1000-1:781,250) in BSA/PBS. 100 µl of either of the rabbit antibodies were added to the respective wells and the plates were incubated at $4^{o}C$ for 12 h. The plates were washed with PBS-T for 5 times and 100 µl of (1:40,000) antirabbit IgG-biotin conjugate (Sigma Chemicals, St. Louis, USA) was added to each well and plates were incubated for 2 h at room temperature. The microtiter plates were washed and 100 µl of (1:200) avidin-alkaline phosphatase (Sigma Chemicals, St. Louis, USA) was added to each well. The enzyme reaction was induced by the addition of 100 µl of p-nitrophenyl phosphate 1 mg/ml in 10% diethenolamine buffer (pH 9.7). At 30 min, 25 µl of 3 N NaOH was added to stop the reaction. The absorbances in all the wells were read at

405 nm, using an ELISA reader (Titertek, Multiscan, Flow Laboratories, USA). The end-point titers in ELISA were also recorded by visual examination.

Results

	Titer
CFA against rabbit polyvalent antibody to **M tuberculosis**	1:390,625
CFA against rabbit antibody to antigen 5	1:78,125
Antigen 5 against rabbit polyvalent antibody	1:3125
Antigen 5 against rabbit antibody to antigen 5	1:3125
CFA against normal rabbit serum	Nil
Antigen 5 against normal rabbit serum	Nil
CFA against antirabbit IgG-biotin conjugate	Nil
Antigen 5 against antirabbit-biotin conjugate	Nil

3.6.3 Evaluation of antibodies by Western blot method

140 µg of CFA and 80 µg of **M tuberculosis** antigen 5 were subjected to SDS-PAGE (74). After the electrophoresis, the resolved proteins in the gels were electrotransferred onto the NCP strips (124). The transfer buffer contained 0.025 M Tris, 0.192 M glycine in 20% methanol (pH 8.3). A current of 0.8 mA per cm^2 for one h was provided during the electrotransfer of antigens from the gels to NCP.

After the electrotransfer a NCP strip was stained with 0.1% amidoblack (in 25% isopropanol and 10% acetic acid) and then destained in a solution containing 25% isopropanol and 10% acetic acid. After ascertaining the presence of the protein in the NCP, the immobilised proteins in the NCP were treated with 2.5% skim milk in PBS for 1 h at 40°C. The NCP strips were washed with PBS and incubated individually with rabbit polyvalent antiserum to CFA and rabbit antiserum to **M tuberculosis** antigen 5 (1:200) for 2 h at 40°C. The NCP strips were washed several times in PBS and incubated with (1:1000) antirabbit IgG-biotin conjugate for 2 h at 40°C. The strips were again washed in PBS and incubated with (1:200) avidin-alkaline phosphatase for 2 h. The colour reaction was developed by immersing the NCP strips in a substrate containing - O-dianisidine tetrazotized (0.25 mg/ml) and β naphthyl acid phosphate (0.25mg/ml) dissolved in 0.06 M sodium borate buffer (pH 9.7) and Magnesium sulphate $2H_2O$ (1.2mg/ml). After the development of the colour the NCP strips were washed first in a solution of methanol, acetic acid and distilled water (5:1:5) followed by a final rinse in distilled water. Appearances of CFA and antigen 5 against their respective antibodies are shown in Figure 3.IV.

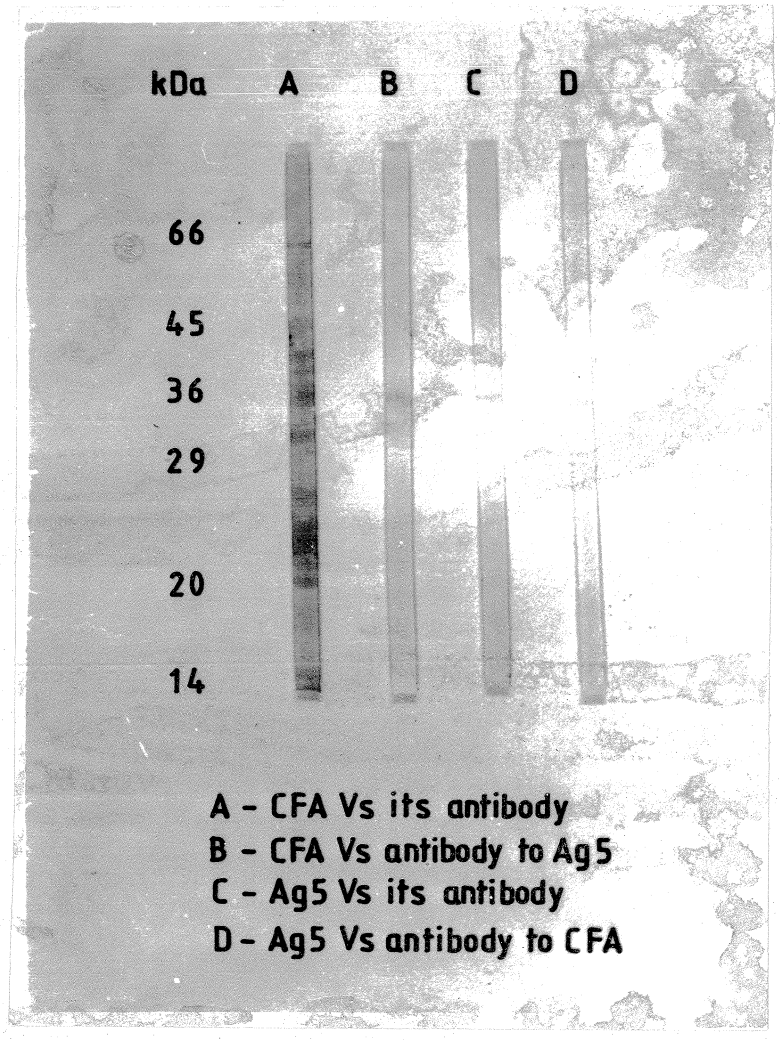


Fig. 3.IV Showing the evaluation of the rabbit antibodies to CFA and antigen 5 by the Western blot method.

Interpretation:

1. (a) The polyvalent rabbit antibody has reacted with CFA of **M tuberculosis** and 9 protein bands were seen. The molecular weights of these proteins were 78 kDa, 72 kDa, 63 kDa, 56 kDa, 43 kDa, 35 kDa, 31 kDa, 24 kDa and 18 kDa. (b) NCP containing CFA demonstrated a single band when incubated with rabbit antibody to **M tuberculosis** antigen 5. The molecular weight of this band was found to be 35 kDa.

2. **M tuberculosis** antigen 5 gave single band when treated with rabbit antibody to antigen 5 and the molecular weight of the protein was found to be 35 kDa.

3. Antigen 5 is one of the immunodominant antigens of **M tuberculosis.**

3.7 Detection of antibody to PPD and M tuberculosis antigen 5 in CSF of patients by an indirect ELISA

An indirect ELISA was standardised in the laboratory (44). The wells in the microtiter plates were individually coated with 100 µl of PPD and antigen 5 (20 µg/ml). The plates were incubated in a humid chamber at room temperature for 2 h. The contents in the plates were discarded and the plates were washed three times with PBS-T. The unreacted sites in the wells were blocked by the addition of 1% BSA/PBS for 1 h following which the plates were again washed

thoroughly. The CSFs from TBM and control groups were serially diluted with BSA/PBS (through the range of 1:10-1:640) before they were added in the respective wells of the microtiter plates. The plates were incubated for 12 h at +4°C. The plates were then subsequently washed with PBS-T and 100 µl of (1:1000) anti-human IgG-alkaline phosphatase conjugate was added and plates incubated at room temperature for 2 h following which they were washed with PBS-T for 5 times. 100 µl of substrate (1 mg of p-nitrophenyl phosphate in 1 ml of 10% diethenolamine buffer pH 9.7) was added. After 30 min, 25 µl of 3 N NaOH was added to stop the reaction. CSF sample from a culture positive, autopsy proven case of TBM was used as the positive control. The blank wells contained antigen 5, enzyme conjugate and the substrate. The microtiter plates were read both visually as well as with an automated ELISA reader at 405 nm. The assay was repeated if the end-point did not give consistent reaction and the observer bias was eliminated by repeating the same CSF specimens on two different occasions. The end-point reaction at all dilutions in the CSF specimens of tuberculous and control subjects were recorded for statistical evaluation.

3.7.1 Detection of antibody to PPD and M tuberculosis antigen 5 in the serum by an indirect ELISA

An indirect ELISA (44) was standardised to detect the IgG antibody to PPD and **M tuberculosis** antigen 5 in the

sera of patients with TBM, non-TBM, non-meningitic neurological diseases, pulmonary tuberculosis, leprosy and normal healthy controls. Microtiter ELISA plates were coated individually with 2 µg/well of PPD and **M tuberculosis** antigen 5. The plates were incubated for 2 h at room temperature. Following which, they were washed with PBS-T and quenched with 1% BSA in PBS for 1 h. The plates were again washed and 100 ul serially diluted (1:1000-1:5000) sera from all the patients were added and the plates were incubated for 12 h at 4°C. The plates were then washed and 100 µl of (1:1000) antihuman IgG-alkaline phosphatase was added and incubated for 2 h at room temperature. The plates were washed and incubated with 100 µl of the substrate (1 mg of p-nitrophenyl phosphate). After 30 min of incubation, 25 µl of 3N NaOH added to stop the reaction. The microtiter plates were read both visually as well as with an automated ELISA reader. The end-point titers in the sera of patients with TBM, Non-TBM, non-meningitic neurological disorders, pulmonary tuberculosis and leprosy were individually recorded for the statistical evaluation.

3.7.2 Detection of antibody to M tuberculosis antigen 5 in CSF by Dot-Iba

Dot-Iba was carried out (103) on NCP strips. NCP strips measuring 7.5 cm x 12.5 cm were cut and fixed to a

plastic slide holder. Ten evenly distributed dots were marked on the NCP with a lead pencil for the orientation purposes. 2 µl of **M tuberculosis** antigen 5 (1 mg/ml) was placed over the dots. The NCP was incubated at 40°C for 2 h and immersed in 1% BSA/PBS for 1 h following which the NCP strips were washed and blotted dry.

CSF samples from patients with TBM, non-TBM and non-meningitic neurological disorders were serially diluted (1:2 to 1:16) in BSA/PBS. 2 µl of CSFs from all the patient groups were placed over the antigen dots. After incubation for 1 h at room temperature the NCP strips were washed three times with PBS-T and blotted dry. The NCP strips were then immersed for 1 h in (1:1000) antihuman IgG-alkaline phosphatase conjugate. The strips are then washed thoroughly with PBS-T, and immersed in 5 ml of a freshly prepared substrate consisting of O dianisidine tetrazotized (0.25 mg/ml) and β -naphthyl acid phosphate (0.25 mg/ml) dissolved in 0.06 M sodium borate buffer pH 9.7 containing Magnesium sulphate $2H_2O$. At 30 min, the reaction was stopped by pouring off the substrate and washing the NCP first in a solution of methanol, acetic acid and water (5:1:5) followed by a final rinse in distilled water. A positive reaction was indicated by the development of a purple to purple-pink circle around the dot.

3.7.3 Detection of antibody to M tuberculosis in CSF by Western blot method (124)

The components in CFA was separated by SDS-PAGE and they were transferred onto NCP as described earlier. The NCP strips were then washed in PBS and quenched in 2.5% skim milk for 1 h at 40°C. The NCP strips were incubated with CSF specimens (1:10 dilution) from all the patients for 2 h at 40°C. The NCPs were washed with PBS and then incubated with (1:1000) antihuman IgG-alkaline phosphatase conjugate for 2 h at 40°C. The strips were washed several time with PBS. The colour reaction was developed by the addition of a substrate containing O dianisidine tetrazotized (0.25 mg/ml), β -naphthyl acid phosphate (0.25 mg/ml) and Magnesium sulphate (1.2 mg/ml) dissolved in 0.06 M sodium borate buffer (pH 9.7). The reaction was stopped by the addition of a mixture containing methanol, acetic acid and distilled water (5:1:5) followed by a rinse in water. A positive reaction was indicated by the development of purple-pink bands.

3.8 Detection of mycobacterial antigen concentration in CSF by an inhibition ELISA

Inhibition ELISA procedure (131)

Before the application of the assay in clinical specimens, the assay was standardised by incubating equal

volume of rabbit (1:5000) polyvalent antibody to CFA with different concentrations of CFA (1-10,000 ng/ml) for 12 h at + 4°C. This antigen-antibody complex was centrifuged at 5000 rpm for 10 min and 200 µl of clear supernatant was then transferred to each well of a microtiter ELISA plate. The microtiter plate was presensitised with CFA (2 µg per well in 0.1 M carbonate - bicarbonate buffer pH 9.6) and subsequently blocked with 1% BSA in PBS. Following 12 h incubation, the plates were washed with PBS-T and 200 µl of a 1:40,000 dilution of antirabbit IgG-biotin conjugate was added. The plates were incubated for 12 h at room temperature. The plates were then washed with PBS-T and 200 µl of avidin-alkaline phosphatase was added to each well and the plates were incubated at room temperature for 2 h. The colour reaction was developed by the addition of 200 µl of p-nitrophenyl phosphate (1 mg in 1 ml of 10% diethanolamine buffer) and the reaction was subsequently stopped after 30 min by the addition of 50 µl of 3N NaOH. The control wells in the standardisation inhibition ELISA contained CFA, polyvalent antiserum to CFA. Antirabbit IgG-biotin conjugate, avidin-alkaline phosphatase and the substrate. The assay was repeated for six times. The mean absorbance in the test and control wells were recorded at 405 nm in an ELISA reader. Differences between the absorbance recorded in the control and test samples were

plotted against the logarithemic value of antigen concentration. A linear graph was obtained. The differences in the absorbances and the linear graph obtained in the standardisation ELISA are shown in Figure 3-V.

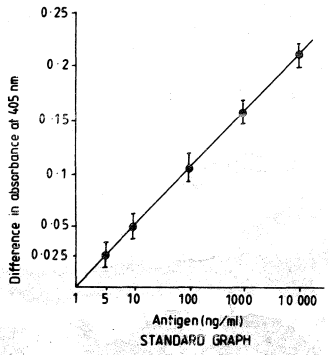
The CSF specimens from patients with TBM and controls were similarly assayed for the circulating mycobacterial antigen under identical laboratory conditions as described in the standardisation ELISA. For every CSF specimen, the assay was performed in duplicate and the mean absorbance was calculated. Antigen concentrations in CSF specimens from all the patient groups were directly quantitated from the standard graph.

3.8.1 Detection of mycobacterial antigen concentration in serial CSF specimen of 8 patients with TBM during ATT (131)

The mycobacterial antigen concentration was studied in 8 patients with TBM in whom 3 to 4 CSF specimens were collected at weekly intervals. These patients were receiving ATT during their hospital stay. The inhibition ELISA was carried out in these CSF specimens as described above and the concentration of mycobacterial antigens in the serial CSF specimens were quantitated.

ABSORBANCE OBTAINED IN THE STANDARDIZATION PROCEDURE FOR INHIBITION ELISA

Antigen (ng/ml)	Absorbance at 405 nm (mean ± SD)	Difference in absorbance between control and antigen
Control	0.460 ± 0.029	–
1	0.460 ± 0.014	0
5	0.435 ± 0.006	0.025 ± 0.0027
10	0.414 ± 0.042	0.046 ± 0.0031
100	0.355 ± 0.041	0.105 ± 0.0038
1000	0.307 ± 0.047	0.153 ± 0.0035
10000	0.245 ± 0.019	0.215 ± 0.0045



Graph of standardisation inhibition ELISA. The difference in the absorbance between control and culture-filtrate antigen is shown.

Fig. 3.V Showing the graph obtained during the standardisation of an inhibition ELISA.

3.8.2 Detection of mycobacterial antigen in CSF by a Western blot method

Proteins in the CSFs were resolved by SDS-PAGE (74). Electrotransfer to the NCP was performed (124) with a semidry blotting apparatus (LKB Novablot). After the electrotransfer the unreacted sites in the NCP strips were incubated with 2% normal goat serum and then quenched with 2.5% skim-milk dissolved in PBS for 1 h at 40°C. The NCPs were washed in PBS and then incubated with rabbit antibody to CFA (1:200) for 2 h at 40°C. The NCP were again washed and incubated with (1:1000) anti-rabbit IgG-biotin conjugate for 2 h at 40°C. The NCPs were washed and incubated with (1:200) avidin-alkaline phosphatase conjugate for 2 h at 40°C. The NCP were then washed four times and incubated in an enzyme-substrate solution for 10 min. The substrate consisted of a mixture containing O dianisidine tetrazotized (0.25mg/ml) and β naphthyl acid phosphate (0.25 mg/ml) dissolved in 0.06 M sodium borate buffer and 1.2mg/ml magnesium sulphate pH 9.7. After the development of the enzyme reaction the NCPs were washed initially in a solution of methanol, acetic acid and distilled water (5:1:5) followed by a final rinse in distilled water.

3.9 Detection of CICs in CSF

Detection of CICs in the CSFs of patients with TBM, non-TBM and non-meningitic neurological disorders were carried out (13).

3.9.1 Isolation of CICs:

CICs in the sera and CSF sepecimens of patients with TBM and control groups were isolated by a PEG precipitation method. 0.8 ml of CSF and serum each were mixed with an equal volume of 12% PEG in 0.1 M sodium borate buffer (pH 8.4) and were incubated for 12 h at +4^{o}C. Immune complexes in the sera and CSFs were precipitated by the centrifugation at 5000 rpm for 30 min. The pellets were washed twice with a PEG washing solution (PEG 2.5% in 0.1 M sodium chloride. 0.25 M sodium borate, 0.08 M EDTA and 0.1 M Tris pH 7.1) and were resuspended in 0.1 M PBS (pH 7.4). PEG precipitate from 0.8 ml of CSF and serum were resuspended in 150 ul of PBS.

A sandwich ELISA and an indirect ELISA were carried out to confirm the presence of **M tuberculosis** antigen 5 and antimycobacterial antibody in the of immune complexes of CSFs.

3.9.2 Detection of M tuberculosis antigen 5 in CICs by a sandwich ELISA

A sandwich ELISA method (130) was standardised in the microtiter ELISA plates. The wells of the microtiter plates were coated with rabbit IgG (5 µg) to **M tuberculosis** antigen 5 and incubated at 37°C for 2 h. Following which the plates were washed with PBS-T for 30 min. The PEG precipitates from tuberculous and control groups were serially diluted through the range of 1:10-1:640 in PBS-BSA and 100 µl of each were added in the plate and they were incubated for 3 h at 37°C. The plates were thoroughly washed and incubated with 100 µl of (1:1000) antihuman IgG-alkaline phosphatase conjugate for 3 h at 37°C. 1 mg of p-nitrophenyl phosphate in 1 ml of 10% diethanolamine buffer (pH 9.7) was used as the substrate. The reaction was stopped after 30 min by the addition of 25 µl of 3 N NaOH. The absorbances in all the samples were measured at 405 nm with an ELISA reader.

The "cut-off" point was determined to be at 1:80 as it gave the best discrimination between tuberculous and nontuberculous groups. The mean and SD of the absorbances at 1:80 were calculated for tuberculous and control groups. A test was considered positive for the presence of **M tuberculosis** antigen 5 in PEG precipitates if an

absorbance in a test sample was more than the mean plus 2 SD of the mean antigen concentration in the control group.

3.9.3 Detection of mycobacterial antibody in CICs by an indirect ELISA

An indirect ELISA (44) was carried out for the detection of antimycobacterial antibody in the CICs of serum and CSF of patients in the tuberculous and control groups.

2 µg of **M tuberculosis** antigen 5 was used to coat each well of a microtiter ELISA plate. After incubation, the plates were washed with PBS-T and blocked with 1% BSA in PBS. The PEG precipitates from tuberculous and control groups were serially diluted in 1% BSA through a range of 1:10-1:160. 100 µl each of the samples added in their respective wells and the plates were incubated for 12 h at +4^{o}C. The plates were washed with PBS-T and the amount of antibody to **M tuberculosis** antigen 5 present in PEG precipitate was determined by the addition of 100 µl of (1:100) antihuman IgG-alkaline phosphatase conjugate. The plates were washed with PBS-T and 100 µl of p-nitrophenyl phosphate was added. The enzyme reaction was stopped by the addition of 25 µl of 3 NaOH and the absorbance in all samples were measured at 405 nm in an ELISA reader.

The "cut-off" point was determined to be 1:80 as it gave the best discrimination between tuberculous and control groups. Tuberculous group showing an absorbance at 1:80 that was above the mean in the control group plus 2 SD were considered positive for the presence of antimycobacterial antibody in CICs.

3.10 Quantitation of albumin and IgG in sera

The quantitations of albumin and IgG in serum were carried out by a single radial immuno diffusion method (82). 12 ml of 1% agar gel solution in barbitone buffer (0.1 M pH 8.6) was poured onto the clean glass tubes. The gel solution was heated to 56^{o}C. 0.6 ml of antihuman albumin and 0.25 ml of anti-human IgG. (Sigma Chemical, St. Louis) were added to the individual tubes. The agar-gel antibody mixtures are then uniformly poured on to a petridish (Falcon optilux:Becton). The petridishes were kept in a moist chamber and 48 wells were cut at 5 mm distance, using a LKB template. The wells were filled with 5 µl of test sample and standards (Sigma Chemicals, St. Louis). For albumin and IgG estimations, the serum was diluted 1:100 and 1:10 in PBS respectively. The petri-dishes were incubated for 48 h at +4^{o}C and the diameter of the precipitin rings around the wells was measured in test, standard and controls. A standard graph was plotted and the concentration of albumin

and IgG in the unknown test samples were calculated from the standard graph. The results were expressed in mg% after multiplying the values with the dilution factor.

3.10.1 Quantitation of albumin and IgG in CSFs

The quantitations of albumin and IgG in CSF specimens were carried out by a single radial diffusion method, using low concentration partigen plates (82). 12 ml of 1% agar-gel solution in barbitone buffer (0.1 M pH 8.6) was prepared and stored in sterile glass tubes at +4^{o}. The agar solution is heated to 56^{o}C and 0.15 ml of antihuman albumin and 0.08 ml of antihuman IgG were added to the respective tubes. They were uniformly poured onto a petri-dish. The petri-dishes were kept in a moist chamber and 24 wells were cut at 5 mm distance from each other. The wells were filled with 10 µl of test CSF and standards. The petri-dishes were incubated for 48 h at +4^{o}C and the diameter of the precipitin ring was measured in test CSFs and standards. A standard graph was plotted and the concentration of albumin and IgG in CSFs samples were calculated directly from the standard graph. The results were expressed in mg%.

3.10.2 Calculation of CSF-IgG index (122) and mycobacterial antibody index (1,69)

This was carried out by using the following formula:

$$\text{CSF IgG index} = \frac{\text{CSF IgG}}{\text{Serum IgG}} \cdot \frac{\text{CSF albumin}}{\text{Serum albumin}}$$

$$\text{Mycobacterial antibody index} = \frac{\text{CSF mycobacterial antibody titer}}{\text{Serum mycobacterial antibody titer}} \cdot \frac{\text{CSF albumin}}{\text{Serum albumin}}$$

3.11 Mantoux's Test (Intradermal tuberculin test)

Tuberculin PPD was supplied by BCG Vaccine Laboratory, Madras. Mantoux's test was carried out as per the instruction given by the manufacturer. 0.1 ml tuberculin PPD was injected intracutaneously into the inner aspect of the forearm. During the inoculation, a lenticular, sharply protuberant vesicle developed at the site of injection. The reaction was read after 48 h. The test is considered positive when a red spot (erythema) or an infiltration (induration) of at least 10 mm diameter is visible around the injection sites.

3.12 Demonstration of mycobacterial antigen in intracranial tuberculomas by an immunohistochemical method

Immunohistochemical studies for the demonstration of mycobacterial antigen in tuberculous lesions were performed

(55) using a peroxidase-antiperoxidase method. Four micron-thick paraffin sections from tuberculous and non-tuberculous intracranial granulomatous lesions were subjected through a series of three preliminary incubations of (a) 3% hydrogen-peroxide and methanol (1:5) for 30 min. (b) concentrated hydrochloric acid and methanol (1:500) for 10 min. and (c) 10% normal rabbit serum diluted in 0.05 M Tris buffered saline (pH 7.4) for 10 min. Sections were incubated for 5 h at +4°C with rabbit IgG to **M tuberculosis** (5-10 µg/ml). Subsequent incubations were, 20 min at room temperature with 1:100 swine anti-rabbit IgG (Dakopats, Gostrup, Denmark) and then with rabbit peroxidase-anti-peroxidase complexes. The sections were washed thoroughly with Tris-buffered saline containing 1% normal rabbit serum after each incubations. The substrate consisted of 2 mg of diaminobenzidine tetrachloride (Sigma Chemicals, St. Louis) dissolved in 4 ml of 0.05 M Tris buffer (pH 7.4). 5 ml of 3% hydrogen peroxide were added just prior to use. Sections were incubated in the substrate for 5-10 min at room temperature. Finally, sections were counterstained with haematoxyline, dehydrated and mounted. The sections were examined under the microscope for the presence of colour reaction within the cytoplasm of macrophages and giant cells of the granuloma. In order to

evaluate the specificity of the reaction, normal rabbit IgG was substituted instead of rabbit IgG to **M tuberculosis.** This substitution gave consistently negative results in all the specimen of tuberculoma. This will suggest that localisation of mycobacterial antigen in the granulomatous lesions was specifically brought by the addition of rabbit IgG to **M tuberculosis .**

RESULTS AND DISCUSSION

CHAPTER IV

4.1. Results of the antibody based assays

4.1.1 Results of an indirect ELISA using PPD antigen

The data on IgG antibody titers to PPD antigen in the CSF specimens from 18 'definite' and 57 'probable' cases of TBM, 75 patients with non-TBM and 75 patients with non-tuberculous neurological diseases are shown in Table 4.I. Based on the standardisation ELISA, a 'cut-off' point was selected at 1:160 for scoring a test CSF specimen positive for tuberculous aetiology.

In the 18 'definite' cases of TBM, the CSF antibody titers ranged between 1:160-1:640 and the mean absorbance at 1:160 was 0.82 (0.7-1.3). In 45 out of 57 'probable' cases of TBM, the IgG antibody titers in CSFs ranged between 1:160-1:640 and the mean absorbance at 1:160 was 0.87 (0.72-1.4).

The CSFs in 47 out of 55 patients with partially treated pyogenic meningitis gave negative results in the ELISA and hence a possibility of a tuberculous aetiology was excluded in these patients. However 8 out of 55 CSF specimens from patients with partially treated pyogenic

TABLE - 4.I

Data showing the results of the indirect ELISA to detect IgG antibody against PPD antigen in CSFs

Parameters	TBM		Non-TBM			Non-meningitic neurological disorder
	Definite TBM (n=18)	Probable TBM (n=57)	Pyogenic meningitis (n=55)	Viral encep: (n=17)	Fungal meningitis (n=3)	Control group (n=75)
End-point titer						
1 : 20-1:80	18	57	47	16	2	75
1:160-1:640	18	45	8	0	1	0
Mean absorbance						
1:160	0.82	0.87	0.31	0.21	0.51	0.15
Sensitity %						
1:160-1:640	100	79	0	0	0	0
Specificity %						
1:160-1:640	0	0	85.5	100	66.6	100

1:160 end-point titer was taken as 'cut-off' point for scoring a test CSF as positive.

meningitis (Streptococcal pneumoniae n = 3; Pseudomonas aeruginosa n = 1, no organisms isolated in CSFs n = 4) gave positive results in the ELISA at 1:160 end-point titer. 17 CSF specimens from patients with viral encephalitis gave negative results. One out of 3 cases of fungal meningitis gave positive result and none of the CSFs from 75 patients with non-meningitic neurological diseases gave positive results.

In the earlier studies PPD antigen was used in the ELISA to detect antimycobacterial antibodies in the CSFs of patients with TBM. In these studies, a commercial PPD was used. Kalish et al (62) studied three culture proven patients with TBM and 33 other patients with chronic meningitis. Kalish et al observed, elevated levels of IgG antibody to PPD in the CSFs of three patients with TBM. They also studied the serial CSFs and sera from one of the three patients of TBM and observed a peak CSF antibody level on the 44 days of the disease. The peak serum antibody level occurred on the 66 day of the disease. It was interesting to note from that the serum antibody level in patients with TBM was lower than the CSF antibody levels. Kalish et al also found a decrease in the antibody level as the patients with TBM improved clinically. In 33 patients with chronic meningitis, the CSFs did not contain antibody to PPD. However, this study did not highlight the results

in culture negative patients with TBM. This is relevant because the utility of an ELISA should be critically evaluated in culture negative patients with TBM. Secondly the need to establish the tuberculous aetiology in culture negative patients with TBM is much more essential than in culture positive patients with TBM.

Kalish et al also observed a decrease in the antibody level following the ATT in one patient with TBM. It will be necessary to attribute the reasons for the decrease in the antibody titer particularly when IgG antibody is synthesised within the CNS in patients with TBM. In our data we had an opportunity to analyse the IgG antibody levels in serial CSFs of 20 patients with TBM. These patients were receiving the ATT during their period of hospitalization and we did not observe a decrease in the CSF antibody titer in any one of these patients. The antibody titer in majority of the patients with TBM remained same and in four patients an increase in the antibody titer was observed. It is also relevant to emphasise that there was no corresponding increase in the serum antibody titer in patients with TBM during the therapy.

Prabhakar et al (99) observed false negative results in 5 out of 10 'definite' cases of TBM with PPD antigen and therefore this antigen was not used further in their study.

Instead of PPD they used a commercial BCG and recorded sensitivity of 48% and a specificity of 92%. BCG antigen though carried a higher specificity, it was found to be less sensitive. Hernandez et al (53) applied BCG antigen in a study of TBM. They studied 20 CSF specimens from patients with a bacteriological diagnosis of TBM, 31 patients with acute pyogenic meningitis, 20 patients with viral meningitis. 19 children without infection in the CNS were selected as control. In their study IgG and IgM antibodies against BCG were detected in the CSFs, using an indirect ELISA method. The results showed 100% specificity and 100% sensitivity for patients with TBM. Hernandez et al emphasised simultaneous detection of IgG and IgM antibodies against BCG antigen as well as the use of covalently attached antigen may be responsible for the 100% sensitivity in the ELISA.

In none of the above studies neither BCG nor PPD antigen were prepared in the laboratory. It needs to be emphasised that commercial PPD and BCG antigens have mostly clinical and epidemiological applications and batch to batch differences in their composition are known to occur. Thus commercial PPD or BCG antigens are not ideal antigens to be used in a sensitive test like ELISA. In order to substantiate this, we compared the results of ELISA with tuberculin PPD prepared in our laboratory with commercial

PPD and BCG antigens. Tuberculin PPD prepared in our laboratory showed no false negative results in 'definite' cases of TBM and the test gave 100% sensitivity in all the definite cases of TBM. In order to improve the specificity of the ELISA a higher 'cut-off' point (1:160) was chosen. Despite, false positive results were observed in 14.5% patients with partially treated pyogenic meningitis. Nevertheless tuberculin PPD has definite advantages over commercial PPD or BCG in the ELISA. Moreover tuberculin PPD is stable and can be very easily prepared in any laboratory and does not require any expensive equipment or technical skill.

4.1.2. Results of an indirect ELISA using M tuberculosis antigen 5.

The data on IgG antibody titer to antigen 5 in the CSF specimens of 18 'definite' and 57 'probable' cases of TBM, 75 non-TBM patients and 75 patients with non-meningitic neurological diseases (control groups) are shown in Table 4.II. Based on the standardization of the assay,a 'cut-off' point was chosen at 1:80 for scoring a test CSF specimen positive for tuberculous aetiology. None of the 75 CSF specimens of patients with non-meningitic neurological diseases gave a positive result at 1:80 end-point titer in the ELISA. The mean absorbance recorded in these specimens was 0.14. Four out of 55 CSFs from partially treated

TABLE - 4.II

Data showing the results of the indirect ELISA to detect IgG antibody against Antigen 5 in CSF

Parameters	TBM		Non-TBM			Non-meningitic neurological disorder
	Definite TBM (n=18)	Probable TBM (n=57)	Pyogenic meningitis (n=55)	Viral encep (n=17)	Fungal meningitis (n=3)	Control group (n=75)
End-point titer						
1:20-1:40	18	12	51	0	0	75
1:80-1:640	18	45	4	0	0	0
Mean absorbance						
1:80	0.62	0.78	0.23	0.18	0.20	0.14
Sensitity %						
1:80-1:640	100	79	0	0	0	0
Specificity %						
1:80-1:640	-	-	92.8	100	100	100

(1:80 end point titer was taken as a 'cut-off' point for scoring a test CSF as positibe)

patients with pyogenic meningitis gave positive reactions at 1:80 end-point in the ELISA. However 51 out of 55 patients with partially treated pyogenic meningitis had CSF antibody titers between 1:20 and 1:40 and hence the tuberculous aetiology was ruled out in these patients. None of the CSF specimens in patients with viral meningitis and fungal meningitis gave positive reaction at 1:80 in the ELISA and hence no false positive results were seen in these patients.

In all the 18 CSF specimens of patients with 'definite' TBM, the antibody titer in CSF ranged between 1:80-1:640 and the mean absorbance was 0.62 (0.56 ± 1.24). In 45 out of 57 probable cases of TBM, the IgG antibody titer in CSF ranged from 1:80-1:640 and the mean absorbance was 0.78: (0.65 ± 1.26)

M tuberculosis antigen 5 has been widely used in the serodiagnosis of patients with pulmonary tuberculosis (9,38,81). However there is only one published report which emphasised the application of **M tuberculosis** antigen 5 in the immunodiagnosis of TBM. Coovadia et al (26) evaluated the sensitivity and specificity of the ELISA using antigen 5 and they compared the data with other parameters viz. the radioactive bromide partition test and estimation of ADA activity in the CSF specimens of patients with TBM. Their study consisted of 38 children with TBM, 49 children with

non-tuberculous neurological diseases and 14 children with pulmonary tuberculosis with no clinical evidence of meningitis. ELISA in their study was found to be highly specific with antigen 5, but it was relatively less sensitive for the diagnosis of TBM. The sensitivity of the ELISA was found to be only 53% in patients with TBM. Coovadia et al consider that the presence of excess of antigen or antigen-antibody complexes in CSF, may be responsible for the low sensitivity of the assay. They also found antibody titers in CSF reflect neither the severity of the disease nor antibody estimation is useful for monitoring response to the ATT. Coovadia et al have concluded that radioactive bromide partition test possess greater diagnostic value in distinguishing TBM from viral and pyogenic meningitis and that the radioactive bromide partition test carry greater diagnostic significance than ELISA in patients with TBM.

In this study we evaluated the specificity and sensitivity of the ELISA for the detection of IgG antibody to **M tuberculosis** antigen 5, in 57 CSF specimens from culture negative patients with TBM. The results were compared in terms of IgG antibody titers in the CSFs with that of 18 culture positive patients. In 45 out of 57 culture negative patients with TBM, the antibody titers were

1:80 or above and thus the assay showed a sensitivity of 79%. None of the CSFs in patients with non-meningitic neurological diseases had shown a positive reaction in the ELISA at 1:80. However false positive reaction was observed in 4 out of 55 patients with partially treated pyogenic meningitis. The sensitivity of the ELISA in our data was much higher than with those reported by Coovadia et al. In their study, radioactive bromide test was found to have a higher sensitivity and specificity than ELISA. We have had no experience of carrying out radioactive bromide partition test in our laboratory. We consider that this technique can not be performed in routine laboratories in developing countries like India where the laboratory resources and expertise are limited. Secondly this test cannot be used for a routine diagnosis of tuberculosis in every patient with TBM. Thirdly hazards of handling radioactive materials may restrict the utility of this test.

4.1.3. Comparison of CSF geometric mean antibody titer with clinical data of patients with TBM and controls

For the calculation of geometric mean antibody titer, the serial dilution data were normalised by the logarithmic transformation of the data using the formula $X = \log (\text{titer})/10$ where the titer is taken as the reciprocal of the end-point dilution.

Table 4.III shows the comparison of CSF geometric mean antibody titer with the clinical data in patients with TBM and controls. The CSF geometric mean IgG level were significantly higher in patients with TBM than in controls. The geometric mean antibody titer in TBM was 82.4 while it was 4.6 in the control group. An intradermal tuberculin test (Mantoux's test) was considered positive if the reaction diameter was more than 10 mm. Based on this criteria 12 patients in the control group had a mean reactive diameter of 12.2 mm and the geometric mean antibody titer in them was 5.2. In 24 patients in the control group, the mean reactive diameter was 8.2 mm and the geometric mean antibody titer in them was 4.9. Among the 75 patients with TBM, data on intradermal tuberculin test was done only in 50 patients. In 20 patients, the mean reaction diameter was 8.5 mm and the geometric mean antibody titer in them was 84.5. In 30 patients with TBM the mean reaction diameter was 12.6 mm and geometric mean antibody titer in them was 89.6.

The geometric mean antibody titer in 30 patients who received ATT for more than one month, was 88.6 while this was found to be 82.1 in 25 patients with TBM who received the ATT for less than one month. The differences in the geometric mean antibody titer in CSFs were not statistically

TABLE 4.III

Data showing correlation between CSF geometric mean antibody titer and clinical data

Clinical data		Geometric mean antibody titer in patients with TBM	Geometric mean antibody titer in controls
A.	Tuberculin reactor status		
	> 10 mm	89.6	5.2
	< 10 mm	84.6	4.9
B.	Duration of ATT		
	> One month (n=38)	88.6	-
	< One month (n = 25)	82.1	-
C.	Definite cases of TBM (n = 18)	86.5	-
D.	Probable cases of TBM (n = 57)	82.6	-

significant in 'definite' and 'probable' cases of TBM. Thus the geometric mean antibody titers to **M tuberculosis** antigen 5 in CSFs of patients with TBM did not show any correlation between tuberculin reactor status or duration of chemotherapy in patients with TBM. The antibody titers during the course of the therapy remained unaltered in majority of the patients with TBM and in four patients with TBM, the CSF antibody titer to **M tuberculosis** antigen 5 was increased during the ATT.

4.1.4. Results of the detection of antibody to M tuberculosis antigen 5 using Dot-Iba in CSF

The data for the detection of IgG antibody to **M tuberculosis** antigen 5 by the Dot-Iba method are shown in Table 4.IV. In all the patients with 'definite' TBM, the assay gave a positive reaction at titer 1:16. No false negative reaction was seen in any of the 18 patients with 'definite' TBM. Of the 57 patients with 'probable' TBM, the assay yielded positive reaction in 42 patients at 1:16. In the remaining 15 patients the antibody titer ranged between 1:2 and 1:8 In patients with non-TBM and non-meningitic neurological diseases colour reaction was not observed at 1:16. The 'cut-off' point was therefore set at 1:16 as this gave the best discrimination between tuberculous and non-tuberculous subjects. Thus Dot-Iba showed a sensitivity

TABLE - 4.IV

Data showing the results of the DOT-Iba for the detection of IgG antibody against M tuberculosis antigen 5 in CSF

Patient groups	Antibody titer			
	1:2	1:4	1:8	1:16
1. TBM (n = 75)				
Definite TBM (n=18)	18	18	18	18
Probable TBM (n=57)	8	5	2	42
2. Non-TBM (n = 75)				
a) Pyogenic meningitis (n=55)	30	22	3	0
b) Viral encephalitis (n=17)	0	0	0	0
c) Fungal meningitis (n=3)	2	1	0	0
3. Non-meningitic neurological disorders (n=75)	0	0	0	0

of 73.6% and a specificity of 100% for tuberculous aetiology in 'probable' cases of TBM.

The ELISA methods hold definite promise as an aid in the diagnosis of TBM. However several technical difficulties are often encountered in the ELISA. The results of ELISA can be influenced by the variability of the amount of antigen absorbed onto the surface of individual wells in the microtiter ELISA plates. Secondly the ELISA plates show batch to batch differences and may also show "perimeter" effect. Thirdly for the interpretation of the results, an ELISA reader is required. ELISA readers are expensive and many laboratories in developing countries can not afford to equip an ELISA reader. In order to overcome these technical difficulties we developed a simple Dot-Iba for the detection of antimycobacterial antibody in the CSF specimens. The Dot-Iba can be used as a rapid screening test for the diagnosis of TBM and it required only 6h to perform the test. Besides this, Dot-Iba has several advantages over the conventional ELISA method. The NCP has been shown to possess better binding capacity than the polystyrene plates (51). The results of Dot-Iba can be read visually and there is no need for an expensive equipment such as an ELISA reader. More importantly, the NCP can be retained for a retrospective study in patients with TBM.

4.1.5 Results of the demonstration of antibody to M tuberculosis antigen in CSF by Western blot method

The results of western blot method to demonstrate the presence of antimycobacterial antibodies in CSFs of patients with TBM and control groups are shown in Figure 4.I. As it is apparent from the figure that several non-specific antibodies are present in the CSF of patients in the non-TBM group. Careful interpretation of the data revealed that the CSFs of patients with TBM contained a antibody which has specifically reacted with 35 kDa antigen present in the culture filtrates of **M tuberculosis.** This specific antibody conspicuously absent in any of the CSFs belonging to control groups of patients. Thus by a western blot method specific antibody to 35 kDa antigen of **M tuberculosis,** was demonstrated. Therefore western blot method can also be used to distinguish patients with TBM and patients in the control groups.

4.2 Results of antigen based assays

4.2.1 Results of the detection of mycobacterial antigen in the CSF by an inhibition ELISA

The data obtained in an inhibition ELISA for the quantitation of mycobacterial antigen in the CSF specimens of patients with TBM, non-TBM and non-meningitic

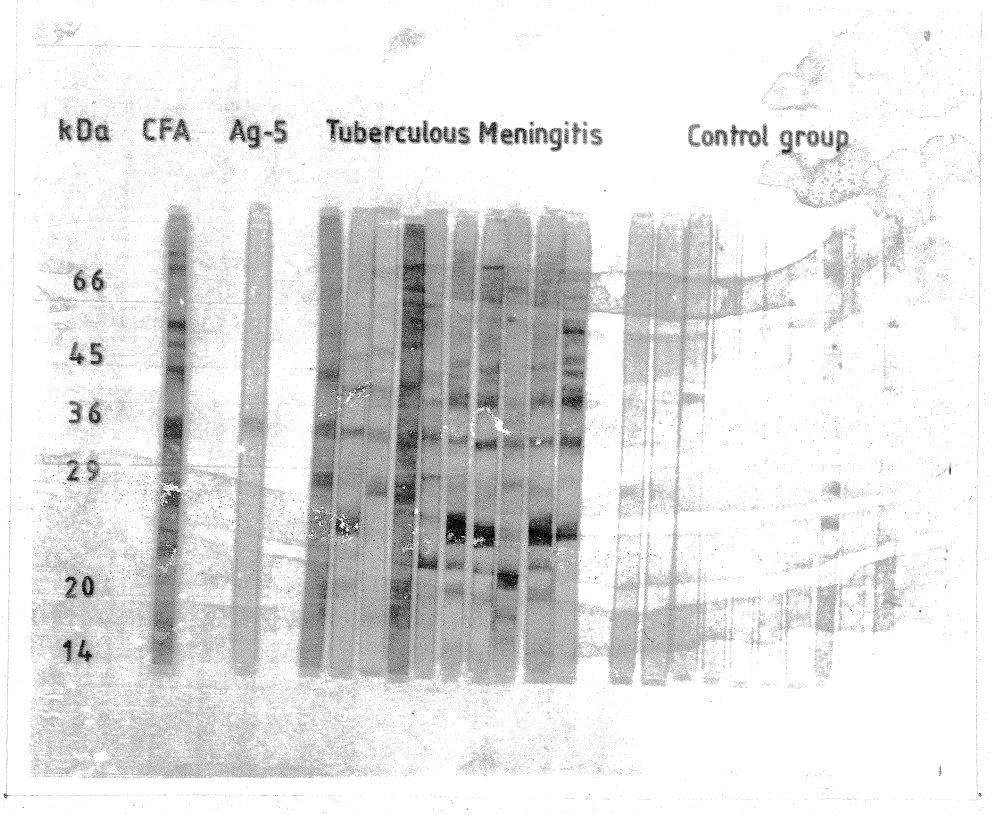


Fig. 4.I Showing the Western blot method to demonstrate the presence of specific antibody in the CSFs of patients with TBM. This antibody reacted with 35 kDa protein present in the CFA. Note the absence of this specific antibody in the CSFs of patients in the control group.

neurological diseases are shown in Table 4.V. In the 75 CSF specimens from patients with non-TBM and 75 CSF specimens from patients with non-meningitic neurological diseases the mean antigen concentration in CSF was 1.45±1.3 ng/ml. A test in the inhibition ELISA was considered positive for tuberculous aetiology if the antigen concentration in CSF was greater than 5.35 ng/ml i.e. mean antigen concentration in the control plus 3 SD. By this criteria none of the CSF, in the control groups of patients was positive for tuberculous aetiology. The CSFs in all the 18 'definite' cases of TBM were positive for tuberculous aetiology and the concentration of mycobacterial antigen in CSFs of patients with 'definite' TBM was in the range of 14-290 ng/ml (mean 158.8±15.4 ng/ml). In the 47 out of 57 'probable' cases of TBM, the mycobacterial antigen concentration in the CSFs was in the range of 15-280 ng/ml (mean 151.6±18.2 ng/ml). In the remaining 10 'probable' cases of TBM, the antigen concentration in the CSF was below 5.35 ng/ml and therefore they were considered to be negative for tuberculous aetiology. Thus inhibition ELISA carried a sensitivity of 100% in 'definite' cases of TBM and 82.4% in 'probable' cases of TBM. More importantly it showed no false positive results in the control groups of patients and thus inhibition ELISA was considered as a specific method for

TABLE - 4.V

Data showing the results of inhibition ELISA for the detection of M tuberculosis antigen in CSF

Parameters	TBM		Non-TBM			Non-meningitic neurological disorder
	Definite TBM (n=18)	Probable TBM (n=57)	Pyogenic meningitis (n=55)	Viral enceph: (n=17)	Fungal meningitis (n=3)	Control group (n=75)
Antigen conc > 5ng/ml	18	47	0	0	0	0
Range of antigen conc (ng/ml)	14-290	15-280	1.2-3.7	1.2-3.7	1.2-3.7	1.2-3.7
Mean antigen conc (ng/ml)	158.8	151.6	2.8	1.45	1.45	1.45
Sensitivity %	100	82.4	0	0	0	0
Specificity %	-	-	100	100	100	100

establishing a tuberculous aetiology in a patient of meningitis due to an unknown aetiology.

4.2.2 Correlation between the antigen concentration in the CSFs and clinical status of 8 patients with TBM during ATT

The mycobacterial antigen concentrations were also measured in 8 patients with TBM in whom CSF specimens were collected for 3 to 4 times during their hospital stay. These patients were receiving ATT at the time of CSF analysis. The antigen concentration in the serial CSF specimens of 8 patients with TBM are shown in Table 4.VI.

The antigen concentration in CSFs showed a gradual decrease between 3 and 4 weeks following the institution of ATT. There was a positive correlation between the clinical recovery and antigen concentration in these 8 patients at the time of their discharge from the hospital. Therefore estimation of mycobacterial antigen in the serial specimens of CSFs in a patient with TBM can be used as a marker to monitor anti-tuberculous chemotherapy. There are only few reports which highlights the estimation of mycobacterial antigen concentration in the serial CSF specimens of patients with TBM during therapy. Kadival et al (60) applied Sandwich ELISA in the retrospective study and

TABLE 4.VI

Data showing the correlation between mycobacterial antigen concentration in CSFs and clinical status in 8 patients with TBM during ATT

S.No.	**M tuberculosis** in CSF specimen	Antigen concentration in the serial CSF specimens (ng/ml)				Outcome, sequelae
		1	2	3	4	
1.	Positive	82.5	32.6	20.5	15.8	alive, right hemiparesis
2.	Positive	38.6	26.5	17.8	14.9	improved, none
3.	Negative	70.9	39.8	19.0	9.5	improved, none
4.	Negative	40.5	31.6	22.5	8.0	improved, none
5.	Negative	158.4	70.5	32.0	-	alive, optic atrophy
6.	Negative	165.5	65.8	28.1	-	improved, none
7.	Positive	62.0	22.4	18.5	11.5	alive, seizure
8.	Negative	180.5	110.5	51.5	-	alive, hydrocephalus

observed a significant difference in the antigen levels in the treated group of patients with TBM. However they did not define the antigen concentration in the serial CSF specimens in individual patients. Owing to the retrospective nature of the study, no correlation between antigen level in CSF and clinical status was made in their study. In our study positive correlation was observed between antigen concentration in serial CSF specimens and clinical status at the time of follow up.

Earlier reports on the detection of mycobacterial antigens in CSF specimens in patients with TBM include latex agglutination test (72), sandwich ELISA (134), reverse passive haemagglutination (21). Chandramuki et al (21) applied a reverse passive haemagglutination method to detect a soluble non-protein mycobacterial antigen in the CSF specimens of patients with TBM. The sensitivity of the assay in their study was 78% and 73% respectively for the culture positive and culture negative patients with TBM. However the assay also gave false positive results in 21% patients with pyogenic meningitis. In a subsequent study Watt et al (134) detected mycobacterial antigen in the CSF specimens by a sandwich ELISA. They evaluated the specificity of the ELISA in non-tuberculous subjects and compared the sensitivity of the ELISA with the direct

demonstration of **M tuberculosis** in patients with TBM. Watt et al also reported false positive reaction in patients with bacterial meningitis. It needs to be emphasised that false positive results in an ELISA will not only lead to an incorrect diagnosis and inappropriate therapy but also can result in the ceasation of any further efforts to investigate these patients for other causes of meningitis. With an inhibition ELISA we have been able to eliminate the false positive results in all the non-tuberculous subjects. There are paucity of reports about the use of inhibition ELISA for the detection mycobacterial antigen in the CSFs of patients with TBM. Bal et al (8) used an inhibition ELISA in their study and they observed maximum saturation of inhibitory capacity of antigen at 25ng/ml and that the assay cannot estimate the antigen concentration if CSF contained more than 25ng/ml. There are subtle differences in the methodology in this study than that of Bal et al. By the application of two antibodies in the assay (primary antibody to **M tuberculosis** and antirabbit IgG-biotin conjugate) saturation of inhibitory capacity of antigen in the standardisation ELISA was enhanced and that the assay can estimate the antigen concentration up to 1000ng/ml. Arti Ramkisson (102) used a competition inhibition ELISA for the detection of mycobacterial antigen. In 59 CSFs from patients with the bacterial, viral infection as well as TBM,

the antigen was present in significant concentration only in the CSFs of patients with TBM than in other group of patients. They also emphasised the utility of the competitive ELISA for the laboratory diagnosis of TBM.

4.2.3 Detection of mycobacterial antigen in CSF by Western blot method

The mycobacterial antigens present in the CSFs of patients with TBM when treated with the rabbit antibody to **M tuberculosis**, showed two bands in NCP. The molecular weights of these antigens are 52 kDa and 27 kDa of which 27 kDa (Figure 4.II). These two antigens were present in all the 18 'definite' cases of TBM and 47 out of 57 'probable' cases of TBM. These two mycobacterial antigens were absent in 75 patients with non-TBM and 75 patients with non-meningitic neurological diseases.

There is one earlier report by Wadee et al (132) who studied the body fluids viz. CSF, ascitic fluid, and pleural fluids by SDS-PAGE. The western blots method detected the presence of 43 kDa of **M tuberculosis** in tuberculous subjects and such an antigen was absent in the body fluids of patients with non-tuberculous diseases.

In our study we were able to detect two mycobacterial antigens in the CSF patients with TBM. By the

application of two antibodies in the assay viz. - rabbit antibody to **M tuberculosis** and antirabbit IgG-biotin conjugates, the sensitivity of the assay could be increased to 100% in 'definite' patients with TBM and 82.4% in probable cases with TBM.

We could not detect any band of 35 kDa in the CSF specimens of patients with TBM. One of the reasons may be that 35 kDa porotein is present in very small quantities in CSFs of these patients and therefore Western blot method is unable to detect 35 kDa band. However we did find two bands in the region of 52 kDa and 27 kDa in CSFs of patient with TBM which were absent in the CSFs of patients in the control group. One possible explanation could be that these proteins are related to the leukocyte enzyme-alkaline phosphatase. Since a substrate for alkaline phosphatase was used in the procedure these bands figure out prominently in NCP. Moreover a Western blot method cannot be routinely applied in every CSF sample for the demonstration of mycobacterial antigen in the CSFs. We suggest that further studies is necessary to characterise the precise nature of the two bands in the NCPs.

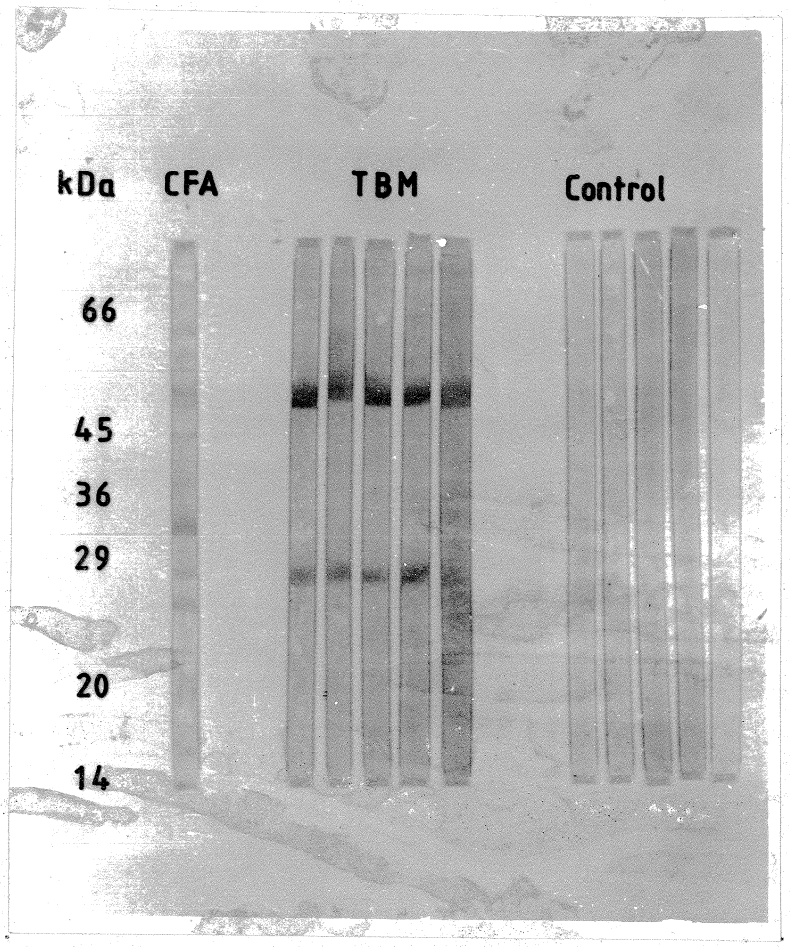


Fig. 4.II Showing the Western blot method to demonstrate the presence of 52 kDa and 27 kDa mycobacterial antigens in the CSFs of patients with TBM. Note the absence of these two mycobacterial antigens in the CSFs of control group.

4.3 Comparison between the results of bacteriological and indirect ELISA methods

4.3.1 Correlation between the results of bacteriological method (culture) to detect M tuberculosis and an indirect ELISA method to detect antibody to M tuberculosis antigen 5 in CSFs of patients with TBM.

A retrospective study was made of the correlation between the culture of **M tuberculosis** and the detection of IgG antibody to **M tuberculosis** antigen 5 in the CSFs by means of an ELISA. **M tuberculosis** was cultured from the CSFs in 18 out of 75 patients with a clinical diagnosis of TBM, IgG antibody to **M tuberculosis** antigen 5 was demonstrated in significant titers in all the culture positive cases of TBM. Thus a positive correlation was observed between culture of **M tuberculosis** and detection of IgG antibody in the CSFs in all the 'definite' cases of TBM.

Table 4.VII shows the correlation between the results of bacteriological method for **M tuberculosis** and IgG antibody to antigen 5 in the CSFs by an indirect ELISA. **M tuberculosis** was isolated in 18 patients of TBM in their initial lumbar CSFs. The cultures were reported to be negative in the subsequent CSFs collected at different time interval in a same patient. On the other hand IgG antibody

TABLE 4.VII

Data showing the comparison between the results of bacteriological method and indirect ELISA in CSF of patients with TBM

Parameters	Bacteriological method	Indirect ELISA
No of CSFs tested	75	75
No of CSFs with positive results	18	63
No of CSFs with positive culture and positive ELISA	18	18
No of CSFs with negative culture and positive ELISA	57	63
No of CSFs with negative culture and negative ELISA	57	12
Duration of the test	6 wks	24 h
Sensitivity %	24	84

to **M tuberculosis** antigen 5 was present in significant titers in all the CSF specimens collected at different time intervals in the same patient with TBM. The sensitivity of the culture technique in patients with TBM was only 24% while the sensitivity of an indirect ELISA method was 84%. Culture methods usually required 6-8 weeks to grow **M tuberculosis**, while an indirect ELISA method required only 24h to predict a tuberculous aetiology in CSFs of patients with a clinical suspicion of TBM. More importantly the indirect ELISA gave positive results in the CSFs of patients in whom **M tuberculosis** was isolated and no false negative results were observed in ELISA of culture positive patients with TBM.

4.3.2 Correlation between the bacteriological methods to detect M tuberculosis and an indirect ELISA to detect antibody to M tuberculosis antigen 5 in lumbar, cisternal and ventricular CSFs in patients with TBM.

The data for the detection of AFB in Z-N stained CSF smears, culture of **M tuberculosis** and IgG antibody to **M tuberculosis** antigen 5 in lumbar, cisternal and ventricular CSFs are shown in Table 4.VIII. It is apparent that lumbar CSF did not demonstrate AFB in any CSFs and positive cultures for **M tuberculosis** were obtained only in 2 out of 8 CSFs of patients with TBM. On the other hand

TABLE 4.VIII

Data showing the bacteriological method to detect M tuberculosis and indirect ELISA method to detect antibody to M tuberculosis antigen 5 in CSFs of patients with TBM

Material	Bacteriological method		Indirect ELISA
CSF	Z-N stain	Culture	Geometric mean antibody titer
Lumbar CSF (n=8)	0/8 (0%)	2/8 (25%)	96.5 (100%)
Cisternal CSF (n = 8)	6/8 (75%)	7/8 (87.5%)	104.8 (100%)
Ventricular CSF (n=8)	4/8 (50%)	6/8 (75.0%)	97.2 (100%)

Figures in parentheses represent the percentage of results

cisternal CSF was positive for AFB in 6 patients and positive cultures were obtained in 7 out of 8 patients. In ventricular CSF, AFB was seen in four patients and 6 had positive culture for **M tuberculosis**. The cisternal and ventricular CSFs were subjected to culture and they showed 3 to 4 discrete colonies in L-J slopes while lumbar CSF showed one colony. These bacteriological data suggests that lumbar CSF contain low density of tubercle bacilli than cisternal CSF in patients with TBM. The geometric mean IgG antibody titer to **M tuberculosis** antigen 5 in lumbar, cisternal and ventricular CSF were 96.5, 104.8 and 97.2 respectively and are not statistically different from one another ($p > 0.05$).

In our study **M tuberculosis** was isolated only in 24% of patients with TBM. In the published reports (96,129) the isolation rates of **M tuberculosis** in CSFs of patients with TBM ranged between 15-20%. Several reasons may account for the low isolation rates of **M tuberculosis** in patients with TBM. CSF specimens in these patients are usually obtained by the lumbar route and the lumbar CSF contains a lower concentration of **M tuberculosis** than in cisternal CSF. Secondly tubercle bacilli are embedded in the dense exudate in the basal cisterns and leptomeninges. These exudates forms a barrier for tubercle bacilli to circulate in the lumbar CSF. Thirdly and perhaps more importantly, most of

the patients with TBM received a course of ATT before they are referred to a specialised centre for neurological disease. Lumbar CSF in partially treated patients with TBM seldom contain AFB and hence smears and cultures are invariably negative for **M tuberculosis.** Though positive cultures were more frequently obtained in cisternal CSF than lumbar CSF, for a routine bacteriological diagnosis, CSFs from either cisternal or ventricualar routes cannot be obtained in vast majority of patients with TBM. However the IgG antibody titer in lumbar, cisternal and ventricular CSF are present in significant concentrations and the antibody titers in lumbar, cisternal and ventricular CSFs are not statistically different from one another. In contrast to the low isolation rates of **M tuberculosis,** antibody to **M tuberculosis** antigen 5 in lumbar CSF is present in patients with TBM. Therefore we suggest that the lumbar CSF in a patient with suspected TBM should be simultaneously submitted for culture and ELISA tests. This will be useful for the early laboratory diagnosis of TBM.

4.4 Antibodies to M tuberculosis in Serum

4.4.1. Data showing the concentration of albumin and IgG in serum and CSF. CSF:IgG index; and mycobacterial antibody-index in patients with TBM, non-TBM and non meningitic neurological disorders

Table 4.IX shows the data on the serum albumin, IgG and CSF albumin, IgG levels in patients with TBM as well as

TABLE - 4.XX

Data showing the concentration of albumin and IgG in the sera and CSFs; CSF-IgG index and mycobacterial antibody index

Parameters	TBM	Non-TBM	Non-meningitic neurological disorders	Normal range
No of specimens	75	75	75	-
Serum albumin (mg%)	3898 ± 776	3747 ± 730	3870 ± 783	3200-4500
Serum IgG (mg%)	1879 ± 493	1711 ± 324	1609 ± 470	800-1600
CSF albumin (mg%)	107 ± 98	58 ± 55	27 ± 13.7	10-40
CSF IgG (mg%)	49 ± 6.2	20 ± 2.4	6 ± 5.6	2-4
CSF-IgG index	0.83 ± 0.43	0.75 ± 0.40	0.21 ± 0.19	0.1-0.6
Mycobacterial antibody index	40 ± 3.8	2.23 ± 0.15	0.18 ± 0.1	0.1 - 0.5

p value of CSF albumin between TBM and non-TBM (p .>0.05)

p value of CSF IgG between TBM and non-TBM ($p < 0.05$)

p value of CSF IgG index between TBM and non-TBM ($p > 0.05$)

p value of mycobacterial antibody index between TBM and non-TBM ($p < 0.001$)

in the control groups. The IgG levels in patients with TBM were higher than the IgG levels in the non-TBM group. This may be due to the intrathecal synthesis of immunoglobulins by lymphocytes present in the meningeal exudates. In order to ascertain whether the IgG antibodies are produced intrathecally in patients with TBM, we calcualted CSF-IgG as well as mycobacterial antibody indices. The mycobacterial antibody index was significantly increased in patients with TBM than the patients in the control group. The CSF-IgG index was higher in patients with TBM as well as in some patients with chronic meningitis. An increase in the mycobacterial antibody index suggest, a intrathecal synthesis of IgG antibody against mycobacterial antigen. Therefore it is unlikely that, passive transfer of serum IgG antibody to **M tuberculosis** into CSF takes place in patients with TBM. In order to strengthen this observation further, patients with pulmonary tuberculosis without meningitis were studied and they showed low mycobacterial antibody index (128). Kalish et al (62) also observed high level of binding of IgG antibody to PPD in CSF, when simultaneous serum levels were low in their patients. They also found elevated CSF-IgG as well as anti-PPD indices in their patients with TBM.

4.4.2. Comparative analysis of the results of an indirect ELISA for antibody to PPD and M tuberculosis antigen 5 in CSFs and sera of patients with TBM and non-meningitic neurological disorders

Table 4.X shows the comparative analysis of the results of indirect ELISA for IgG antibody to PPD and **M tuberculosis** antigen 5 in CSFs and sera of patients with TBM as well as patients in the control group. Our data on the comparative analysis of IgG antibody to PPD and **M tuberculosis** antigen 5 in sera and CSFs of patients with TBM substantiate the studies of Kalish et al. In our data 84% of patients with TBM showed IgG antibody to antigen 5 in their CSFs while only 40% of patients with TBM showed antibody in their sera. None of the patients with non-meningitic neurological diseases showed IgG antibody to antigen 5 in their CSFs. In non-TBM group 12% patients showed IgG antibody to PPD and 5.3% of patients demonstrated IgG antibody to **M tuberculosis** antigen 5 in their CSFs, suggesting the presence of cross reacting IgG antibodies in CSFs of patients with non-TBM.

The IgG antibody titer to PPD and **M tuberculosis** antigen 5 in the sera of patients with non-TBM, non-meningitic neurological disorders and healthy controls

TABLE - 4.X

Data showing the comparative analysis of the results of indirect ELISA for IgG antibody to PPD and M tuberculosis antigen 5 in CSFs and sera

End-point - titer	PPD		**M tuberculosis** antigen - 5	
	CSF	Serum	CSF	Serum
	1:160	1:1000	1:80	1:1000
TBM	63/75 (84%)	30/75 (40%)	63/75(84%)	30/75 (40%)
Non-TBM	9/75 (12%)	16/75 (21.3%)	4/75 (5.3%)	15/75 (20%)
Non-meningitic neurological disorders	0/75 (0%)	14/75 (18.6%)	0/75 (0%)	12/75 (16%)
Healthy controls	-	9/50 (18%)	-	7/50 (14%)

Figures in parentheses represent the percentage of results

were more or less equal suggesting either sensitisation of the population with BCH vaccination, or previous exposure to tuberculous infection (125).

4.4.3 Results of indirect ELISA for the antibody to PPD and M tuberculosis antigen 5 in the sera of patients with pulmonary tuberculosis and leprosy and healthy controls

Table 4.XI shows the results of indirect ELISA for serum IgG antibody to PPD and **M tuberculosis** antigen 5 in patients with pulmonary tuberculosis and leprosy. At a dilution end-point 1:1000, 100% positivity was observed in the sera of patients with pulmonary tuberculosis to PPD and **M tuberculosis** antigen 5. In the healthy individuals, the serum antibody to antigen 5 was present in 18% with the PPD antigen and 14% with **M tuberculosis** antigen 5. In leprosy, the serum antibody was positive in 30% each with PPD and **M tuberculosis** antigen 5.

Daniel et al (38) carried ELISA for IgG antibody to PPD and **M tuberculosis** antigen 5 for the serodiagnosis of tuberculosis in 41 patients with active tuberculosis, 19 patients with inactive tuberculosis and 59 healthy control subjects. They observed, with both antigens, sera from persons with inactive tuberculosis showed titers which did not differ significantly from those of the control subjects.

TABLE - 4.XI

Data showing the results of indirect ELISA for the detection of antibody to PPD and M tuberculosis antigen 5 in sera at 1:1000 dilution end-point

Patients	PPD	**M tuberculosis** Antigen-5
Pulmonary tuberculosis	50/50 (100%)	50/50 (100%)
Leprosy	15/50 (30%)	15/50 (30%)
Healthy controls	9/50 (18%)	7/50 (14%)

Figures in parentheses represent the percentage of results.

Patients with active tuberculosis showed high antibody titers than patients with inactive tuberculosis. This difference was greater with antigen 5 than with PPD. Daniel et al concluded that ELISA with **M tuberculosis** antigen 5 provides a diagnostic test which is more sensitive and specific than ELISA with PPD. In our study, 100% positivity for antibody to PPD and **M tuberculosis** antigen 5 was obtained in patients with pulmonary tuberculosis, because all these patients were in the active stages of the disease. 30% of positivity was obtained in the sera of patients with leprosy which indicates that IgG antibody in the sera of patients with leprosy cross-react with antigen 5 in the ELISA.

4.5 Isolation and characterisation of CICs in the CSFs of patients with TBM and control groups

Table 4.XII shows the data of **M tuberculosis** antigen 5 in the CICs of patients with TBM and non-TBM groups. The mean absorbance obtained in the ELISA (at 1: 80 end-point) for antigen 5 in patients with non-TBM was 0.195±0.11. A test was considered positive for the presence of antigen 5 in the CICs if the absorbance was more than 0.415 (mean absorbance in control + 2SD). By this criteria 12 out of 42 patients with TBM showed the presence of antigen 5 in their CICs. 10 of these patients received ATT for less than one week while 30 patients with TBM received

TABLE 4.XII

Data showing M tuberculosis antigen 5 and antimycobacterial antibody in CICs of patients with TBM and controls

Patient group	*Sandwich ELISA-Mean absorbance at		**Indirect ELISA Mean absorbence at	
	1 : 40	1 : 80	1 : 40	1 : 80
TBM Group (n = 42)				
< 1 week therapy (n=12)	0.68 ± 0.09	0.62 ± 0.13(n=10)	0.81 ± 0.10	0.79 ± 0.13(n=10)
> 2 weeks therapy (n=30)	0.43 ± 0.06	0.38 ± 0.12(n=2)	0.84 ± 0.21	0.76 ± 0.11(n=18)
Non-TBM Group (n=40)				
Stroke (n=8)	0.36 ± 0.08	0.22 ± 0.12(n=0)	0.51 ± 0.13	0.45 ± 0.08(n=0)
Pyogenic meningitis(n=18)	0.49 ± 0.11	0.20 ± 0.14(n=0)	0.62 ± 0.09	0.49 ± 0.12(n=0)
Cryptococcalmeningitis(n=2)	0.42 ± 0.13	0.17 ± 0.11(n=0)	0.61 ± 0.08	0.38 ± 0.16(n=0)
Viral encephalitis (n=12)	0.48 ± 0.18	0.18 ± 0.01(n=0)	0.44 ± 0.07	0.48 ± 0.14(n=0)

* cut-off value 0.415 at 1:80
** cut-off value 0.706 at 1:80 for scoring a test CSF as positive

ATT for 2-4 weeks. Out of these 30 patients, antigen 5 in the CICs was present only in two patients.

The antibody to **M tuberculosis** in the CICs was demonstrated in 28 out of 42 patients with TBM. In the non-tuberculous subjects the antimycobacterial antibody titer ranged between 1:20-1:40. But at a 'cut-off' point 1:80, none of the CSFs in non-TBM group were positive for the presence of antibody to **M tuberculosis** antigen 5 in their CIC.

In order to evaluate the significance of CICs in patients of TBM, a study was undertaken to isolate and characterise the CICs in the CSF specimens. Secondly role of CICs in the pathogenesis of TBM was also evaluated. The CSFs of 30% of patients with TBM, demonstrated the presence of mycobacterial antigen in the CSFs and the antigen concentration in the CICs decreased during the ATT. In 70% patients with TBM, CICs are not formed because of the nonavailability of mycobacterial antigen in optimal quantitities for the formation of immune complexes because the immune-complexes are formed by the free antigen and antibody in CSF specimen. The sera of patients with TBM did not contain immunne-complexes and therefore it is unlikely that passive transfer of immune complexes from serum to CSF takes place in patients with TBM. The CICs particularly in

CSFs of untreated patients with TBM are likely to be formed in high quantities and this can result in the deposition of immune complexes around the wall of major intracranial arteries. This will lead to the neurological complications like hemiplegia, seizures and brain-stem dysfunctions. All these complications are known to occur in patients with TBM. The results of this study also emphasise that ATT should be initiated in the early stages of the TBM so that CICs are not formed during the course of the disease and thus occurrence of neurological complications of patients with TBM can be prevented.

4.6 The results of the demonstration of mycobacterial antigen in intracranial tuberculoma by an immunohistochemical method.

The paraffin sections of all the intracranial tuberculoma were initially examined under the haemetoxyline and eosin stains. The salient histopathological features were characterised by the presence of Langhan's giant cells, epithelioid cells and lymphomononuclear cells (Figure 4.III).

Table 4-XIII shows the data of the immunohistochemical method for the demonstration of mycobacterial antigen in ten intracranial tuberculous granulomas. The results of the

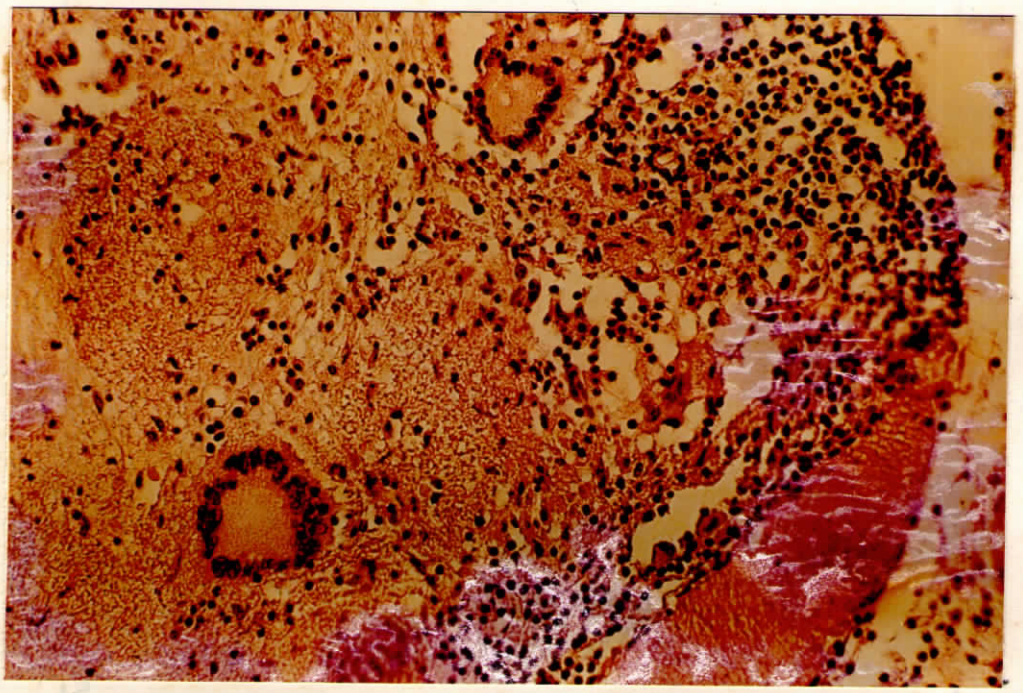


Fig. 4.III An intracranial granulomatous lesion consisting of Langhan's type of giant cells, epithelioid cells and lymphomononuclear cells (Haematoxyline and eosin x 160).

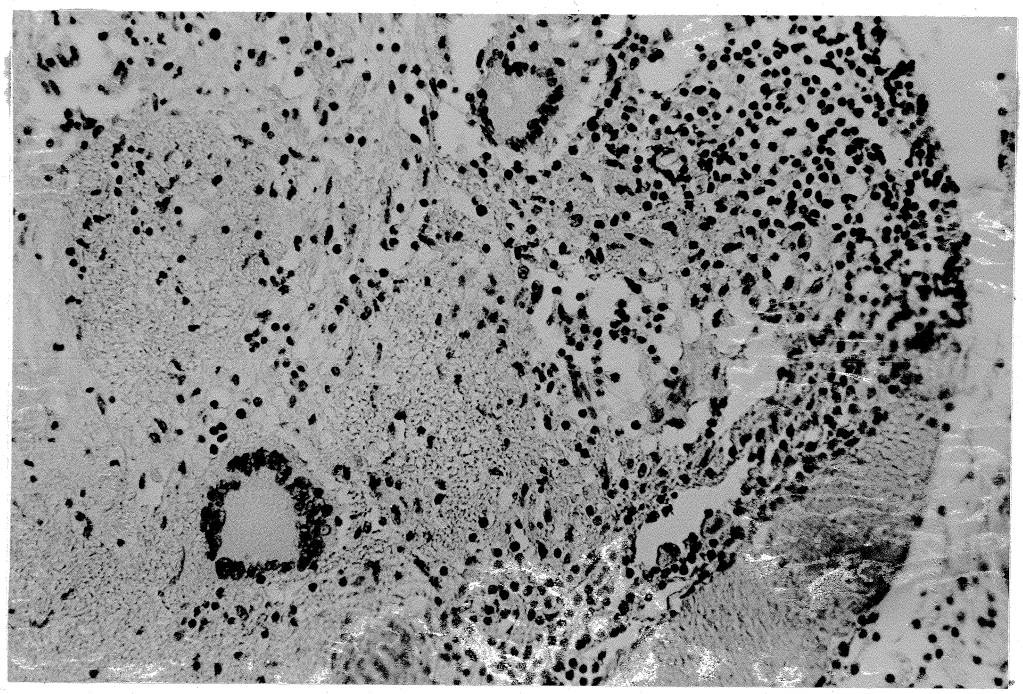


Fig. 4.III An intracranial granulomatous lesion consisting of Langhan's type of giant cells, epithelioid cells and lymphomononuclear cells (Haematoxyline and eosin x 160).

TABLE - 4.XIII

Data on immunohistochemical technique and bacteriological methods in intracranial tuberculoma

Case No	Anatomical location of tuberculoma	Mycobacterial antigen		Z-N stain for AFB	Culture for **M tuberculosis**
		In caseous zone	In non-caseous zone		
1.	Posterior fossa (intracerebellar)	++	++	-	+
2.	Intraventricular (Lt. lateral ventricle)	+	++	-	-
3.	Rt. Occipital	++	++	-	-
4.	Rt. frontal	+	++	-	-
5.	Rt. posterior frontal	+	++	-	-
6.	Posterior fossa mass	++	+	-	+
7.	Posterior fossa mass	+	++	-	-
8.	Intrapontine	+	+	-	-
9.	Rt. tentorium	+	+	-	-
10.	Posterior fossa	+	++	-	-

immunohistochemical methods was compared with Z-N staining method in the paraffin sections of tuberculoma.

In the Z-N stained paraffin sections, AFB were not demonstrated in any of the intracranial tuberculoma while mycobacterial antigen was demonstrated in the paraffin section of all the ten cases of tuberculoma. There were two types of distribution of mycobacterial antigen in tuberculous lesions. In the non-caseating zones, the mycobacterial antigen was characteristically seen within the cytoplasm of the macrophages, and Langhan's giant cells. The mycobacterial antigen appeared as diffuse staining granular brownish-pink material (Figure 4.IV). The second type of distribution of mycobacterial antigen was observed in the caseous zones in which clusters of mycobacterial antigen staining masses contrasted sharply with remainder of amorphous necrotic debris (Figure 4.V). In both caseous and non-caseous zones, characteristic size and shape of the AFB were not identified. This could be due to the phagocytosis of the bacilli within the granuloma. In none of the non-tuberculous (fungal) granulomatous lesions, characteristic intracytoplasmic localisation of mycobacterial antigen was present (Figure 4.VI). The fungal granulomas were selected as a controls and they resembled histopathological features of a tuberculous granuloma. Thus

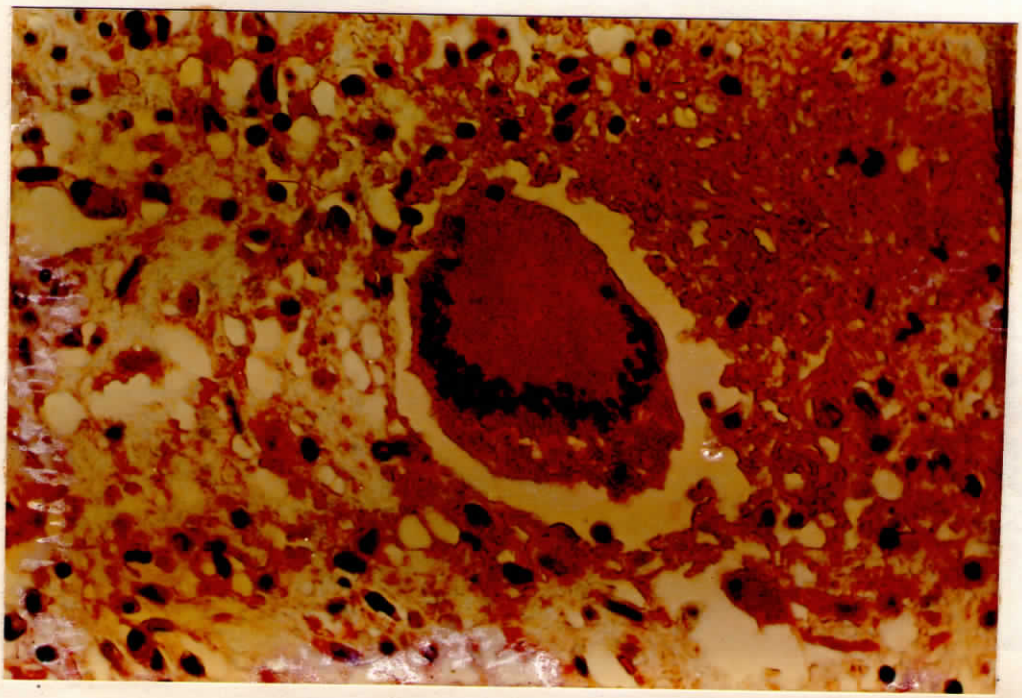


Fig. 4.IV An immunohistochemical method to demonstrate the presence of granular, pinkish-brown material within the cytoplasm of Langhan's type of giant cells in an intracranial tuberculous granuloma (Peroxidase-antiperoxidase method x 160).

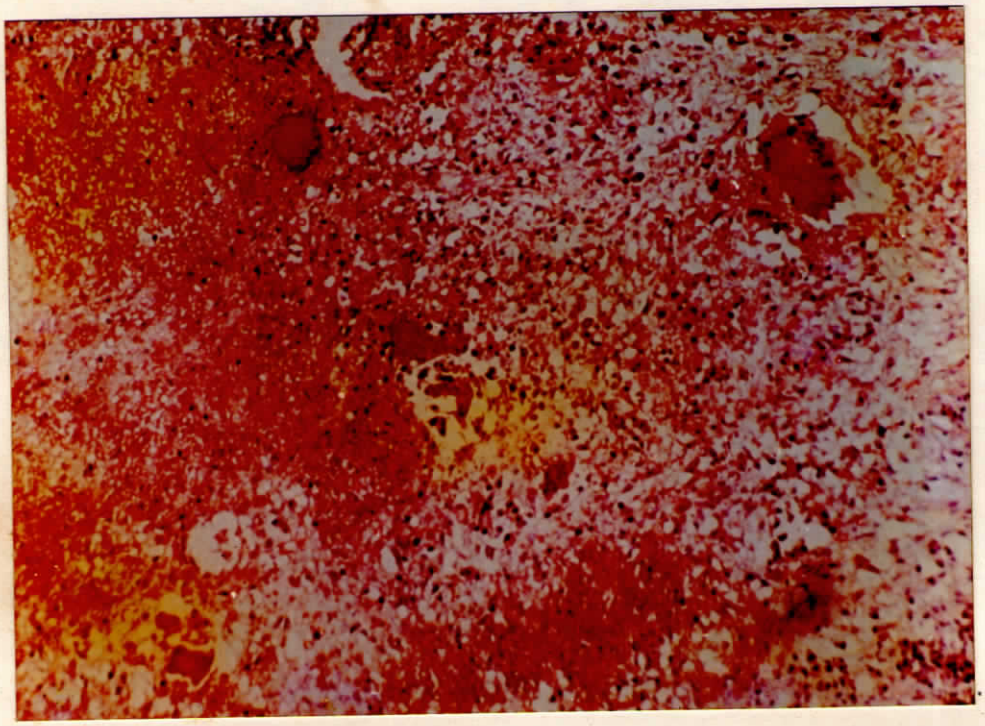


Fig. 4.V Photomicrograph showing clusters of mycobacterial antigen staining masses which are demarcated from the caseous zone in an intracranial tuberculous granuloma (Peroxidase - antiperoxidase method x 160).

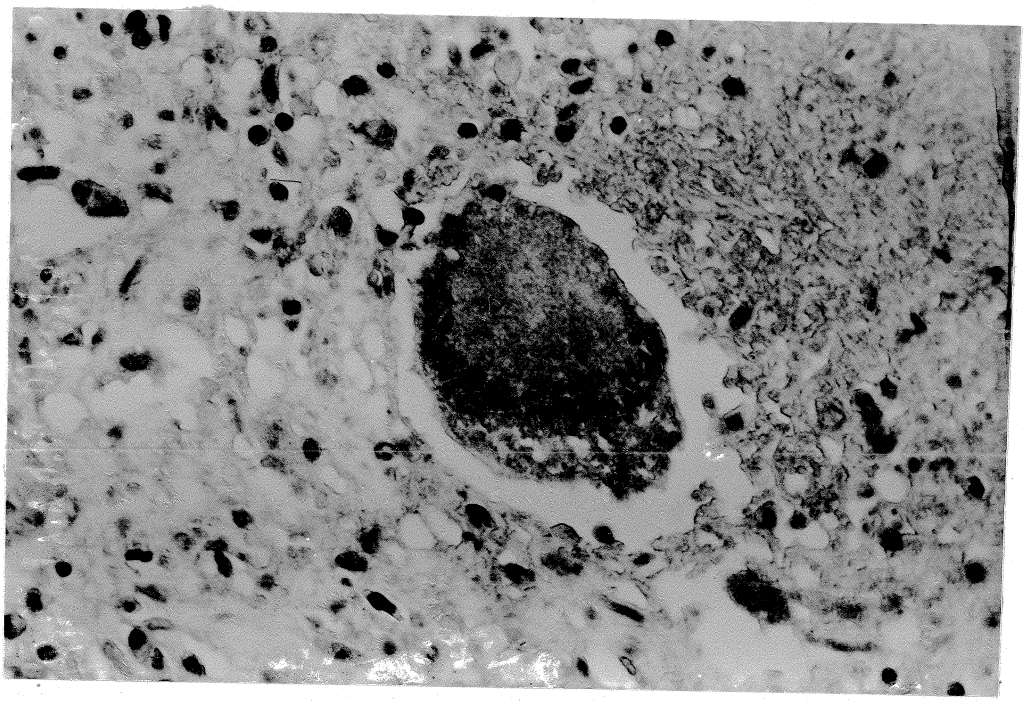


Fig. 4.IV An immunohistochemical method to demonstrate the presence of granular, pinkish-brown material within the cytoplasm of Langhan's type of giant cells in an intracranial tuberculous granuloma (Peroxidase-antiperoxidase method x 160).

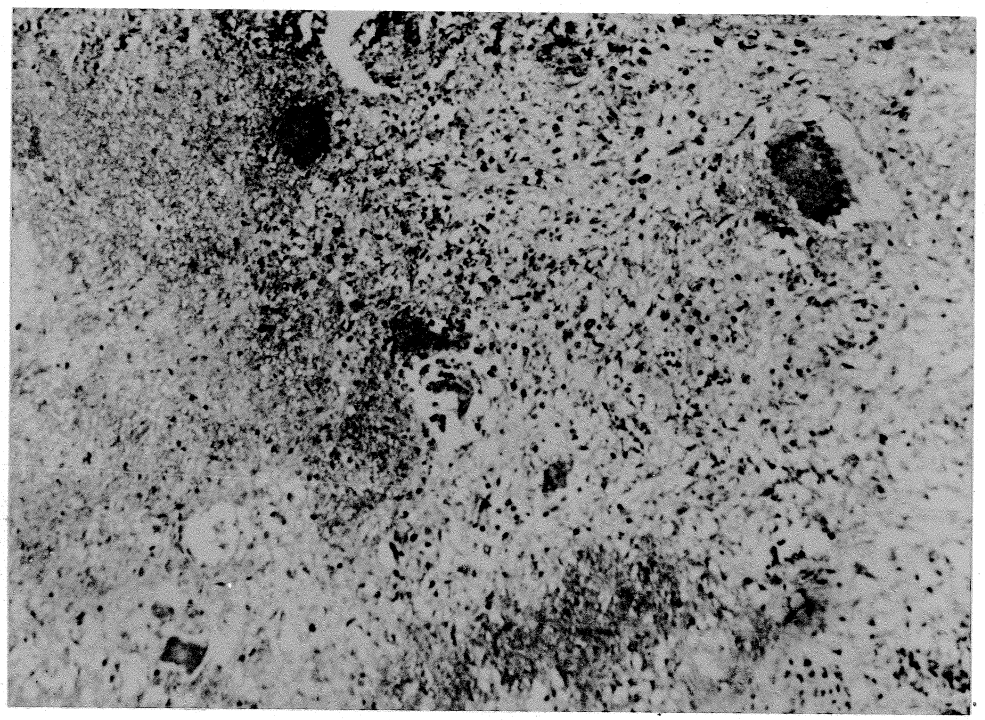


Fig. 4.V Photomicrograph showing clusters of mycobacterial antigen staining masses which are demarcated from the caseous zone in an intracranial tuberculous granuloma (Peroxidase - antiperoxidase method x 160).

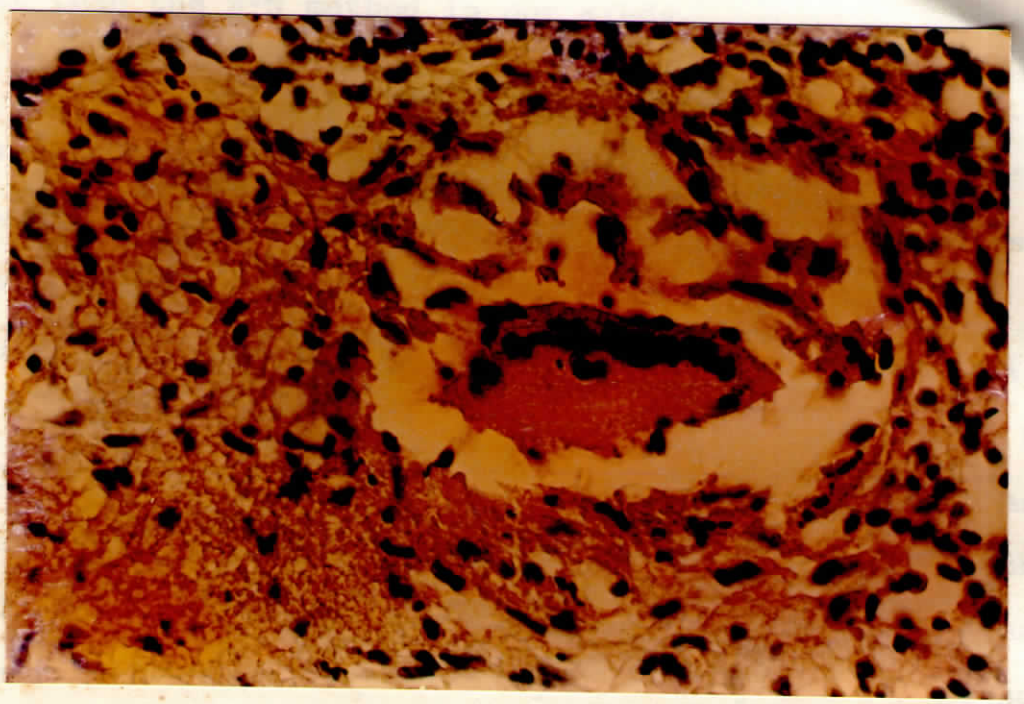


Fig. 4.VI Photomicrograph showing a fungal granuloma simulating a tuberculous granuloma. Note the absence of pinkish-brown material in the cytoplasm of the giant cells (Peroxidase antiperoxidase method x 160).

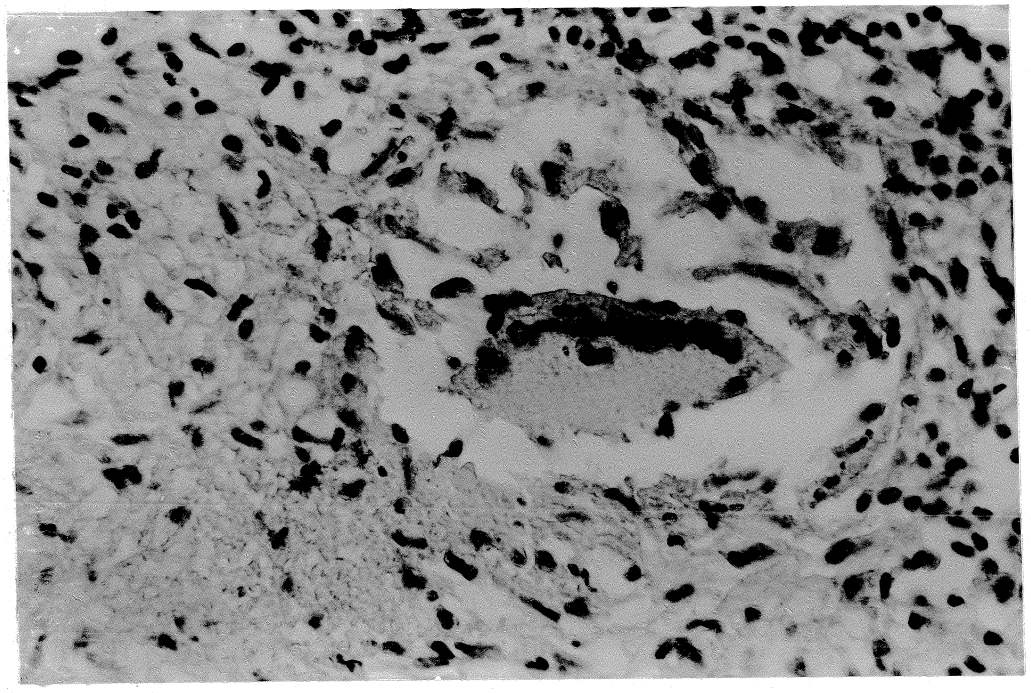


Fig. 4.VI Photomicrograph showing a fungal granuloma simulating a tuberculous granuloma. Note the absence of pinkish-brown material in the cytoplasm of the giant cells (Peroxidase antiperoxidase method x 160).

immunohistochemical methods carried not only a high degree of specificity but also carried a sensitivity that was greater than the Z-N method in our study.

Intracranial tuberculomas are one of the common neurosurgical specimens that are sent for the histopathological examination. The diagnosis of tuberculosis is usually made by the salient histopathologial features in the paraffin sections which include foci of caseation necrosis and formation of a granuloma, composed by epithelioid cells, Langhan's giant-cells and lymphomononuclear cells. AFB are seldom found in the paraffin sections of tuberculoma. Two important reasons may be ascribed for the low positivity of AFB by the Z-N method in the paraffin sections of cases due to tuberculous granuloma. Firstly, majority of the patients with tuberculosis received ATT prior to the surgery and as a result of which number of the viable tubercle bacilli are few in the tuberculous lesions. Secondly the AFB are engulfed and phagocytosed by the macrophages in a tuberculous granuloma. As a result of which morphology of the bacilli becomes distorted and cannot be identified under a microscope. In the Z-N preparation altered morphology of the bacilli are not identified and thus ~~often~~ false negative results are often reported in the Z-N stained paraffin

sections of tuberculous granuloma. It is important to note that caseating granulomas are also known to occur with non-tuberculous infections of CNS particularly due to the fungal infections. It is therefore essential to distinguish a caseating intracranial granulomatous lesion due to tuberculous infection from that of a fungal infection. This distinction is necessary for the further management and therapy in the patients. In the absence of a reliable bacteriological confirmation of tuberculosis, an immunohistochemical method was applied in this study to achieve the above objective. Polyvalent rabbit antibody to **M tuberculosis** was used as a primary antibody. The linking and labelling antibodies were swine antirabbit IgG and rabbit peroxidase-antiperoxidase complexes respectively. By the conventional immunohistochemical method, mycobacterial antigen was demonstrated in all the cases of intracranial tuberculoma. The mycobacterial antigen was characteristically seen within the cytoplasm of macrophages and giant cells. In granulomatous lesions due to fungi and parasites, mycobacterial antigens were not seen within the cytoplasm of the giant cells.

Humphrey et al (55) used a rabbit polyclonal antibody to **M tuberculosis** in their immunohistochemical analysis of 59 specimens of pulmonary tuberculosis and they emphasised

usefulness of this technique for establishing the specific mycobacterial aetiology of a ceseating pulmonary granuloma. In a control of 50 patients with non-mycobacterial diseases, mycobacterial antigens were not demonstrated. The immunohistochemical staining method may at times give positive reaction in lesions caused by atypical mycobacteria because the antigens in atypical mycobacteria may react with polyvalent antiserum against **M tuberculosis.** Using a Dot-Iba, Barbolini et al (10) evaluated the specificity of MAbs against the CFA from 7 different atypical mycobacterial strains. However, none of these MAbs were found to be specific or unique for **M tuberulosis.**

Immunohistochemical technique can therefore be applied in the routine laboratory diagnosis of intracranial tuberculosis granuloma because it is more sensitive than a conventional Z-N method and secondly this technique will distinguish tuberculous from a non-tuberculous infection of CNS. The technical aspect of an immunohistochemical method is simple for a routine application in the laboratory. The immunological reagents are stable at $+4^{o}C$ and can be used for at least six months.

SUMMARY AND CONCLUSIONS

CHAPTER V

5.1 Restatement of problem

TBM is one of the common infectious diseases of the CNS among hospitalised patients in our country. A hospital statistics of this referral institution for neurological diseases indicates that TBM constitute 37% of all infectious diseases of CNS. TBM is a potentially curable disease and a complete neurological recovery in patients with TBM entirely depends upon an early diagnosis and prompt institution of ATT. Any delay in making the diagnosis of TBM and delay in the commencement of ATT will result in the neurological complications as well as sequelae of the disease. The **'gold standard'** applied for the laboratory diagnosis of TBM is the demonstration of the causative agent of the disease **M tuberculosis** in CSF specimens by the bacteriological methods. In this study despite, the best possible laboratory and technical efforts **M tuberculosis** was isolated only in 24% of patients who were clinically diagnosed as TBM. In the remaining 76% patients with TBM, CSFs were analysed on more than one occasions and **M tuberculosis** was not demonstrated by the bacteriological methods. Besides the lower sensitivity, the bacteriological

methods are cumbersome, time consuming and require at least 6-8 weeks to grow the tubercle bacilli. Thus culture method is not useful in establishing an early laboratory diagnosis of TBM. Because of the delay in the diagnosis as well as the delay in the institution of appropriate ATT drugs, a significant percentage of patients with TBM manifest neurological complication as well as a sequelae of the disease. These factors may account for the high mortality and morbidity rates that are currently prevalent in patients with TBM. Thus alternate methods other than bacteriological method are essential for the laboratory diagnosis of TBM, so that an early laboratory diagnosis of TBM can be established in patients with TBM.

5.2 Description of the procedures and major findings

In order to achieve the above objective we have established an ELISA as well as western blot methods in our laboratory to detect the specific antibody to **M tuberculosis** in CSF specimens as well as an inhibition ELISA to measure the concentration of mycobacterial antigen in CSF specimens of patients with TBM. The sensitivity of both ELISA methods were critically evaluated in all the culture positive cases of TBM and the specificity of the ELISA was also evaluated in larger samples of patients with non-tuberculous neurological diseases.

In this study both the indirect and inhibition ELISAs gave positive results in all the 18 culture positive cases of TBM and thus an excellent correlation was observed between the results of ELISA and culture methods. The ELISAs did not yield false negative result in any one of the culture positive patients with TBM. This observation strengthen the possibility that ELISA can be used in the laboratory diagnosis of TBM. While the culture methods required 6-8 weeks time, the ELISA method required only 48 hours to predict a tuberculous aetiology in patients of meningitis due to unknown aetiology. The CSFs of the 18 culture positive patients were used as positive controls in the ELISA, whenever the assay was performed in batches of test CSF specimens. Based on the results of correlation between the ELISA and culture methods, 57 culture negative patients with TBM were assayed for measuring the IgG antibody to mycobacterial antigens, and also for the detection of circulating mycobacterial antigens in CSF. The assay yielded 82% sensitivity for tuberculous aetiology and 47 out of 57 culture negative patients were classified as TBM on the basis of ELISA. These patients were given the benefit of ATT for four weeks during their hospital stay. 81.2% patients showed a definite improvement in their clinical status. The neurological status in these patients showed good correlation with the biochemical cytological

picture of the CSF. At the time of follow-up these patients showed clinical recovery and none of the patients manifested any sequelae of the disease. Thus application of ELISA method has helped in making a diagnosis of TBM in vast majority of culture negative patients with TBM.

Among the three mycobacterial antigens used in the ELISA viz. CFA, PPD, antigen 5 we found that **M tuberculosis** antigen 5 was more specific for the diagnosis of TBM.

Estimation of mycobacterial antigen in the CSF by the inhibition ELISA is a novel technique. The assay is not only sensitive but also carry a very high degree of specificity for the diagnosis of TBM. Presence of mycobacterial antigens in CSF suggest that the disease is in the active stages. The mycobacterial antigen concentration tend to decrease with the duration of ATT and therefore the inhibition ELISA is not only useful in the laboratory diagnosis of TBM but also can be used to monitor the schedule of ATT in patients with TBM.

Both indirect and inhibition ELISA methods can be used in the rapid laboratory diagnosis of TBM because they are reliable and reproducible. Both ELISA methods carry a sensitivities, higher than the sensitivity of the bacteriological methods.

Besides, the diagnostic utility of ELISA in the CSFs, an attempt was also made in this study to demonstrate mycobacterial antigen in the intracranial tuberculous lesions by an immunohistochemical method. This method was essential because AFB could not be demonstrated in the tuberculous lesions by Z-N method. Using an immunohistochemical method mycobacterial antigen was demonstrated in the tuberculous lesions and thus helped in making a diagnosis of tuberculosis. Secondly, an immunohistochemical method was also helpful in distinguishing a tuberculous granuloma from a fungal granuloma, because a distinction between these two diseases was difficult at the light microscopic level.

5.3 Conclusions

1. The results of this study indicates that ELISA methods have potential application in the laboratory diagnosis of TBM.

2. In this study, the results of the ELISA showed good correlation with the bacteriological methods in all the 'definite' patients with TBM.

3. ELISA methods are rapid, reproducible, and carries higher sensitivity than bacteriological methods in our study.

4. Estimation of mycobacterial antigen by an inhibition ELISA is more specific for the diagnosis of TBM as this assay did not show any false positive results in non-tuberculous subjects.

5. The mycobacterial antigen concentration in CSF decreases during ATT in patients with TBM and therefore estimation of mycobacterial antigen in serial CSF specimens of patients with TBM can be used to assess the clinical response to ATT.

6. **M tuberculosis** antigen 5 is more specific than PPD and CFA, therefore antigen 5 should be preferred in an indirect ELISA for the detection of antimycobacterial antibodies in the CSFs.

7. Antimycobacterial antibodies are present in the CSFs of patients with TBM and their titers do not decrease during the therapy.

8. Western blot technique can detect the presence of a specific antibody to **M tuberculosis** antigen 5 in the CSFs of patients with TBM. This method will be useful in differentiating patients with TBM from patients in the control group.

9. Mycobacterial antigens can be demonstrated in tuberculous lesions by an immunohistochemical technique. This method can differentiate a granulomatous lesion

caused by **M tuberculosis** from that of granulomatous lesion caused by a fungus. Immunohistochemical method carries higher sensitivity than Z-N staining method in our study. Immmunohistochemical method therefore should be used as a routine test in a surgical pathology laboratory for establishing a tuberculous aetiology of a granulomatous lesion.

5.4 Recommendation for further investigation.

Currently in our country there are more than 110 Medical institutions. A large number of patients with TBM are admitted in these Medical College Hospitals. In a significant proportion of these patients, the diagnosis and treatment for tuberculosis is often delayed for the want of specific bacteriological confirmation. The results of our study strongly indicates that ELISA method can be used as an alternative method for the laboratory diagnosis of TBM. We propose to bring a diagnostic kit and supply them to all the Medical College hospitals in our country for the early diagnosis of TBM. The ELISA diagnostic kits will be equipped with stable reagents and can be used for atleast one year. The technical aspects of ELISA can be easily performed in any routine laboratories attached to these Medical College hospitals.

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APPENDIXES

APENDIX - A

Publications from this Thesis

1. Mathai A, Radhakrishnan VV, Rao B. Isolation of **M tuberculosis** antigen 5 by immunoadsorbent affinity chromatography and its diagnostic application in tuberculous meningitis. **Indian J Med Microbiol** 1989; 7:49-53.

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APPENDIX - B

PREPARATION OF REAGENTS

B.1 Acrylamide solution

Acrylamide = 25.2g

Bisacrylamide = 0.8g

Dissolved in 70 ml of deionised water. When the acrylamide is completely dissolved, add water to a final volume of 100 ml. Filter the solution under vacuum through a 0.45 um membrane. Store stock acrylamide at $4^{o}C$ in a dark bottle.

B.2 Carbonate bicarbonate buffer

Sodium carbonate = 1.59 g

Sodium bicarbonate = 2.93 g

Sodium azide = 0.2 g

Total volume made upto 1 litre by dissolving in distilled water, pH 9.6.

B.3 Diethanolamine buffer

Diethanolamine = 97 ml

Sodium azide = 0.2 g

Magnesium chloride $6H_2O$ = 0.1 g

Dissolved in 800 ml distilled water pH adjusted to 9.8 by dil. HCl, made upto 1 litre.

B.4 Phosphate buffered saline

Disodium hydrogen phosphate $2H_2O$	= 1.44 g
Potassium dihydrogen phosphate	= 0.2 g
Potassium chloride	= 0.2 g
Sodium chloride	= 8.0 g

Total volume made upto 1 litre by dissolving in distilled water, pH 7.4.

B.5 Phosphate buffered saline - Tween 20

Phosphate buffered saline	= 1000 ml
Tween - 20	= 0.5 ml

B.6 Sodium acetate (0.1M)

Dissolved sodium acetate 8.2 g in 800 ml distilled water. Adjust the pH to 5.0 by the addition of acetic acid. Make up the final volume to 1000 ml. Store at $4^{o}C$.

B.7 Sodium borate buffer (0.1M

Dissolve di-sodium tetraborate 38.14 g in 800 ml distilled water. Adjust the pH to 9.0 by the addition of dilute HCl. Make up the volume to 1000 ml. Store at $4^{o}C$.

B.8 Sodium bicarbonate buffer (0.1M)

Dissolve sodium bicarbonate 8.4 g in 800 ml distilled water. Adjust the pH to 9.0 by 0.1 M sodium carbonate. Make up the final volume to 1000 ml. Store at $4^{o}C$.

APPENDIX-C

LIST OF ABBREVIATIONS

ADA	Adenosine deaminase
AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
ATT	Antituberculosis therapy
BCG	Bacille Calmette-Guerin
CFA	Culture filtrate antigen
CIC	Circulating immune complex
CMI	Cell-mediated immunity
CNS	Central nervous system
Conc	Concentration
CSF	Cerebrospinal fluid
Dot-Iba	Dot immunobinding assay
DTH	Delayed-type hypersensitivity
EDTA	Ethylene diamine tetra acetic acid.
ELISA	Enzyme-linked immunosorbent assay
Enceph	Encephalitis
HRP	Horse-radish peroxidase
IEP	Immunoelectrophoresis
IFA	Incomplete Freund's adjuvant
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect haemagglutination assay

IL-I	Interleukin - 1
IL-2	Interleukin - 2
kDa	Kilodalton
LJ	Lowenstein-Jensen
MAb	Monoclonal antibody
MAbs	Monoclonal antibodies
MAF	Macrophage activating factor
MIF	Macrophage inhibitory factor
M	Mycobacterium
MTSE	Soluble extract of Mycobacterium tuberculosis
NCP	Nitrocellulose paper
NK	Natural killer
OT	Old tuberculin
OVA	Ovalbumin
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween
PEG	Polyethylene glycol
PPD	Purified protein derivative
RaHG	Rabbit antihuman gammaglobulin
RIA	Radioimmunoassay
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SRF	Skin reactive factor
TBM	Tuberculous meningitis
Z-N	Ziehl-Neelsen
µM	Micromoles

APPENDIX-D

PROFORMA FOR RECORDING THE CLINICAL DATA AND LABORATORY INVESTIGATIONS IN PATIENTS WITH TUBERCULOUS MENINGITIS, NON-TUBERCULOUS MENINGITIS AND NON-MENINGITIC NEUROLOGICAL DISORDERS

Name of the patient H.No. Age Sex

Date of Admission Date of Discharge/death

1. **Symptoms**

   Fever
   Headache
   Vomiting
   Convulsions
   Altered Sensorium
   Focal Neurological deficits
   History of Tuberculosis

2. **General Examinations**

   Temperature/Pulse/BP
   Lymphadenopathy

3. **CNS Examination**

   Consciousness
   Cranial nerve palsies
   Fundus
   Other neurological deficits
   Signs of meningeal irritation

4. **CVS/GIT/RESP System**

5. **Investigations**

   1. TLC
   2. Hb
   3. ESR
   4. Blood Sugar

5. Serum albumin
6. Serum IgG
7. Bacteriology of any site of infection
8. Chest X-ray
9. Mantoux test
10. Biopsy if any
11. CT scan head

6. **CSF Chart**

1. Pressure
2. Total Count
3. Lymphocyte
4. Protein
5. Sugar
6. Albumin
7. Immunoglobulin-G
8. Gram stain/AFB stain/India ink
9. Culture for Pyogenic/AFB/Fungus
10. ELISA for M tuberculosis

7. **Treatment and duration**

Antitubercular drugs
Anti-pyogenic drugs

8. **Hospital Course**

improved/deteriorated/expired

9. **Autospy findings**

10. **Diagnosis of TBM**

1. Confirmed by AFB isolation of CSF.
2. Presumed by TB focus in the body.
3. Presumed by therapeutic response.
4. Confirmed at autopsy.
5. Confirmed by ELISA.