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

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SUMMARY

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

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

INTRODUCTION

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

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CENTRAL NERVOUS SYSTEM (CNS) TUBERCULOSIS:

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

TUBERCULOUS MENINGITIS (TBM):

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

LABORATORY DIAGNOSIS OF TBM:

The laboratory diagnosis of TBM can be made from

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

a) Cytological and biochemical parameters in CSF:

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

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

b) Bacteriological methods

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

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

Acid-fast bacilli in CSF smear

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

CSF culture for M tuberculosis

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

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

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

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

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

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

c) Biochemical methods

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

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

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

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

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

d) Immunodiagnostic approach

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

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

e) Molecular biologic approach

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

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

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

Cytological:

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

a) The leukocyte count may be normal.

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

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

ten significantly alters the cytopathological picture.

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

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

Biochemical

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

Bacteriological methods

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

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

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

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

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

Biochemical methods

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

Immunodiagnostic approaches

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

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

Molecular biologic methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Broad outlines of this study:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

surgical specimens of intracranial granulomatous lesions due to fungal infections.

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

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

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

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

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

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

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

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

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

TBM and in patients with chronic pyogenic meningitis.

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

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

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

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

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

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

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

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

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

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

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

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

The results of this study would highlight

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

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

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

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

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

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Public Health Significance of Leptospirosis – A Neglected Disease in India

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SUMMARY

Leptospirosis is the most widespread zoonosis in the world. The existence of leptospirosis in India was proved in 1929 through studies conducted in Andaman Islands. Since 1988 outbreaks of febrile illness with haemorrhagic tendencies locally known as Andaman Haemorrhagic Fever have been occurring in the islands. In 1993 the case of the disease was established as leptospires. Leptospirotis is also common in many states of the country, particularly those in the east and west coast. A study conducted by an ICMR Task Force showed that leptospirosis exists in most parts of the country. Epidemiologically there are four distinct form of leptospirosis viz. rural, urban, recreational and disaster-related. The transmission cycle of leptospirosis involves carrier animals, environment and human beings. Control can be achieved by intervention measures targeting several points on the transmission cycle. However, measures targeting human beings are the only feasible ones in the present situation. The public health significance of leptospirotis has been overlooked for several decades and the disease has emerged as an important health problem. A multi-sectoral approach is necessary to combat this environmentally acquired infection.

INTRODUCTION

Leptospirosis is the most widespread zoonosis in the world (1). It is caused by spirocheates belonging to various pathogenic species of the genus Leptospira. Leptospirosis affects human beings and many other species of vertebrates. It can present in a wide spectrum of clinical manifestations in human beings. The syndrome of icteric leptospirosis with renal involvement is referred to as Weil's disease. Another recognized clinical form is that presenting with severe pulmonary haemorrhage (2, 3, 4). Other complications include Acute Respiratory Failure (5), myocarditis (6), meningitis and renal failure (7).

Eighty-five years back the Japanese scientists Inada and Ido identified Leptospira, which was later confirmed by the German scientists Uhlenhuth and colleagues. Since the discovery of

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leptospires and its association with Weil's diseases, several other disease syndromes prevailing in different parts of the world have been added to the list of diseases caused by these bacteria. Leptospirosis occurs in a large number of countries in all the five inhabited continents. In most of the countries, leptospirosis was considered as an uncommon disease till recently and hence was given low priority in health programmes. The International Leptospirosis Society (ILS) made an attempt to compile data on occurrence of leptospirosis in various countries (8). The data shows that on an average 10,000 severe cases requiring hospitalization occur annually all over the world.

Leptospirosis is known to be endemic in Andaman Islands since early years of 20th century. The first report of bacteriologically confirmed leptospirosis in India originated from Andaman Islands in 1931 (9). The disease was common among the free-living convicts of South Andaman who were engaged in rice cultivation and other occupations that expose them to wet conditions. The occurrence of the disease had close association with rainfall and the peak occurrence used to be during the post-monsoon period. No information about the status of leptospirosis in Andamans between 1931 and 1993 is available in literature.

Andaman Haemorrhagic Fever

In the post-monsoon season of 1998, an outbreak of febrile illness with haemorrhagic manifestations appeared, first in Port Blair and other areas of South Andaman and then in Diglipur of North Andaman (Fig 1). The first case was an 18 year old girl, who was admitted to G.B. Pant Hospital, Port Blair with fever, cough and haemoptysis. The provisional diagnosis was miliary tuberculosis based on X-ray findings. In addition to antituberculous drugs she was also put on penicillin. She recovered completely within one week after initiating therapy and her X-ray shadows disappeared. Later similar cases occurred among the labourers camping at a forest camp at Jirkatang in South Andaman. Since the aetiology of the disease was unknown, it was named as Andaman Haemorrhagic Fever (AHF). AHF outbreaks recurred ev-

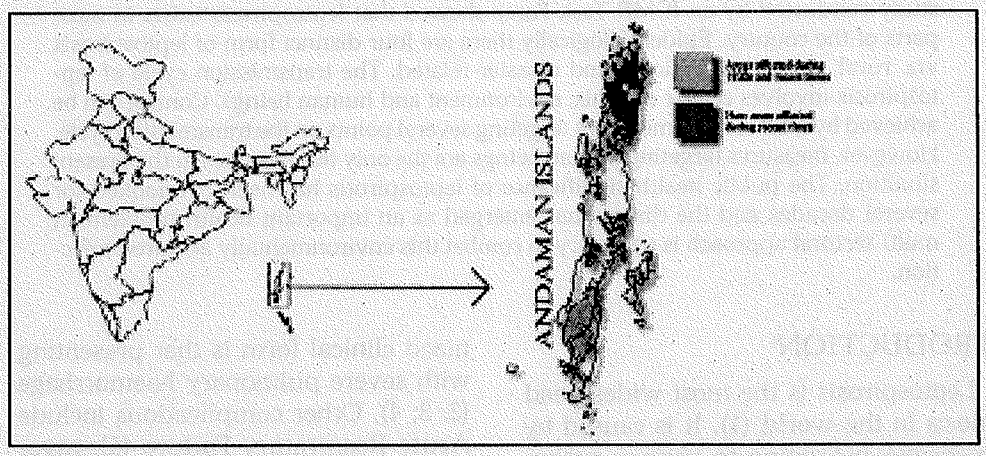

Fig. 1. Map of Andaman islands showing areas affected by AHF outbreak

ery year since then, but the aetiology remained unknown in spite of investigations conducted by several national level organizations. An outbreak occurred in Diglipur in 1993 was investigated by us and the etiology was established serologically as leptospires. This was later confirmed by isolation of leptospires from the blood of AHF patients.

There were a lot of epidemiological similarities between the outbreaks of leptospirosis that occurred during the first half of 20th century and the outbreaks of AHF.

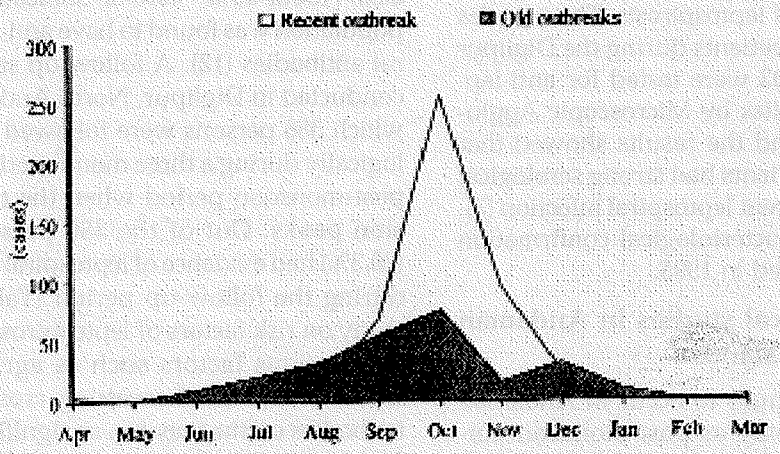

Fig. 2. Seasonal trend in the occurrence of leptospirosis cases during 1921 - 26 and AHF case during 1988 - 93

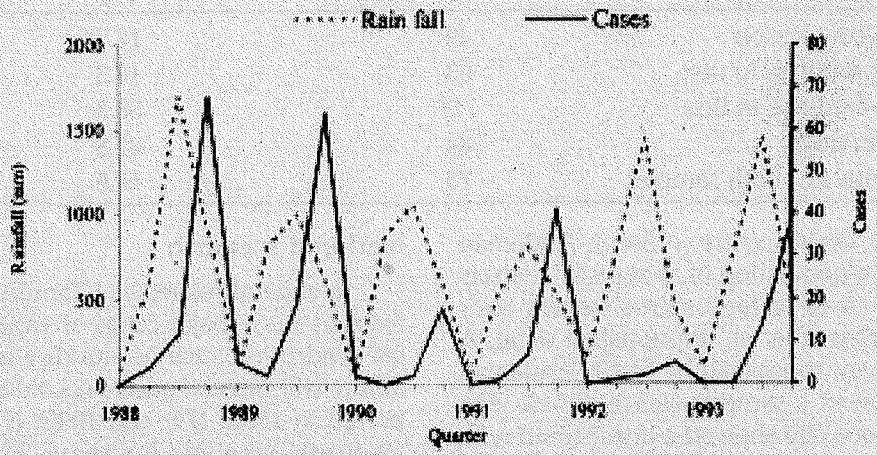

Fig 3. Association between rainfall and occurrence of AHF cases

The seasonal variation (Fig 2), association with rainfall (Fig. 3), occupation of patients and affected areas were similar. However, the important common clinical features of AHF were different from those of earlier outbreaks. Because of the epidemiological similarities between AHF and earlier outbreaks, we considered it worthwhile to investigate AHF for it being a different clinical syndrome of leptospirosis. The samples collected from patients during the Diglipur outbreak in 1993 were tested for anti-leptospiral antibodies by Microscopic Agglutination Test and the results showed that 66.7% of the patients had strong serological evidence of current leptospiral infection (2). Subsequently bacteriological confirmation was also obtained in 1995.

Epidemiological studies in Andaman and Nicobar Islands

AHF continues to occur in Andaman Islands commonly as post-monsoon outbreaks and occasionally as sporadic cases (4). A surveillance system based at a rural primary health centre in South Andaman serving a population of about 9,500 detects 40 - 70 cases of leptospirosis every year (Vijayachari P et al, unpublished data). A serosurvey conducted in North Andaman (10) showed a seroprevalence of 54%. The prevalence rate showed a linear trend with age and was more than 72% in those aged above 30 years. Seroprevalence studies were conducted among the tribes of Andaman and Nicobar Islands also. All the tribes had seroprevalence rates lower than the settler population except Shompens living in the jungles of Great Nicobar, who had a seroprevalence of 53.5% (11). Seroprevalence studies were conducted among the animal population of the islands also. About 30% - 45% of domestic animal population was found to have anti-leptospiral antibodies (12). A follow up study was conducted in Diglipur, North Andaman, in which 386 persons were followed up serologically during a three month period in the post-monsoon period when the transmission peaks. Out of the 386 persons, 113 (29.3%) had evidence of leptospiral infection during the follow-up period (Table 1). A study on risk factors of leptospirosis identified various factors such as agricultural work, forest work, harvesting, crossing water bodies on the way etc. as significant risk factors associated with seropositivity to leptospires (10).

Table 1. Results of serological follow up of 386 persons during peak transmission

Serological result	No. (n=386)	(%)
Sero-conversion	49	12.7
Four-fold rise in titre	63	16.3
Two-fold rise in titre	117	30.3
Fall in titre	84	21.8
Negative in both samples	73	18.9

National scenario

In endemic states like Tamilnadu, leptospirosis is a major cause of various clinical syndromes such as jaundice and renal failure (13). Several outbreaks have been reported in 1980s (14) and 1990s (15). Leptospirosis accounts for about 30% of the cases of pyrexia of unknown origin (PUO),

in Chennai city during monsoons. Leptospirosis has been identified as an occupational hazard of pineapple farmers in Kolancherry in Kerala. During a 10 year period, 976 patients among pineapple worker were identified with a case fatality ratio of 5.2% (16). During the period 1990 - 1998, leptospirosis was suspected in 1909 patients in Kolancnerry and 173 isolates were recovered from the patients (Kuriakose M, personal communication). Leptospirosis outbreaks occur every year in Surat and Valsad districts in Gujarat, in various places in Tamilnadu and Kerala and frequently in the coastal areas of Maharashtra and Karnataka. About 30% of paediatric patients presenting with clinical presentation matching the Indian Leptospirosis Society's working definition for clinically suspecting leptospirosis were confirmed to have leptospirosis based on a rapid diagnostic test (17).

Several outbreak occurred in various parts of the country were investigated. In 1999, after the super cyclone an outbreak of febrile illness with haemorrhagic tendencies occurred at several villages in Orissa that were submerged in flood waters for several days. An investigation was carried out in these villages and it was found that an outbreak of leptospirosis with an attack rate of about 14% occurred in these villages after the cyclone (18, 19). In 2000, an outbreak of a mysterious illness occurred in Mumbai and Thane following heavy rainfall and floods. We investigated these outbreaks and established the leptospiral etiology.

The Indian Council of Medical Research (ICMR) constituted a Task Force on leptospirosis to assess the disease burden due to leptospirosis in the country. The Task Force conducted a nationwide study to estimate the proportion of leptospirosis cases among all the fever cases that fulfilled a case definition. Thirteen centres in different parts of the country participated in the study. The results of the study showed that about 14.7% of the fever cases included in the study had leptospiral infection as diagnosed by a rapid diagnostic test. The proportion of leptospirosis cases was higher in states in western and eastern coasts compared to northern and central parts of the country (Fig 4). Patient

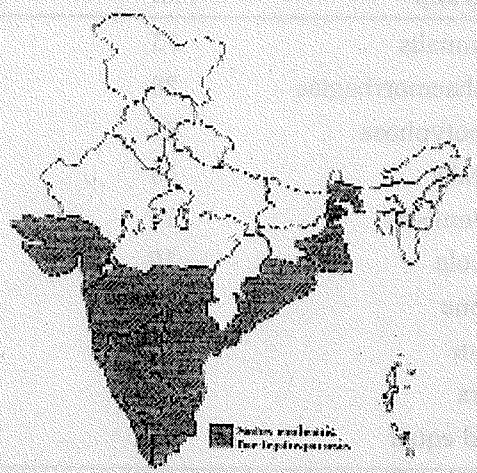

Fig 4. Map of India showing endemic states

inclusion showed a peak in July- August months (Fig 5), which is the peak monsoon season in most parts of the country. The commonest infecting serogroup (as per MAT titre) was Autumnalis followed by Icterohaemorrhagiae, Grippotyphosa and Australis (Table 2). Serogroups Autumnalis, Australis, Ballum and Grippotyphosa were present in all the 11 serogroup tested in MAT were present in Southern region.

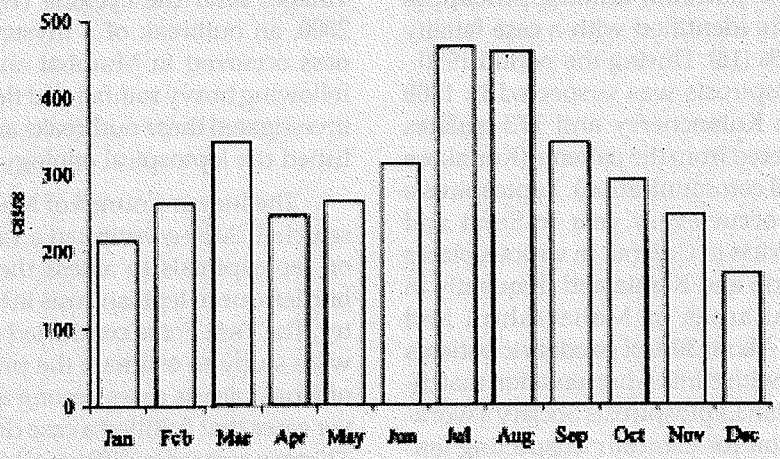

Fig. 5. Month-wise patient inclusion in ICMR Task Force study on leptospirosis

Table 2. Commonest infecting serogroups (as per MAT titres)

Sl No	Serogroup	No.	(%)
1	Autumnalis	73	27.7%
2	icterohaemorrhagiae	38	14.4%
3	Grippotyphosa	25	9.5%
4	Australis	25	9.5%
5	Hebdomedis	19	7.2%
6	Canicola	10	3.8%
7	Pomona	10	3.8%
8	Ballum	9	3.4%
9	Others	10	3.8%
13	Mixed equal	45	17.0%
	Total	**264**	**100.0%**

Clinical presentation

The severity of illness in leptospirosis can vary from mild flu like illness to severe and fatal forms with multiple organ failure. The mild form is characterized by non-specific symptoms/signs such as fever, headache and myalgia with very few patients showing symptoms/signs of organ involvement, whereas in severe forms symptoms/signs of organ system involvement are common (Table 3). There are two major clinical types of severe leptospirosis, the hepato-renal type and the pulmonary type (4), though some overlap between these types are occasionally seen. Other complications such as myocarditis, meningitis etc. are less common. Biochemical and pathological abnormalities indicating organ involvement are also more common in severe forms (Table 4).

Table 3. Frequency of different symptoms/signs among mild and severe cases

Symptom/sign	Mild cases (N=70)	Severe cases (n=58)
Fever	100.0	100.0
Headache	79.0	51.7
Body aches	77.6	39.7
Vomiting	39.2	29.3
Muscle tenderness	39.2	82.8
Cough	19.6	70.7
Rigors	16.8	12.1
Abdominal pain	15.4	6.9
Icterus	5.6	51.7
Oliguria	5.6	50.0
Conjunctiva suffusion	5.6	50.0
Hypotension	5.6	39.7
Haemoptysis	4.2	50.0
Lung crackles	0.0	44.8
Subconjunctival haemorrhage	0.0	29.3
Neck stiffness	0.0	12.1
Altered sensorium	0.0	12.1
Hepatomegaly	0.0	6.9

Table 4. Biochemical abnormalities among mild and severe cases

Abnormality	Severe	Mild
Abnormal LFT	51.7%	11.2%
Serum Bilirubin	1.7 - 15.5 mg/dL	1.6 - 2.5 mg/dL
SGOT	47 - 258 IU	
SGPT	52 - 290 IU	
Abnormal RFT	51.7%	1.4%
Blood Urea	53.1 - 301.6 mg/dL	
Serum Creatinine	1.7 - 7.4 mg/dL	1.4 - 2.3 mg/dL
Abnormal LFT & RFT	45.6%	1.4%
X-Ray Shadows	62.1%	1.4%

Transmission cycle

Leptospirosis transmission involves carrier animals, human beings and environmental vehicles of transmission (Fig. 6). The natural habitat of leptospires is the renal tubules of their animal host. Almost every known species of rodent, marsupial and mammal can be carrier and excretor of leptospires (20). Although leptospires are susceptible to environmental factors, such as drying, acidic or highly alkaline pH, low humidity, salinity and presence of detergents and other bactericidal chemicals, under favourable circumstances they can survive for long periods in water and wet soil. Lep-

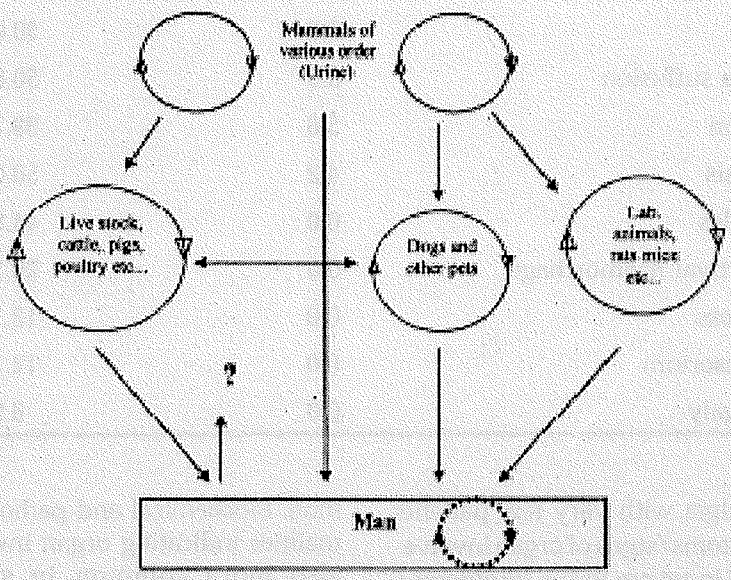

Fig. 6. Schematic diagram of transmission cycle of leptospiro

tospires can infect human beings when they come into contact with environment contaminated with urine, tissue or body fluids of carrier animals. Although direct infection from carrier animals to humans occurs occasionally, indirect infection through environmental vehicles contaminated with leptospires is far more common and hence epidemiologically more important. Therefore, leptospirosis can be viewed as an environmentally acquired infection.

Various occupational groups are at high risk of leptspiral infection either because their occupation requires close contact with animals or because they are occupationally exposed to possibly contaminated soil and surface water. These occupational groups include agriculaural workers involved in wet as well as dry farming, sewage workers, forest workers, butchers, veterinarians, miners, fresh water fishermen, sports persons involved in water sports etc. However, in many tropical countries with wet, warm and humid climate where water-logging is common, whole communities could be at risk. The risk increases substantially when flooding occurs as a result of natural disasters, thus making leptospirosis a hazard during the aftermath of natural disasters such as cyclones and floods.

Epidemiological patterns

There are four epidemiological forms of leptospirosi viz. rural, urban, recreational and a sequelae of natural disasters.

Rural leptospirosis: Rural form is usually associated with farming activities, particularly wet farming such as rice. People working in flooded fields get exposed to leptospires in the ground water or wet soil while working in water-logged fields leading to outbreaks. This form is commonly associated wit agricultural cycles. Leptospirosis is a known health hazard of rice farmers in countries such as Indonesia and Thailand. High incidence of leptospirosis has been recorded in Thai provinces with large populations of farmers (21). Outbreaks have occurred in Korea on several occasions when the fields were flooded before harvest (22). The outbreaks of AHF in Andamans is also an example of this epidemiological form (2).

Urban leptospirosis: The urban form is a result of the poor environmental hygiene in the cities and towns. People get exposed to over-flowing sewers that are often contaminated with leptospires excreted by carrier animals. During rainy season, the sewage canals over-flow onto roads posing risk to whole communities living in such areas. Outbreak of leptospirosis in Mumbai and Thane in 2000 and 2005 following heavy rainfall and flooding was an example of this epidemiological form of leptospirosis. Urban epidemiological form is also seen in Chennai city, where during monsoons every year the incidence of leptospirosis increases sharply.

Recreational leptospirosis: The recreational form usually occurs in developed countries or among people from developed countries who visit tropical countries and participate in water-related recreational activities such as canoeing and swimming. Although rarely reported in India, outbreaks following water sports events have been reported in other Southeast Asian countries such as Malaysia (23) and in developed countries including Japan (24) and USA (25).

Leptospirosis following natural disasters: Natural disasters such as floods often trigger large outbreaks of leptospirosis. Such disasters lead to a closer contact between animals and human beings. Floodwaters are often contaminated with urine from carrier animals and people exposed to such waters contract the infection. During the past decade several outbreak have been reported

in India (18, 19) and other tropical countries following natural disasters such as cyclones and floods.

Control of leptospirosis

Control of leptospirosis involves breaking the transmission cycle at any of the target points (fig 7). Primarily there are four points along the transmission cycle that can be targeted. These are measures targeting reservoir host, those targeting carrier hosts, environmental measures and host (human being) targeted measures. Measures targeting carrier animals and environment can be formulated after sufficient information on the specific nature of transmission cycle existing in a community is generated, whereas measures targeting humans can be instituted without a prior knowledge of the actual transmission dynamics. The possible measures targeting human beings are promoting protective gear at workplace, vaccination and chemoprophylaxis. Community acceptance of protective gear may be poor in the present settings. Till now, no vaccine effective against the multitude of serovars of leptospires is available. It is unlikely that such a vaccine will be developed in the foreseeable future. Chemoprophylaxis, though not practicable in endemic situations, could be of use during epidemics. A study conducted at Andaman Islands has shown that chemoprophylaxis, though not effective in preventing infection, offers 54% protection against symptomatic leptospirosis and reduces severity (26).

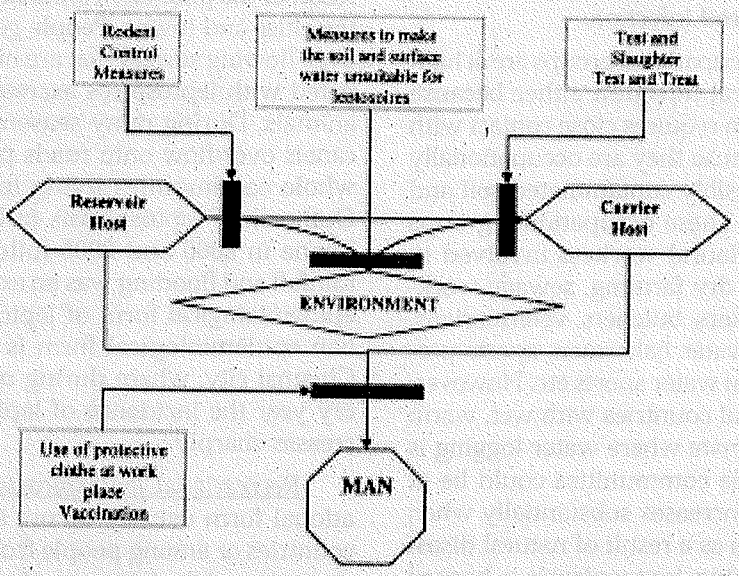

Fig 7. Target points for intervention for control of leptospirisos

CONCLUSIONS

For several decades we have overlooked the importance of leptospirosis as a potential public health problem. The pathogen, its survival characteristics, our environment and lifestyle of people all make leptospirosis an inevitable hazard of people of

tropical developing countries. A major chunk of our population lives under perpetual threat of this infection. About 60% of the 744 million strong work force of the country is engaged in agriculture and a significant section of this workforce is engaged in cultivation of water-intensive crops such as rice. The farming techniques are by and large conventional leading to unprotected exposure of the agricultural workers to possibly contaminated soil and surface water. We have large population of free-grazing and stray animals that can pollute the environment with the pathogen. The city-dwellers are at no less risk. Environmental sanitation of the cities and towns is poor. Drainages are often blocked resulting in flooding of the roads even during a light rain. Overflowing sewers contaminate the flood waters and the people exposed to this become at risk of contracting the infection. The disease causation is multi-factorial and hence intervention strategies should target multiple factors, which can be achieved only by an inter-sectoral collaboration involving health, agricultural, animal husbandry and environmental sectors. The need of the hour is a networking of multiple sectors and organizations for a joint effort to control this long overlooked infection.

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Role of the preoptic area in sleep regulation

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SUMMARY

Alteration in the amount of sleep with changes in ambient temperature, brain temperature or any part of peripheral and core body temperature, suggests a close relationship between these two regulations. A strong reason for this belief is the fact that the hypothalamus, especially the medial preoptic area (mPOA), participates in the regulation of sleep and body temperature. Thermosensitive neurones of the mPOA have been implicated not only in the regulation of body temperature but also in sleep. Simultaneous changes in sleep and body temperature, produced either on lesion or on stimulation of the mPOA, have given reasons to suggest that these two functions are controlled by the same set of neurones of the mPOA. It is proposed that the function of the mPOA is not restricted to regulation of sleep and body temperature, and their interlinking. But it may be essential for the homeostatic regulation of energy balance of the body, in response to alterations in the environmental and body temperature, on the one hand, and sleep-wakefulness, on the other.

INTRODUCTION

The sleep related changes in body temperature (1, 2, 3), and the effects of environmental temperature on sleep (4, 5) had given rise to the thought that the regulation of sleep and of body temperature are generally intimately related. The 24 hour periodicity of our environment, which can be assumed to have developed to optimise the adjustment to this periodic change, has a marked influence on our physiological functions, including rest, activity, sleep and thermoregulation. Another strong reason for the belief that the regulation of sleep and of body temperature are related is the fact that the preoptic area (POA), especially the medial preoptic area (mPOA) participates in the regulation of sleep and body temperature (6, 7, 8, 9). In experimental animals the temperature of the POA can be selectively changed. Increasing and decreasing the POA temperature can produce an increase and decrease of sleep (10, 11). More over, the neurones of the POA that show increased or decreased activities with temperature

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have been implicated in the regulation of sleep (12). These observations have been proposed to support the hypothesis that sleep is modulated by thermosensitive elements of the brain (2, 4, 13). It was also suggested that the thermoreceptors in the POA might provide an input to the sleep-regulating mechanisms situated in this area (14). Most of the recent findings on this subject have come from the studies on rats. Rat is a good animal for the study of the interrelationship between thermoregulatory and sleep regulatory mechanisms, as it shows several episodes of sleep and wakefulness within 24-hour period. They show polycyclic changes in body temperature with the alteration in vigilance state. In these animals, the normal body temperature that varies with the time of the day, which is under the control of a circadian mechanism, and the alteration in body temperature change with episodes of sleep, can be observed separately. The role of the mPOA in the interrelationship between thermoregulatory and sleep regulatory mechanisms in rats, which has given rise to the formulation of several hypotheses on sleep function, will be given due focus in this review.

Brain regions above the brain stem, namely the mPOA of the hypothalamus, play an important role in the regulation of Slow Wave Sleep (SWS). The most important brain regions in the hierarchy of neural structures regulating the body temperature are anterior hypothalamic-preoptic area, and the posterior hypothalamus. The mPOA, which forms the part of the anterior hypothalamic-preoptic area, plays the most important role in both physiological and behavioural thermoregulatory responses (9, 15, 16). The heat loss or gain measures are initiated by the degree of activity of the temperature receptors in the anterior hypothalamic-preoptic area. However, the temperature signals from the peripheral areas of the body, especially from the skin and certain deep body tissues (the spinal cord and the abdominal viscera), also alter the "set-point" of the hypothalamic temperature control centre. The "set-point" increases as the skin temperature decreases, and when the skin temperature is high, the "set-point" decreases. The posterior hypothalamus, which can be described as the sympathetic centre, controls the vasoconstriction of the skin blood vessels.

Apart from the subconscious mechanisms for body temperature control, the body has yet another temperature-controlling mechanism that is even more potent. This is the behavioural control of temperature. Whenever the internal body temperature becomes too high, signals from the brain temperature controlling areas give the person a psychic sensation of being overheated. Conversely, whenever the body becomes too cold, signals from the skin, and probably also from the deep body receptors, elicit the feeling of cold discomfort. Therefore, the person makes appropriate environmental adjustments to re-establish comfort. Indeed, for man, this is the only really effective mechanism for body heat control in severely cold environs. But to study the neural mechanism involved in these regulations and interrelation we depend a lot on the information obtained from lower animals like rats.

Body temperature changes

Lesion studies in rats had provided information that proved invaluable in understanding the thermoregulatory

function of the POA. The electrolytic lesions of the POA, which destroyed the cells and fibres of passage, produced hyperthermia (or increased body temperature) with impaired heat defence abilities in rats (17). It was suggested that the hyperthermia resulted from impaired heat defence abilities. This lesion effect could be either due to the destruction of the POA neurones, nerve fibres of passage and the afferent terminals. Use of neurotoxins like N-methyl D-aspartic acid (NMDA), which could selectively destroy the neurones, leaving most of the nerve fibres and the afferent terminals intact, provided a very useful tool for further investigations in this field. After selective destruction of the mPOA neurones (using local injection of NMDA) there was increase in body temperature. This increase was more marked during the initial one or two weeks. This was followed by a phase during which the body temperature was reset at a level that was higher than normal but lower than that during the initial week after the lesion. The shift in core temperature could be either due to a failure in thermoregulatory ability, or a change in the "set temperature" for thermoregulation. This hyperthermia produced by the NMDA lesion of the mPOA was without impaired heat defence abilities (9). The thermoregulatory ability was tested by noting the changes in the rectal temperature of the rats when they were kept for two hours inside hot (37°C) and cold (6°C) chambers. The mPOA lesion did not produce any change in the response pattern of rectal temperature on heat exposure (Fig.2). This showed that the ability of the animal to regulate its body temperature, when exposed to a hot environment, was not affected. On the other hand, its ability to maintain a stable rectal temperature, on cold exposure, was affected after the mPOA lesion, as the rectal temperature showed greater reduction in the lesioned animals than in the normal ones. Though the rectal temperature was drastically lowered during the initial half an hour of exposure to cold, it was maintained at this lowered level on continued exposure to a cold environment. So the mPOA neuronal lesion produced an increase in the range of thermostat setting, rather than a failure in thermoregulation per se. In other words, in the mPOA lesioned rats, there was a change in the "set temperature" for thermoregulation, and they were able to defend their temperature within this reset range.

Sleep changes

There was a reduction in the time spent in all the stages of sleep, and an increase in wakefulness after the mPOA lesion. All the stages of sleep were reduced throughout the 22 days of study. There was greater suppression of S2. There was more reduction in daytime sleep, resulting in a change in night-day sleep ratio.

There was a significant decrease in their duration of SWS episodes. The decrease was found in the duration of both S1 and S2 episodes. The number of SWS episodes showed a trend of increase. This was primarily due to an increase in short duration episodes. The number of short duration SWS episodes were increased on all the days after the lesion. There was also an increase in the number of awake episodes.

Reduction in the PS episode duration was significant only on the 2nd day, though its frequency was significantly reduced on the 2nd, 4th and 22nd days after the lesion. The reduction in the frequency and duration of the PS episodes, after the lesion, during the light and dark periods, followed the trend recorded in the 24h data.

Body temperature variation with sleep-wake cycles

Body temperature shows variation with the circadian rhythm and sleep-wake cycles. Both these variations were affected after lesion of the POA. Animals with polycyclic sleep-wakefulness are ideally suited for the study of body temperature variation with sleep-wake cycles. In animals with monocyclic pattern of sleep-wakefulness, the variation in body temperature with sleep-wake cycles do coincide with the circadian changes. On the other hand, in polycyclic animals like rats and hamsters it is possible to see the changes in body temperature with sleep-wake cycles, as it does not coincide with the circadian rhythm.

The amplitude of the circadian rhythm of the body temperature was shown to be much larger than normal in golden hamster with POA lesions (18). The body temperature of the rats shows cyclic variation with sleep-wake cycles (3). The average duration and amplitude of these cycles were 5-7 min and 0.26-0.29°C respectively. The magnitude of body temperature variations was increased after the mPOA lesion. The mPOA could be involved in the fine tuning of the set point for thermoregulation ie, to prevent large deviations from the normal thermal set point, by promptly activating appropriate thermoregulatory responses. Without the mPOA, these responses would not be as effective as in the normal, and the ultradian and circadian deviations would therefore be much larger. Change in ambient temperature could be a greater challenge for rats with mPOA damage, than for the normal. There was higher ultradian variation in body temperature of the lesioned rats when they were exposed to changes in ambient temperature. A delayed compensatory response in the mPOA-damaged rats would have produced the exaggerated temperature fluctuations. The increased amplitude of the body temperature variations after the lesion indicates the possibility that the mPOA thermoregulatory system may oppose rather than defend the ultradian and circadian alterations of body temperature in normal rats (3). This could also suggest the possibility that the larger deviation in the body temperature may also contribute towards the increase in wakefulness.

Temporal sequence of changes in body temperature and sleep during the postlesion period

Hyperthermia during the first week after the mPOA lesion was severe. This was followed by a constant mild hyperthermia during the subsequent weeks (8, 9). On the other hand, there was reduction in sleep after mPOA lesion, and there was no variation in the magnitude of reduction in sleep throughout the post-lesion period. Thus, there was no temporal correlation between sleep and temperature changes after the mPOA lesion. This certainly shows that there are neurones in the mPOA which play a role in the regulation of sleep and body temperature. Though this observation does not support the multimodal neurone theory, it does not totally disprove this possibility. It also suggests that the change induced in one parameter is not totally dependent on the other parameter. At the same time, one cannot rule out the possibility that the compensatory measures might have contributed to the differences in the sleep and temperature changes.

a. The changes in body temperature and sleep on local injection of neurotransmitter agonists and antagonists at the mPOA

If changes in either sleep or body temperature can be elicited (without affecting the other parameter), by selective stimulation of different sets of neurones, it can be put forward as an argument in favour of the assumption that these two functions are controlled by different sets of neurones. Selective stimulation of different sets of neurones could be achieved by chemical stimulation of the mPOA. The changes in sleep and body temperature were studied in free moving animals, after the injection of neurotransmitters and their antagonists at the POA, through chronically implanted cannulae. Injections at the POA usually produced alterations in both sleep and body temperature more easily from the mPOA than the lPOA. Direction of changes in these two parameters indicate the following possibilities.

1. Carbachol (acetylcholine agonist) and noradrenaline (NE) administration at the mPOA produced hypothermia and arousal (19, 20, 21, 22, 23, 24). This may indicate that sleep and body temperature were altered by the same set of neurones of the mPOA. Application of alpha adrenergic antagonists phenoxybenzamine and phentolamine at the mPOA produced opposite changes in sleep and body temperature, ie there was injection bound sleep and hyperthermia (19, 24). These findings after application of adrenergic antagonists supported the possible role of noradrenergic system at the mPOA in the regulation of sleep and body temperature. It could be also taken to indicate that a tonic activity of noradrenergic system is responsible for the maintenance of wakefulness and normal body temperature (20, 21). So it is reasonable to assume that sleep and body temperature were altered by the same set of neurones of the mPOA, and they are under tonic influence of inputs coming to this area, like the inputs through noradrenergic system.
2. Detailed analysis of sleep and body temperature responses elicited by the above mentioned drugs casts doubts on the assumption that these changes are brought about by the same set of neurones of the mPOA. The neurotransmitters and their antagonists, injected at the mPOA, did not always produce simultaneous alterations in sleep and body temperature (22, 24, 25). Arousal induced by Carbachol and NE outlasted the reduction in body temperature (22, 24). Sleep induced by phenoxybenzamine and phentolamine was far shorter than the duration of temperature change (22). Moreover, there were instances when only one of these parameters (sleep or body temperature) was only altered. Administration of serotonin at the mPOA produced hyperthermia without any change in S-W (26). Alpha-2 agonist (clonidine) administration at the mPOA produced arousal (27), but it was not effective in producing any change in temperature (28, 29). The initial short-lasting rise in temperature after the injection could be attributed to the non-specific effect of handling and injection. So, it may be suggested that the mPOA controls sleep and temperature through independent, but overlapping, neuronal circuits. This conclusion, which is primarily based on studies in our laboratory, is also supported by the observations of Krueger and Takahashi (30).

3. Further support for the assumption that there are separate controls of sleep and body temperature came from the studies in which various noradrenergic agents were applied at the mPOA (31, 32). In an area innervated by noradrenergic fibres, locally applied NE could act on both post-synaptic and pre-synaptic receptors (33). A pre-synaptic site of action of hypothalamically-injected NE was suggested (34). Studies using alpha-2 adrenergic agents provided some insight into the mechanism of action of NE. Application of NE at the mPOA in normal rats produced arousal and hypothermia. NE injected at the mPOA can act on alpha-1 or alpha-2 adrenergic receptors, apart from beta-receptors, and many other receptors about which very little is known. Alpha-2 receptors are predominantly present in pre-synaptic terminals. Alpha-2 agonist (clonidine) administration at the mPOA produced arousal (27), but it was not effective in producing any change in temperature (28, 29). Clonidine injection can result in the activation of pre-synaptic alpha-2 receptors, and bring about decreased release of endogenous NE at the synaptic cleft (29). Clonidine injection into the mPOA resulted in the activation of pre-synaptic alpha-2 receptors, on both the groups of noradrenergic afferents, but it brought about a decreased release of endogenous NE in those neurones in which there was a tonic release. This decreased release of endogenous NE produced arousal in sleeping animals (27). Clonidine also acted on the inactive terminals, which synapse on the temperature regulatory neurones. Since these fibres normally secrete very little NE, there was no change in the body temperature when this drug was applied. Yohimbine, an alpha-2 antagonist, blocks the pre-synaptic receptors and facilitates the release of endogenous NE from nerve terminals. Post-synaptic action of the released NE on alpha-1 receptors, induces sleep in normal animals (27). Yohimbine failed to exert facilitated release of NE from those nerve terminals that synapsed on the temperature regulatory neurones, since they are normally inactive. Hence, there was no change in the body temperature on application of this drug.

4. Before it is concluded that there are two different sets of neurones controlling these two functions, the possibility of one of these changes affecting the other has to be considered. It is also well known that sleep (i.e. SWS) is associated with a fall, and arousal with a rise, in body temperature (1, 2, 3). So, any drug that produces sleep (especially SWS) can be expected to produce a fall in body temperature, and that which produce arousal with a rise in body temperature. In the above-mentioned drug induced changes, alpha-adrenergic antagonists phenoxybenzamine and phentolamine produced sleep and rise in body temperature. Carbachol and NE produced arousal and fall in body temperature. Thus the changes in the body temperature (hypothermia with arousal and hyperthermia with sleep) would not have resulted from the changes in sleep-wakefulness (S-W).

This can be put forward as a strong argument in favour of the assumption that different sets of neurones are controlling these two functions.

5. After ruling out the possible influence of S-W changes on body temperature, the changes in S-W, which might have been induced by the alterations in body temperature, need to be considered. Here, one cannot rule out the possibility that the induced change in body temperature may have affected the S-W (35). It is possible that the wakefulness may have resulted from a decrease in body temperature, induced by the central injection of drugs. It has been shown that systemic injection of phentolamine produces reduction in sleep and fall in body temperature. There was no reduction in sleep, when the fall in body temperature was prevented (35). It was suggested that the reductions in sleep, observed after the injection of the drug, could have resulted from a fall in body temperature, rather than from a direct action of the drug on the arousal inducing system. This argument can be extended to state that the changes in S-W, induced by the above-mentioned drugs, were influenced by the changes in body temperature. It could even be asserted that the drugs (neurotransmitter agonists and antagonists) produced change only in body temperature, and not in S-W. This is not likely to be true, as can be seen from the subsequent sections.

6. From the evidences put forward so far, it is reasonable to assume that α_1-adrenergic system at the mPOA is involved in sleep and hypothermia. Changes induced in body temperature and sleep-wakefulness on microinjection of an α_1 agonist, methoxamine (0.5, 1 and 2 µmols), into the mPOA were studied in rats. Methoxamine injection produced hypothermia, but there was no major change in sleep-wakefulness during the 3 hrs after the drug injection, except for a short period (15 min) of sleep after 120 min of injection. Coinciding with the maximum fall in body temperature (at 30 min after the injection), there was a short period of wakefulness when methoxamine was administered at higher doses. There was no change in sleep latency after the drug injection. Hypothermia induced by methoxamine might have masked the hypnogenic action of this drug. The study indicates that the α_1 adrenergic receptors participate in the preoptically mediated thermoregulatory measures which bring down the body temperature. It also suggests that the α_1-adrenergic system at the mPOA is involved in sleep.

b. The roles of noradrenergic terminals in the mPOA in regulating sleep and body temperature.

A clear indication regarding separate control of sleep and body temperature came from the studies in which noradrenergic agents were applied at the mPOA, in animals with and without lesion of the noradrenergic fibres projecting to the mPOA (31, 32). It was suggested that the Clonidine and NE injection at the mPOA could result in the activation of pre-synaptic alpha-2 receptors, and bring about decreased release of endogenous NE at the synaptic cleft. In order to test this proposition further, NE was locally administered at the mPOA in rats, whose

noradrenergic fibre terminals were degenerated.

The noradrenergic terminals in the POA come mainly from the lateral tegmental noradrenergic cell groups in the medulla (36, 37). The fibres of the medullary noradrenergic group ascend through the ventral noradrenergic bundle (VNA) to reach the POA. So, the noradrenergic fibres in the POA can be destroyed by injecting 6-hydroxy dopamine at the VNA (29, 38, 39). NE injection at the mPOA induced sleep in the VNA lesioned animals. As the pre-synaptic adrenergic receptors were not available at the mPOA in these rats (as the noradrenergic terminals had already degenerated), the response elicited must have been due to the action of NE on the post-synaptic receptors (38).

Application of NE at the mPOA in the rats with noradrenergic fibre lesion brought about sleep and decreased body temperature. It could be argued that the decreased body temperature was a result of sleep. It could be also argued that the decreased body temperature and sleep are actively produced by multimodal neurones of the mPOA, and that thermoregulation and sleep regulation are inter-linked at this area of the brain. But, local application of clonidine and yohimbine, in the rats with noradrenergic fibre lesion, further clarified our concept (27, 29). Though arousal was produced in normal rats by the injection of clonidine, at the mPOA, it did not have the same effect on the rats with noradrenergic fibre lesion. Clonidine did not alter the rectal temperature in normal rats but it induced hypothermia in the lesioned rats. Injection of yohimbine, at the mPOA, induced sleep in rats with intact noradrenergic fibres. However, the sleep inducing effect of this drug was very much attenuated in the lesioned animals. There was no significant change in body temperature, in both normal and noradrenergic fibre lesioned animals, after yohimbine administration. On the basis of these findings, it was suggested that there are two separate groups of afferent noradrenergic inputs, ending on the mPOA neurones. One of them, terminating on sleep inducing neurones, is activated during sleep. Those afferents, which synapse on the temperature regulatory neurones, are suggested to be normally inactive and may be activated only when the heat loss mechanism is to be stimulated (29). An intact catecholaminergic pathway within the anterior hypothalamus is required for the rat's physiological control of heat loss in a warm environmental temperature (40). It can be concluded that there are separate sets of noradrenergic terminals for regulation of sleep and body temperature.

Local application of isoproterenol, a beta agonist, into the mPOA, in the VNA lesioned animals, did not produce any significant change in S-W, though it produced arousal in normal rats. Thus, the increase in wakefulness obtained on isoproterenol administration was probably the result of its action on the pre-synaptic noradrenergic terminals (39). The possible involvement of sexual arousal in the isoproterenol-induced increase in wakefulness is discussed in the subsequent section.

Regulation of body temperature at various vigilance states

Body could be divided into two distinct compartments, ie core and shell, for describing the process of thermoregulation. But, when we consider changes in body

temperature, the brain temperature needs to be considered separately from rest of the core. The brain shows temperature changes, which are different from the rest of the body. SWS is associated with a decrease in brain temperature, and REM sleep with an increase, in many mammalian species like rabbit, rat, cat and sheep (3, 41, 42). In this description we will be considering the changes in core, brain and skin temperatures separately. There are several external and internal factors, which either alter, or tend to alter, the body temperature from the "set-point". In these situations, appropriate physiological and behavioural responses are initiated by the brain to bring the temperature back to the "set-point" (10, 11, 43). Changes in brain, core and skin temperatures, associated with transitions in the arousal states, occur in rats throughout the 24-hour diurnal cycle. In the case of body temperature control, we have seen that it is important for the internal core temperature to change as little as possible despite marked changes in the environmental temperature. The skin temperature, in contrast, rises and falls with the temperature of the surroundings, in an awake individual or animal.

Slow wave sleep:- Attempts were made to study the changes in tail skin temperatures during SWS, at different atmospheric temperatures (44). Skin temperature is regulated by sympathetic nerves, which are under hypothalamic control. At 10°C and 21°C, during awake state, the skin is cold as there is partial vasoconstriction of skin blood vessels brought about by the tonic activity of the sympathetic nerves. Falling asleep was accompanied by an increase in skin temperature and vasodilation at these lower temperatures of 10°C and 21°C. At 29°C, the skin is warm during awake state, as the skin blood vessels became intensely dilated. This happens as the sympathetic centres that cause vasoconstriction are inhibited. At this warm temperature, there is no further dilation and increase in skin temperature with sleep onset as the skin vessels are already dilated.

There was a decrease in core temperatures (including brain temperature) during SWS at all the above mentioned temperatures of 10°C, 21°C and 29°C (44). The brain temperature alterations followed the changes in S-W (3). This indicates the strong possibility that these temperature changes result from the alterations in S-W. This leads to the question whether these changes are brought about by a failure in thermoregulation during sleep or is there an active regulatory process which brings about these changes.

Involvement of an active regulatory process has been suggested as there is an alteration in the hypothalamic thermostat during sleep, rather than a failure in thermoregulation. According to this concept, the brain temperature is actively down regulated during SWS. It has been shown that the hypothalamic set points for heat production and heat loss are at a lower level during SWS in the kangaroo rat and the pigeon (43, 45). This has been described as a down regulation of brain temperature. It was proposed that the function of SWS is to cool the brain (13). According to this proposition the heat load accumulated during prior wakefulness determines the SWS intensity by appropriately down regulating the brain temperature (46). It was suggested that there was a lowering of the set point and an increase in heat dissipation with transitions from waking to SWS (10, 45). It was thus suggested that SWS is a part

of the thermoregulatory process that controlled the body and brain temperature.

Taking clues from the active down regulation of body / brain temperature during SWS, it was hypothesised that the SWS-induced brain and body cooling would lower the energy utilisation and reduce cerebral metabolism. In other words, SWS acts as a protection of the brain against the sustained high temperatures of wakefulness. As the mPOA is the most important region of the brain for maintenance of SWS, it is reasonable to assume that this area plays an important role in bringing down the body / brain temperature during SWS. But, the brain temperature variations with sleep-wake changes were not only present, but were even higher in the mPOA lesioned rats (3). So, it was concluded that the mPOA was not involved in the down regulation of brain temperature at various vigilance states.

As there was a reduction in the sympathetic tone and heat production during SWS, it may be assumed that the efficiency of the thermoregulatory mechanism is decreased during this phase of sleep. In man, the largest fall in body temperature, associated with SWS, occurs at the beginning of sleep. This is associated with the change in body posture from an upright position to a recumbent position, and not with the depth of SWS, or stages 3 and 4 of sleep (47). In rats also, the increase of slow wave activity (mean power density in the 0.75-4.0 Hz range) and the decrease of cortical temperature in SWS episodes, were not correlated (48). So, the decrease in body temperature, at least during the initial part of sleep, was independent of SWS (49).

The vigilance dependent changes in the hypothalamic (and brain) temperature of homeotherms are brought about by adjustments in arterial blood flow that could cool the brain. However, there are different mechanisms for brain cooling, i.e. systemic and selective brain cooling. They are affected by the changes in body posture and vasoconstrictor sympathetic outflow related to wake-sleep states (50).

REM sleep:- Core and skin temperature show variations during REM sleep. But, there has been a lot of speculation and debate about the changes in brain temperature. REM sleep was associated with a rise in brain temperature, and the rise was the largest in the cold environment and was attenuated at the warm environment in rats (3, 44). There was no change in brain temperature, when the rats were maintained at 30°C, though there was increase in the brain temperature with a shift from deep SWS to REM sleep at 18°C and 24°C environmental temperature (3). Increase in brain temperature with REM sleep occurs in most mammalian species that have been investigated. But, still there is some doubt about the changes in brain temperature in primates. It has been reported that there was no change in brain temperature in monkeys during REM sleep (51). In human subjects, the tympanic temperature (which could be taken to represent the brain temperature), and even the forehead skin temperature, increase during the REM sleep (52).

Even if it is assumed that the brain temperature alteration during REM sleep is an active process (45), it is likely that the mPOA may not be responsible for this change (3). On the other hand, the posterior hypothalamic lesions produced either a suppression of the increase (or even a decrease) of brain temperature during REM sleep, while skin temperature variations

were not modified. The decrease in cerebral blood flow, which was also always associated with increase in brain temperature, was suppressed after the posterior hypothalamic lesion. So, it was hypothesised that the decrease in brain blood flow depends upon an active vasoconstriction process originating in the posterior hypothalamus (53).

Increase in brain temperature with REM sleep was attributed to an increase in local metabolic rate, and changes in cerebral blood flow (53). During REM sleep, common carotid artery blood flow is spontaneously decreased (54). Simultaneously there is an increase in the amount of vertebral artery blood flowing into the brain (through the circle of Willis). In other words, an increase in brain temperature during REM sleep is characterised by a shift from the carotid artery to the vertebral artery, and probably also to other arterial sources (55). The increase in vertebral artery blood flow appears primarily as an autoregulatory response to the drop in carotid artery blood flow during REM sleep, in response to brain activation in REM sleep (56).

Core temperature decreased and skin temperature increased in the cold, whereas core temperature tended to increase, and skin temperature to decrease, in the heat. This paradoxical peripheral vasomotion during REM sleep supports the previous suggestions on severe thermoregulatory impairment during REM sleep in rats and other species (44). In the cold ambient temperature, deep interscapular (just below the brown fat lobes) temperature decreases during desynchronized sleep. This change in temperature probably results from a depression in sympathetic vasoconstrictor influences, producing blood and brown fat cooling during this stage of sleep (57, 58). But the increase in hypothalamic temperature during this stage of sleep occurs independently of a transfer of heat from interscapular brown fat (57, 58).

It was generally believed that during REM sleep, thermoregulatory responses are virtually absent and that body temperature becomes temporarily dependent on ambient temperature. Therefore, REM sleep has been referred to as a poikilothermic state (59). On the other hand, during REM sleep, sweat gland activity persists though at a lower level than during SWS (60). The observation that REM sleep propensity is highest when core body temperature reaches its lowest physiological level, led to the suggestion that REM sleep represents a regulated mechanism for warming the central nervous system (61). It is difficult to accept that the functions of SWS and REM sleep are to cool and heat the brain respectively, as both the sleep stages were increased with higher ambient temperature (3).

There are some basic differences in REM sleep in animals and humans. REM sleep is the deepest stage of sleep in animals. But, human subjects could be more easily woken up from REM sleep than from SWS. There was an increase in oxygen consumption in human subjects during REM sleep (52). The temperature of the skin of the limb extremities declined at 21°C during REM sleep. Thermoregulation is not likely to be suppressed during REM sleep in humans, unlike in other mammals, as there is peripheral vasoconstriction, increased tympanic temperature and oxygen consumption, and no reduction in REM sleep, when they are exposed to cold (52). Skin temperature showed a small, but significant, increase during REM sleep at

29, 34, and 37°C, but the rectal temperature did not change during REM sleep at any atmospheric temperature. Shivering, which was present during wakefulness at 21°C and 24°C, occurred only occasionally during stages 1 and 2 sleep at 21°C. The increases in oxygen consumption and the absence of marked changes in vasomotor tone during REM sleep in the cold were unexpected (as compared to other mammals), and possibly indicate that this phase of sleep is not as thermally disruptive in humans as in other mammals (62). These differences in thermoregulation should be also viewed along with the differences in REM sleep itself, in man and in other animals.

Effect Of Ambient and Body Temperature on Sleep

Further evidence of a close relationship between sleep regulation and temperature regulation has been derived from experiments in which sleep was analysed after experimental manipulations of ambient temperature, body temperature or brain temperature.

a. Effect of Ambient Temperature on Sleep

Acute exposure to an ambient temperature outside the thermoneutral range has a prominent effect on both temperature regulation and sleep regulation. Though it is possible to define the thermoneutral zone as the comfortable ambient temperature range for human beings, it is difficult to define the same for experimental animals. If the thermoneutral range is defined as the range of ambient temperature in which metabolic heat production is minimal, for the inactive rat, this range is approximately 26-33°C (17, 63). If the absence of behavioural thermoregulation of the rat is taken as a criterion, the range is 18-28°C (63). The maximum REM sleep time is also used to define the thermoneutral temperature. At approximately 30°C, maximum values of REM sleep are obtained (5, 64). REM sleep seems to be more sensitive to changes in ambient temperature than SWS. In the rat a general linear decrease in the percentage of REM sleep from 23°C to 10°C has been reported (44, 65). Thus the REM sleep is reduced during that period in which the regulation of body temperature is suspended. The amount of SWS is also decreased by low ambient temperature (5, 44, 65).

The changes in S-W were studied in rats when they were exposed to different ambient temperatures of 18°C, 24°C and 30°C (5). There was an increase in REM sleep and SWS, and a decrease in wakefulness at higher ambient temperatures. The increase in sleep was primarily due an increase in the duration of sleep episodes.

The increase in the amount of sleep with enhanced ambient temperature may be considered as an adaptation to thermal load aimed at energy conservation (4). REM sleep has been shown to be very sensitive to slight variations in the thermal environment and it varies significantly even within 25°C and 30°C, which have been defined as the thermoneutral zone for rats on the basis of the minimal metabolic rate (66).

It was suggested that when the ambient temperature is low, the central nervous system has to call for an increase in the relative amount of arousal, at the expense of the sleep stages, especially desynchronised sleep, in order to maintain

the body temperature (67). An increase in arousal in cold is necessary for the production of more heat by increasing motor activity. REM sleep, in which the regulation of body temperature is said to be suspended, is incompatible with low ambient temperature, during which appropriate thermoregulatory responses are needed to protect the animals from hypothermia (68). In other words, the functional state of wakefulness enables the organism to optimise thermoregulation.

The changes in S-W were also studied during their exposure to different ambient temperatures after the destruction of the mPOA neurones by NMDA. The mPOA neuronal destruction produced a decrease in sleep at all the three different ambient temperatures. There was a decrease in sleep, particularly the deeper stages of sleep (deep SWS and REM sleep) after the mPOA lesion (8, 69). But, there was a linear increase in sleep with higher temperatures (5). The sleep induced by higher temperatures in the lesioned rats was qualitatively different from that in the normal animals. In normal animals, there was an increase in long duration SWS episodes with higher ambient temperature. But on the other hand, after the mPOA lesion, 30°C ambient temperature produced an increase in the number of short duration SWS episodes. It has been reported that the mPOA is thus important for the maintenance of sleep, as it was the sleep duration, which was primarily affected by the mPOA lesion (8). The warm environment could increase the amount of sleep, even after the mPOA lesion, but the higher ambient temperature was more efficient in initiating sleep rather than in maintaining it. In other words the ability to maintain SWS was affected after the mPOA lesion, and this ability could not be restored by exposure to a warm environment. The findings indicate that the mPOA is essential for sleep maintenance and improving the quality of sleep with higher ambient temperatures.

The decrease in REM sleep frequency might have resulted from a decrease in SWS. REM sleep normally appears after the animal has spent some time in SWS. So, it is possible that the decrease in the duration and frequency of deep SWS, after the mPOA lesion, had resulted in the decreased frequency of REM sleep (8). Though the REM sleep was reduced after the mPOA lesion, the warm environment could prolong the duration of REM sleep episodes, once they were initiated. Thus, the warm environment could influence the REM sleep even in the absence of an intact mPOA. This is understandable, as the major REM sleep generating structures are outside the mPOA.

From the results of this study, it can be concluded that the mPOA is essential to increase sufficiently the duration of sleep episodes (especially SWS) by thermal stimulus, though sleep could be induced through structures other than this area. In other words, the mPOA is essential for organising the sleep architecture (especially SWS), as per the thermoregulatory requirement. It may be mentioned here that one suggested function of the mPOA is to provide a fine-tuning of the energy balance, which will be discussed later (8, 70, 71).

b. Effect Of Body and Brain Temperature on Sleep

Despite thermoregulatory responses, body temperature and brain temperature in the rat increase by more than 1°C over a 24-hour period if the ambient temperature is increased from 21°C to 29°C (44). This

increase in brain temperature and body temperature can evoke an increase in SWS in animals and in human subjects (13, 46, 72, 73, , 74). Even radio frequency diathermic warming of the POA in cats and opossum could induce sleep. Cooling the POA produces huddled posture. Roberts and Robinson (14) have suggested that the POA thermoreceptors may provide an input to the sleep-regulating mechanisms in this area itself. Stimulation of central receptors by changing blood temperature is likely to be an important source of impulses driving the sleep inducing structures of the basal forebrain (75). It was hypothesised that the SWS in mammals and birds is controlled by thermoregulatory mechanisms (13).

Studies have shown that SWS is facilitated when brain temperature exceeds a threshold level (13). This threshold is hypothesised to be determined by responses of preoptic-anterior hypothalamic thermosensitive neurones and to be regulated by both circadian and homeostatic processes. Local warming of the POA produces sleep (14, 76, 77). Preoptic-anterior hypothalamic warming increases EEG delta frequency activity during SWS (78). So, it was suggested that the preoptic-anterior hypothalamic thermoregulatory mechanisms participate in the regulation of the depth of SWS. According to Nakao et al. (79) the SWS is controlled by thermoregulatory mechanisms of the preoptic-anterior hypothalamus. Circadian and homeostatic thermoregulatory processes may be integrated in this brain area.

c. *Effect Of Chronic Exposure to Cold Environment on Sleep*

Changes in S-W induced by acute cold stress may not persist during long-term exposure, as homeostatic regulatory mechanisms may reset the various components of sleep, during the period of thermoregulatory acclimatisation. It was hypothesised that circadian and homeostatic processes regulate the activities of the thermosensitive neurones that control the amount of SWS (13). Continuous recording of S-W for 24hrs, during chronic exposure to cold, along with brain temperature, showed that there was a decrease in sleep, especially paradoxical sleep (PS), during the initial days of exposure to a mild environment of 18°C. Though the sleep parameters came back to the control level by two to three weeks of exposure, the brain temperature remained high, even on continued exposure to cold for four weeks. It is proposed that the elevated brain temperature also played a role in homeostatic restoration of sleep, especially S2 and PS. It was during S2 and PS that the brain temperature was maintained at a higher level, compared to pre-exposure values. This increased brain temperature could be interpreted as a resetting of thermoregulation (or thermostat) to ensure adequate sleep, especially S2 and PS. It has been shown that increasing the body temperature, especially the brain temperature around the preoptic area does induce sleep (4, 11, 13, 80). The adaptive resetting of brain temperature may provide continued stimulus to the sleep generator.

Homeostatic mechanisms would have ensured that the changes in S-W on acute exposure to a low ambient temperature did not persist during long-term exposure. In this study it was observed that the circadian variations in sleep, as well as brain temperature, were disrupted during the acute exposure to a low ambient

temperature During the acclimatisation to low ambient temperature, several factors might have come into operation and reset the various components of sleep and brain temperature. It was observed that there was restoration in circadian variations in S-W and brain temperature by the fourth week of cold exposure. It was suggested that the functional state of wakefulness enable the organism to optimise thermoregulation (44, 68, 80). Though it could be true to some extent during acute cold stress, thermoregulation may be readjusted to ensure homeostatic regulation of sleep during chronic cold exposure.

Preoptic Neuronal Activity as the Basis for Sleep Temperature Interlink

The modulation of the thermo-regulatory responses by the vigilance state could be observed even at the level of neuronal activity. It has been demonstrated that there are neurones in the mPOA involved in the regulation of sleep and body temperature (81, 82, 83). The number of neurones in the preoptic-anterior hypothalamus that were thermosensitive, as well as the thermosensitivity of individual neurones, were reduced in SWS as compared to the wakeful state (84). Most neurones became thermo-insensitive in REM sleep. Thermosensitive neurones of the preoptic-anterior hypothalamic area have been implicated in the regulation of both body temperature and SWS (85). The activation of sleep-related warm-sensitive neurones and the deactivation of wake-related cold-sensitive neurones may play a key role in the onset and regulation of SWS (86). During SWS, a majority of preoptic-anterior hypothalamus warm-sensitive neurones exhibit increased discharge as compared to the wakeful stage. Cold-sensitive neurones exhibit less discharge in SWS, than in wakefulness. Warm-sensitive neurones with increased discharge in SWS exhibited increased thermosensitivity during SWS than in wakefulness. Cold-sensitive neurones with decreased discharge during SWS exhibited decreased thermosensitivity in SWS. In addition, a few neurones that were thermo-insensitive during wakefulness became warm-sensitive during SWS (12).

Warm-sensitive neurones did not exhibit a significant change in thermo-sensitivity during REM sleep as compared to wakefulness and SWS (85). In contrast, cold-sensitive neurones exhibited decreased mean thermosensitivity during REM sleep than in wakefulness. Cold-sensitive neurones as a group did not retain significant thermosensitivity in REM sleep. These findings are consistent with evidence that thermoeffector responses to cooling are lost in REM sleep, whereas some responses to warming are preserved (85).

Osaka & Matsumura (83) examined the effects of NE on the activity of sleep-related neurones in the POA and the neighbouring basal forebrain in the rat. NE and the alpha 2-agonist clonidine generally inhibited sleep-active neurones, whereas the alpha 1-agonist methoxamine and the beta-agonist isoproterenol had no effect on them. Thus, alpha 2-receptors mediated the NE-induced inhibition. NE and methoxamine excited the waking-active neurones, whereas isoproterenol and clonidine did not produce any effect. Accordingly, alpha 1-receptors probably mediated the NE-induced excitation. State-indifferent neurones and REM sleep-active neurones were mostly insensitive to NE. According to Osaka & Matsumura (83), these results suggest that NE promotes wakefulness by inhibiting sleep-active neurones and by exciting waking-active neurones.

Food intake, energy conservation and sleep regulation

It has been hypothesised that hibernation, which is a state showing extreme adaptations for energy conservation, is an evolutionary extension of SWS (87). Phylogenetic and ontogenetic associations between sleep and endothermy are consistent with the hypothesis that sleep evolved in conjunction with endothermy to offset the high energetic cost of endothermy (88). According to them the electrophysiological and thermoregulatory continuum of SWS, circadian torpor and hibernation substantiates a primordial link between sleep and energy conservation. When energy stores decline, energy is conserved by lowering Tb proportionally during sleep or by increasing the daily duration of sleep. Furthermore, these states of hibernation and torpor are entered via SWS (87). These observations prompted some scientists to hypothesise that SWS is an adaptive behaviour for energy conservation in homeotherms (42, 87). But, hibernation is regularly interrupted by short periods during which body and brain temperatures are up regulated to euthermic levels. Though the function of these energetically very expensive episodes is unknown, animals spent most of this time in SWS (89, 90, 91). Sleep, daily torpor and hibernation are no longer considered homologous processes. Animals emerging from these states spend most of their time in sleep, indicating that they were deprived of sleep during torpor. After termination of the torpor-associated hypothermia, there is a compensatory increase in SWS, as it happens subsequent to sleep deprivation.

Earlier reports have shown that the alteration in food intake can disrupt sleep (92). There are reports in the literature that indicate that REM sleep deprivation or total sleep deprivation increases the food intake (93, 94, 95). But the decrease in SWS and REM sleep, resulting from the mPOA lesion, did not produce any increase in food intake and water intake (8). Food deprivation in birds and squirrels resulted in a lowering of the thermoregulatory set point during sleep along with increased SWS (88).

Though there was no significant persistent change in food intake, there was a reduction in the body weight of the rats after the mPOA lesion with NMDA, and electrolytic lesion of the POA (8, 64). Higher locomotor activity and increased body temperature, after the mPOA lesion, produced increased energy expenditure. This might have resulted in a decrease in the body weight because there was no concomitant compensatory addition in energy intake (food intake), in spite of the increase in locomotor activity, rectal temperature and awake period. Therefore, after the lesion, the animal did not recognise low energy reserves, and so it did not bother to conserve energy. Thus, it can be hypothesised that the mPOA lesioned animals had lost the mechanism for the fine-tuning of food intake, in response to the alteration in body homeostasis. The functional integrity of the mPOA may be essential for the regulation of food intake, in response to alterations in the temperature, locomotor activity and S-W. It can also be argued that the mPOA would normally facilitate sleep, an energy-conserving state, when energy reserves are at a critical level (8).

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New Challenges in Gene Delivery *in vivo*

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SUMMARY

Adverse effects of viral vectors, instability of naked DNA, cytotoxicity and low transfection of cationic lipids, cationic polymers and other synthetic vectors are severe limitations in gene therapy now-a-days. An ideal non-viral vector, in addition to targeting to specific cell type, must manifest an efficient endosomal escape, render sufficient protection of DNA in cytosol and help make an easy passage of cytosolic DNA to nucleus. Virus-like size calcium phosphate nanoparticles have been found to succeed many of these limitations in delivering genes to the nucleus of specific cells. This review article has focused some applications of DNA loaded calcium phosphate nanoparticles system as non-viral vector in gene delivery and its potential use in gene therapy as well as highlighted the mechanistic studies to probe the reason for high transfection efficiency of the vector. It has been demonstrated that calcium ions play an important role in endosomal escape, cytosolic stability and enhanced nuclear uptake of DNA through nuclear pore complexes. The special role of exogenous calcium ions to overcome obstacles in practical realization of this field suggests that calcium phosphate nanoparticles is not a "me too" synthetic vector and can be designated as second generation non-viral vector for gene therapy.

INTRODUCTION

Gene therapy is an alternative therapeutic procedure in molecular medicine which can be defined in a most simplified way as a technique by which therapeutic gene can be introduced into the nucleus of a somatic cell and the product of which is responsible for curing or retarding an inherited or acquired disease. The concept of gene therapy was originally developed in early seventies (1). Prolonged studies based on the basic concept of gene therapy and output of some promising data from *in vitro* and animal models studies and through clinical trials have culminated to the birth of several proof-of-the concept results which led the scientists to have high expectations for the practical implementation of the gene therapy technology in the clinic. But unfortunately it has still not come up as a successful tool for the treatment of human diseases. With the promise of specificity and low toxicity it is anticipated that such technology will have a lasting impact on medicine and biotechnology.

The first approved protocol of gene therapy used for human being was in September 1990 (2). During the span of more

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than 14 years since then about 600 gene therapy protocols have been approved and nearly 5000 human beings have been subjected to carry genetically engineered cells into their body (3). The outcome from these trials has established that gene therapy has potential to make revolution in medicine for treating broad array of human diseases and the procedure appears to have very low risk of adverse side reaction unlike drug therapy.

Despite growing body of knowledge regarding the chemistry, biology and pharmacology of genes, the widespread application of DNA based therapeutic remains a daunting challenge. Practical implementation has been more difficult than originally expected. Efficient expression of gene to get therapeutic product is still a major obstacle in further advancement of this field of research. Due to large size, highly anionic in nature and easy degradability by the nucleases, naked DNA results in quite low levels of transduced gene expression (4). In the beginning of gene delivery research in early eighties (5), scientists thought of exploiting natural ability of viruses of delivering genetic materials for the delivery of therapeutic transgenes. Till now this is the most popular way of delivering genes used in gene therapy protocol. However, the inadequacy of the current methodology based on virus as carrier for DNA is attributable to the limitations including immunogenicity, cytotoxicity, restricted targeting of specific cell types, limited DNA carrying capacity, production and packaging problems, recombination and high cost. Most important concerns among them are the stimulation of strong immune responses and the potential for oncogenic transformations (6). These major limitations and eventual clinical failures of viral vectors have led to a shift in focus on synthetic non-viral gene delivery systems. Synthetic vectors, although currently orders of magnitude less efficient than biological vectors, are increasingly being considered as possible alternatives to viral vectors. Unfortunately, the low *in vivo* efficiency often, precludes their use for gene therapeutic purposes; yet, their favourable 'inertness' justifies further exploration of non-viral alternatives (7). Non-viral synthetic carriers such as polymers or lipids are attractive owing to flexibility in design ability to be chemically or biochemically functionalized and tunable toxicity properties. However, the gene expressions using these synthetic carriers are still very inefficient compared to viral vectors. Non-Viral vectors are synthetic vehicles for the introduction of foreign DNA sequences into target cells. Various methods for non-viral gene therapy have been proposed. Some approaches are aimed at developing an artificial virus that attempt to mimic the process of viral infusion using synthetic materials. Other approaches apply the theory and methods of advanced particulate drug delivery to deliver DNA to selected somatic targets. These approaches employ DNA complexes containing lipid, proteins, peptide or polymeric carriers as well as ligands capable of targeting the DNA complex to cell-surface receptors on the target cells and ligands for directing the intracellular trafficking of DNA to the nucleus. These non-viral vectors rely on the basics of supramolecular chemistry in which anionic DNA molecules are condensed into compact, ordered nanoparticles that are 50-200 nm in diameter by complexing DNA with an appropriately designed cationic molecule (8). The polycations reduce the size of the com-

plex, and confer excess cationic charge to the complex, thereby enhancing their cellular uptake by an endocytosis pathway. Since very large DNA molecules can be condensed into compact particles, non-viral vectors permit the incorporation of the gene regulatory regions that may afford better control of gene expression. DNA molecules as large as 2.3 mega base-pair (Mb) can be condensed into compact particles and these particles are suitable for gene delivery (9). Once DNA condensation has been established with synthetic vectors, it is possible to incorporate functional molecules or groups into the carrier entity either by physical entrapment or by chemical conjugation so that cell-specific targeting, cytoplasmic release as well as nuclear localization of DNA can be facilitated (10).

Lipid-based carriers (11), polycationic lipids (12), polylysine (13), polyornithine (14), histones and other chromosomal proteins (15), hydrogel polymers (16), all of which can ionically condense DNA and bind to the cell surface, are found to be ideal candidates for these vector types. But in the use of different types of cationic liposomes, cationic polymers and dendrimers as non-viral vectors for delivery of genes, it has been observed that in addition to cytotoxicity, these carriers do not lead to satisfactory amount of gene expression in the cells. *In vitro* transfection of cultured cells with plasmid DNA is a crucial indication towards the success of DNA as pharmaceutical. Because of the unsuccessfulness of the existing non-viral vectors for providing satisfactory and efficient transfection, these studies also, albeit for different reasons, could not bring the gene therapy technology at the level of clinical practice.

We have recently developed some inorganic phosphate nanoparticles like calcium phosphate, magnesium phosphate, manganese phosphate nanoparticles as safe and efficient carriers of plasmid DNA which manifested quite high degree of transfection efficiencies compared to liposomes and other non-viral vectors (17-19). Calcium phosphate as such is not a new candidate for facilitating gene transfer and subsequent gene expression. One of the early methods of gene transfer in cells in culture involved co-precipitating DNA with calcium phosphate (20). When added to a cell monolayer this insoluble precipitate is taken up by the cell through endosomal pathway and the endosome is broken down in a calcium ion dependent manner, thereby releasing the DNA into the cytosol, which, under suitable circumstances, can be incorporated into the host cell genome. The process by which this transfer of DNA occurs is poorly understood. But it is clear that the division of the recipient cell within 24 hours to 48 hours of gene transfer is important. This method is found to be simple, effective and has been widely used. However, the main drawback of the method of using precipitated calcium phosphate is the extremely low transfection efficiency (10-15%) as compared to that of viral vectors and the difficulty of applying it to *in vivo* studies (21). Moreover, the method also suffers from variations in calcium phosphate-DNA particle sizes, which causes variation among experiments. The transfection efficiency has been found to be dependent on a large number of factors, such as the cell type used, the morphology of the precipitate, pH of the buffers and the time of contact between precipitate and cells. Summing up the results of various studies, it is clearly understood that one of the major impediments of using calcium phosphate precipitate as carrier for nuclear delivery of DNA, like other particulate carriers, is the large

particle size (22) which resulted in the slow transfer of the materials across the cell membranes. Calcium phosphate nanoparticles of average size greater than 300 nm diameters have also been reported to serve, as non-toxic, biocompatible adjuvant for vaccination but these particles are too large to use as carrier for effective DNA delivery (23-24).

Therefore, these two wrongfully conceived myths of low transfection efficiency and inability to apply calcium phosphate precipitates *in vivo* condition have arisen due to large particle size of calcium phosphate and can be overcome by preparing the virus-like size calcium phosphate nanoparticles i.e. diameter of 100nm or less, in which DNA molecules are encapsulated to completely protect the nucleic acid from external hostile environment. It is envisaged that if these two impediments are overcome, the use of calcium phosphate and probably other biocompatible inorganic nanoparticles as gene carriers can become a novel technology in the area of gene therapy and are advantageous over the other viral and non viral vectors in the sense that these vectors are not only safe as well as cost effective but also may overcome many limitations of the other vector types.

With the prospect of using calcium phosphate nanoparticles as carriers, we have involved the use of the aqueous core of the water-in-oil microemulsion as nanoreactor for the preparation of controlled tailor-made size and plasmid DNA encapsulated calcium phosphate nanoparticles (18). These pDNA-loaded nanoparticles have been prepared by forcing the DNA-calcium phosphate co-precipitation inside the aqueous core of the reverse micelles to obtain a highly monodispersed nanoparticles with a narrow size distribution which have been further characterized by their crystal structure, surface charge, aggregational behaviour and pH dependent pDNA release. Upto an extent of 20% w/w of

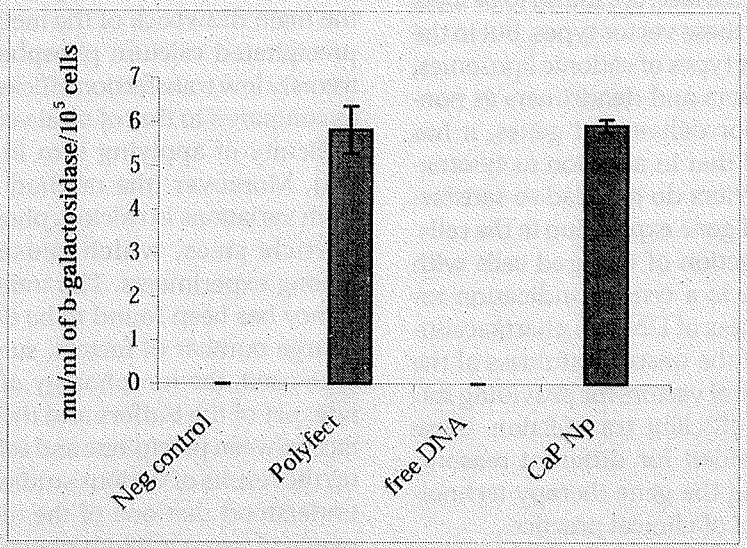

Figure 1 : *In vitro* transfection of pSVβgal plasmid in *HeLa* cell line (vide ref 18). Polyfect as positive control (vide ref 44).

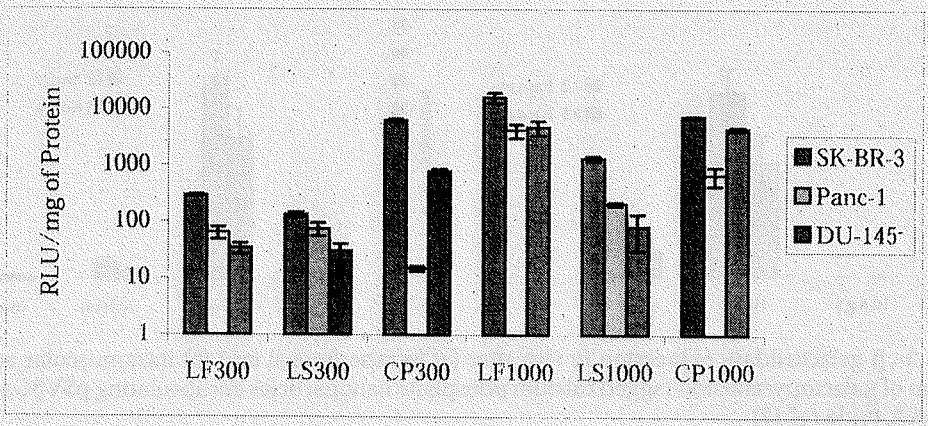

Figure 2 : Luciferase expression in different cell lines using lipofectamine (LF), Liposome (LS) and Calcium Phosphate nanoparticles (CP) as non-viral vectors in presence of transferrin (DNA : Tf = 1:12.5 w/w). DNA quantity 300ng and 1000ng respectively (vide ref 25).

pSVβgal plasmid in calcium phosphate nanoparticles could be loaded. Moreover, these polymer capped nanoparticles can be stored easily in the form of a dry lyophilized powder, which can be readily redispersed in aqueous buffer. It was also demonstrated (18) that the transfection efficiency of these particles in HeLa cell line using pSVβgal as a marker plasmid was found to be more than 100% to that of the polyfect used as control (Figure 1). In DU145 cell lines the transfection efficiency of DNA loaded calcium phosphate nanoparticles was found to be about 1000 times higher compared to that in liposomal system (Figure 2) (25).

One of the important aspects of a gene therapy vector is that it should be targeted to specific cell types. It is possible to attach the liver targeting ligands such as asialoglycoprotein, lactose, galactose etc. on the surface of the nanoparticles by chemically conjugating on the particle surface. It has been demonstrated (17) that the surface modification of pSVβgal loaded calcium phosphate nanoparticles and coated with polyacrylic acid through chemical conjugation of ligand such as p-aminophenyl-1-thio-β-D-galactopyranoside (PAG) are specifically targeted to liver paranchymal cells in vivo (Figure 3a&b) resulting in an enhanced expression of βgalactosidase in the liver.

Calcium Phosphate nanoparticles have been applied as non-viral vector for gene therapy of experimental autoimmune hepatitis (26). Development of disease model was established from significantly elevated levels of IgG, presence of antinuclear antibodies and necroinflammatory changes in the liver tissues and significant depletion of IL-2. Nanoparticles of Calcium phosphate encapsulating the plasmid expression vector of IL-2 (pUMVC3-mIL-2) were synthesized using reverse micelles method (18). More than 60-fold increase in IgG level was observed in disease animal which have been significantly reduced when the animals were treated with pUMVC3-mIL-2 encapsulated calcium phosphate nanoparticles (Figure 4). Fluoresceinated nuclei seen in the liver tissue section of diseased animal indicating the presence of

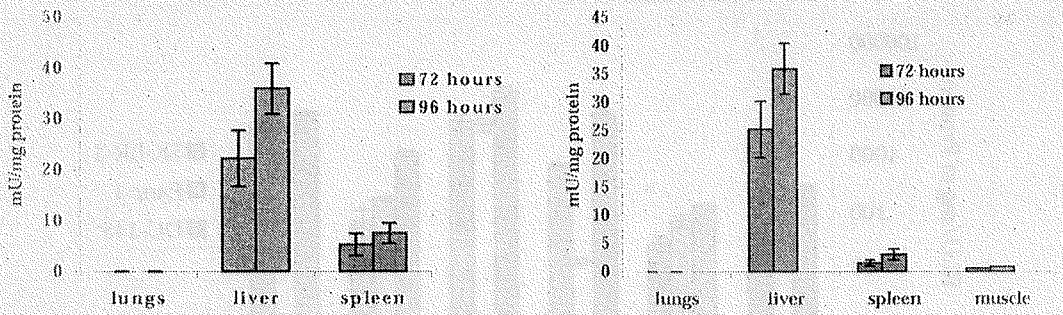

Figure 3 : β-galactosidase expression *in vivo* after (a) intraperitoneal and (b) intramuscular administration of galactopyranoside-tagged calcium phosphate nanoparticles encapsulating pSVβGal plasmid DNA (vide ref 17)

antinuclear antibodies in the serum have been totally eliminated when these animals were treated with pDNA loaded calcium phosphate nanoparticles (result not shown). Histopathological observations of liver sections also indicated a mark regression of inflammatory condition of the tissues. From the results, we have conclusively established the effective therapeutic use of calcium phosphate nanoparticles as non-viral vector *in vivo* for pDNA expression of IL-2.

addition to antibodies because of the increasing recognition of the role and need for CTL in such vaccines. Efforts are also being made to develop vaccines that can induce specific types of T helper responses; Th1 or Th2. DNA vaccines have the potential as new vaccines because of their ability to elicit both humoral and cellular immunity. DNA vaccines are also thought to be potentially safer than traditional vaccines (28). Whilst safety was demonstrated and

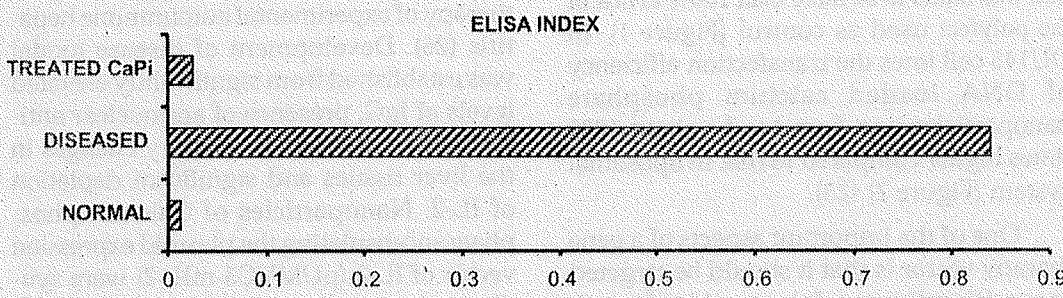

Figure 4 : Elisa index of Anti-liver surface protein antibody in the serum (vide ref 26).

Another interesting application of DNA loaded calcium phosphate nanoparticles reported recently is in DNA vaccines (27). New efforts to develop vaccines emphasized on inducing CD8+ cytolytic T lymphocytes (CTL) responses in

immune responses were generated in many DNA vaccine formulations, overall the potency has been found to be disappointing. A variety of approaches are under evolution to increase the potency of DNA vaccines whilst still retaining their attractive

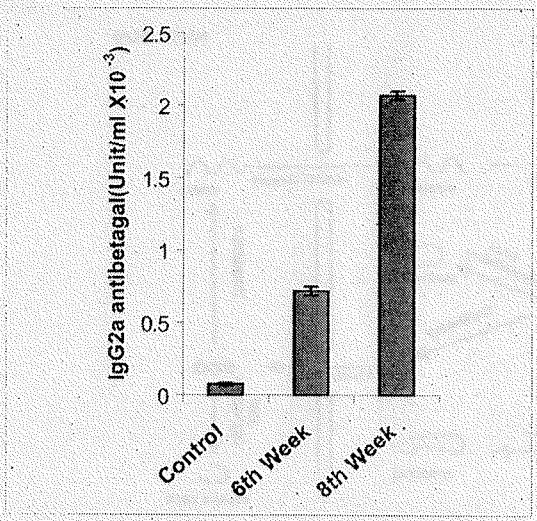

Figure 5(a) : Immunoglobulin (IgG2a) assay in serum of mice injected intraperitoneally with 0.9 μg of pDNA entrapped in calcium phosphate (vide ref 27)

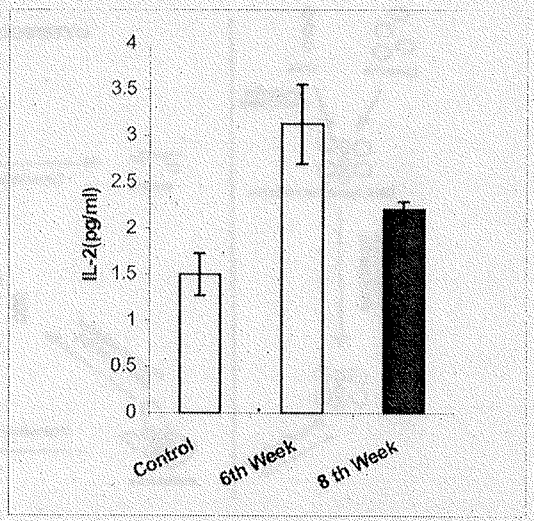

Figure 5(b) : Lymphokine proliferation in Splenocytes of mice *in vitro* (Vide ref 27)

features. Based on the observation of high transfection efficiency by using calcium phosphate nanoparticles as non-viral vector it was thought promising to use these nanoparticles as carriers for DNA vaccines. As a preliminary step an investigation was conducted using pSVβgal plasmid as marker DNA encapsulated in calcium phosphate nanoparticles in murine model. A 10-fold increase of antibody levels in the 6th week post immunization predominated by IgG2a along with immunological synapse formation and lymphocyte proliferation were manifested (Fig. 5). Thus, it is presumed that these calcium phosphate nanoparticles entrapping pDNA have a high potential and a well-defined role in DNA delivery as well as in transfection that can also be used as a further advantage in DNA vaccination.

Now the question is: why calcium phosphate nanoparticles should be preferred to other non-viral vectors? Why calcium phosphate nanoparticles exhibit such a high transfection efficieny? What is mechanism of intracellular trafficking of DNA from the cell surface to the nucleus? According to the current state of knowledge, most of these non-viral DNA delivery systems operate at one of the three general levels: DNA condensation, and complexation, endocytosis and nuclear targeting/ entry (Figure 6).

Negatively charged DNA molecules are usually condensed with different cationic vectors before the entry. Due to its excess positive charge, the vector-DNA complex binds to the negatively charged cell membrane and is subsequently taken up by endocytosis. After endocytosis, the DNA containing particles are largely retained in perinuclear endosomes/ lysosomes. The entrapment of the carrier within these vesicles is one of the major barriers for transfection. The extremely low pH and enzymes within endosomes and

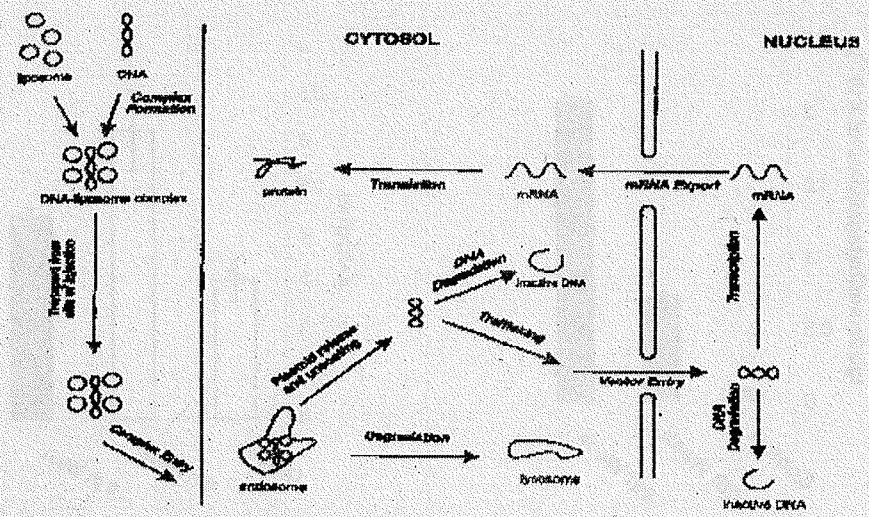

Figure 6 : Schematic illustration of the process involved in gene expression

lysosomes usually bring about the degradation of entrapped DNA inside the carriers. So, endosomal escape is one of the most crucial steps. Ideally, the entire delivered vector should escape from the endosome before degradation along the trafficking route, either by vector fusion with the endosomal membrane or through endosome disruption, in either event, ultimately resulting in the release of the nucleic acid (with or without the protective vehicle) into the cytosol (29).

Several approaches have been used in an attempt to facilitate the cytoplasmic release of the DNA. Ca^{2+} in the form of nascent calcium phosphate microprecipitates (29) and other lysosomolytical agents such as chloroquine, facilitate endosomal/lysosomal release by their fusiogenic and membranolytic activity (30).

In the case of calcium phosphate nanoparticles as delivery system, use of additional compound to disrupt the endosome is not required. This is because, upon internalization, endosomal compartments undergo continuous acidification from the initial cell-surface pH (~7) to an acidic pH of about 5.0.As a result, the dissolution of calcium phosphate nanoparticles in such an acidic endosomal compartment takes place (18) which ultimately leads to an osmotic disbalance and consequent disruption of the endosomal compartment thereby facilitating the release of entrapped DNA into the cytosol.

Exit from the endo-lysolytic pathway however, does not always result in high transfection efficiency. Once the DNA is released in cytoplasm, it must reach the nucleus for expression. There is considerable evidence suggesting that the nuclear membrane presents a barrier to the entry of free or carrier-associated DNA from the cytoplasm (31-33)1. The nucleus of a cell is separated from the cytoplasm by concentric bilayers that form a double membrane structure known as nuclear envelope (34). The nuclear envelope is an integral part of the structural framework of the nucleus (Figure 7), and is involved in organizing

intranuclear events. It serves as a selective barrier, actively transporting proteins required for normal nuclear function and exporting RNA. The movement of molecules across the nuclear envelope is critical for cellular homeostasis, and it allows cells to respond to external events. The outer bilayer of nuclear membrane is continuous with the endoplasmic reticulum. The inner bilayer renders the nuclear structural stability through protein filaments. The inner and outer bilayers fuse at sites throughout the nuclear envelope where large protein pores known as nuclear pore complexes (NPC), are located. The NPCs are the only known pores spanning both bilayers of the nuclear envelope and, as such, represent the most direct pathway into or out of the nucleus. The intermediate space between the two bilayers of nuclear envelope is called cisterna where Ca^{2+} is stored and is released to regulate the passage of molecules through NPC.

After the DNA is released into the cytosol, it may enter the nucleus through the nuclear pore directly or by the support of the nuclear localization signals. The transport of the DNA across the nuclear envelope occurs through the nuclear pores (35). Once within the nucleus, DNA is transcribed into the mRNA, which is then processed and translated into a therapeutic gene product. Nucleocytoplasmic transport from cytoplasm along the NPC proceeds through three mechanisms – (i) unregulated passive diffusion, which is limited to spe-

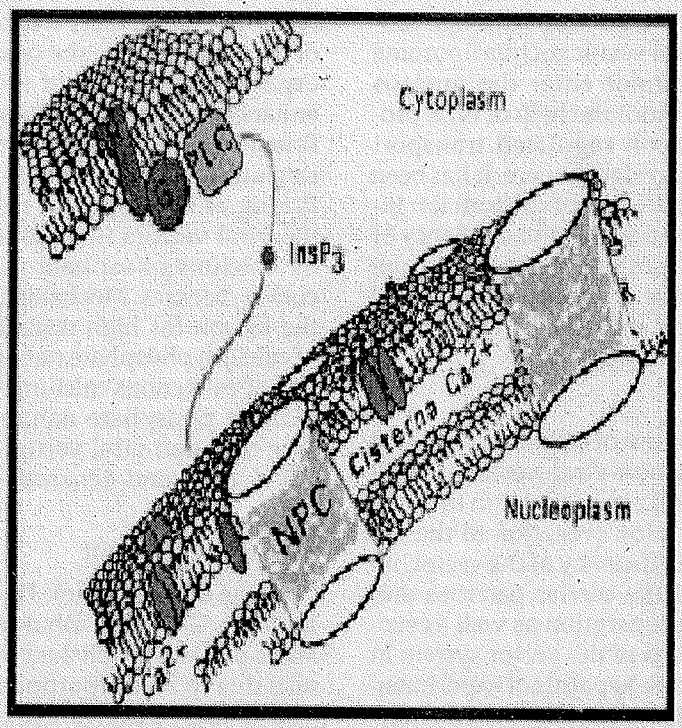

Figure 7 : Schematic of the components of the nuclear membrane involved in CA^{2+} regulated transport through the nuclear pore complex (NPC) (vide ref 34)

cies of less than 10 kDa; (ii) active transport, which is operable for large (> 70 kDa) molecules and requires the presence of a nuclear localization signal (NLS) proteins and the hydrolysis of ATP as an energy source; and (iii) Ca^{2+} regulated transport, which involves intermediate sized molecules (10-70 kDa range) and does not require a NLS or the evolution of ATP. Several experimental evidences suggest that the 'plug on' and 'plug off' of the NPC are always associated with the Ca^{2+} ion dependent mechanism (34). In presence of excessive calcium ions in the cytosol (such as from calcium phosphate nanoparticles), the $InsP_3$ is inactivated through calcium ion mediated complexation so that the $InsP_3$-receptor assisted drainage of cisternal calcium ion to the cytoplasm does not happen. DNA-Ca^{2+} compact complex, under such circumstances, can easily enter the nucleus through NPC. DNA, delivered through conventional non-viral vector such as liposome or a polymer, cannot enter the nucleus through NPC without the help of NLS proteins as calcium ion regulated transport across the NPC is irrelevant here. It has been shown (36) that addition of calcium ion increases the *in vitro* transfection efficiency of pDNA-cationic liposome complexes from 3 to 20 fold and the effect can be inhibited by the presence of EGTA, a calcium complexing chelating ligand.

Conclusion

Although the use of calcium phosphate nanoparticles as non-viral vector is very promising, it is still a new comer in the field of gene therapy. However, due to the the high transfection efficiency of the vector, no adverse effect of the carrier particles and easy method of preparation as well as successful applications of the vector system in curing autoimmune hepatitis of experimental animal models and in DNA vaccine formulation, the areas and the questions that have to be addressed are now being defined. An ideal non-viral vector must manifest an efficient endosomal escape, renders sufficient protection of DNA in cytosol and easy passage of cytosolic DNA to nucleus. Because of the dissolution of calcium phosphate in the low acidic medium of endosomal compartment, the calcium phosphate nanoparticles are dissolved in the endosome and destabilize it through osmotic disbalance and finally deliver the genetic material out in the cytosol. The electrostatic interaction of Ca^{2+} with negatively charged DNA makes the DNA stable against nuclease attack so that DNA can slowly enter the nucleus without any degradation. The efficient nuclear uptake of DNA from the cytosol is also assisted by Ca^{2+} ion through the deactivation of $InsP_3$ so that the nuclear pore complex (NPC) remain wide open thereby allowing DNA-Ca^{2+} complex to pass through it. We discuss here two major areas that foreseeably will be the focal point of investigations in the near future: further experimental proofs of the mechanism of enhanced nuclear uptake so as to make refinement of our understanding the role of calcium ions in the whole process of trafficking of DNA and an attempt to replace the viral vectors in gene therapy protocol by calcium phosphate nanoparticles for curing diseases. Mechanistic study to probe the reason for high transfection efficiency of calcium phosphate nanoparticles and the role of exogenous calcium ions indicate that calcium phosphate nanoparticles is not a "me too" non-viral vector and can be classified as second generation non-viral vector.

Acknowledgement

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Herbal Splendour, Medicinal Health & Modern Medicine

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SUMMARY

Plants have been the main source of healing agents from time immemorial and continued to be the source of drugs even today. Obviously earlier systems of medicines were based on the use of plants as therapy. Ayurvedha, Siddha, Unani and Tribal medicines are mainly based on the use of plants for curing diseases. This review article, projects highlights of the research work on medicinal herbs that were carried out in the Microbiology departments of three Institutes at Chennai - Madras Medical College, Dr. ALM PGIBMS & Presidency College. Many of the plants used as food in India especially, Tamil Nadu have medicinal properties. Besides nutritive value, the therapeutic usefulness various foods which are mainly of plant products are described in "Pathaarthaguna Vilakkam", an ancient compendium on Siddha medicine. Modern science provides evidence for their valid use because the vegetables, fruits, seeds, leaves, roots, have minerals, vitamins, proteins, good cholesterol and pharmaceutically important ingredients, including antimicrobial and immuno-modulatory principles. Modern science enables us to evaluate the efficacy of the claims on the useful products of traditional systems of medicines. It is well known that plants play a pivotal role in Human health.

Many of the drugs in various branches of medicines have their origin from plants. Initial use of plant in extract form was considered as non specific and isolation of specific active principle from the useful plant parts marked a new era in drug development from plants. Collaborative researches involving different disciplines - medical and paramedical have brought out newer information on the physiotherapeutic properties through evaluation on the usefulness of plants by *in vitro, in vivo* experimental studies followed by clinical trials. Getting patent for newer drugs of traditional sciences is strongly supported by DST. *In vitro* antimicrobial highlights of few potential plants from the studies conducted at Chennai revealed that *Phyllanthus amarus, Pongamia pinnata, Terminalia chebula, Terminalia catappa, Ocimum canum, Decalepis hamiltonii, Cassia auriculata, Lawsonia inermis and Plectranthus ambonicus* have appreciable antimicrobial properties and their minimum microbicidal value have been estimated.

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INTRODUCTION

Plants not only provide us food but also medicine. Forty eight percent of drugs in pharmacy are directly obtained form plants. 85% of the Chinese population and 65% of the Indian population rely on plants for medicine.

The earliest mention of the medicinal use of plants was in Rig Vedha which were written between 4000 & 1600 BC. In the Athurvana Vedha, prescription on various use of drugs are mentioned and in Ayurvedha, a upavedha, definitive properties of drugs and their uses in detail are described. Later, Charaka Samita of Ayurvedha (600 BC) list 341 plants and plant products for use in health management Susruta samita described about more medicinal plants. Thus the Ayurvedic drug derived from single plant had increased to 600 species (2).

The WHO had listed over 21,000 plant species for medicinal purposes all over the world. In India 45,000 species of plants are distributed in 16 Agro climatic zones & 15,000 species of these are considered as medicinally important. The number of plants of medicinal value described in Ayurvedha are 7,500, in Sidha – 600(3), in Unani – 550, and in Homeopathy- 400. There are 4,600 ethnic communities in India using plant species for humans and veterinary health care.

Only 10-15% of the existing plants have been evaluated for their biological activity (1). Incidence of resistance gained by microbes for diseases like Malaria, Tuberculosis and Salmonellosis are an eye opener for the need on active research in finding newer drugs. Research on newer drugs of plants is a thrust area of research. Hence research on the *in vitro* and *in vivo* anti-microbial efficacy and other pharmacological properties have been actively pursued through out the world.

Improvement in Evaluation Techniques

In the days of modern science, the evaluation techniques have undergone rapid improvement. Scientific use of chemicals as drugs to control microbial infections had its beginning in 1495 when mercury was used to treat syphilis (4). Use of plant extract as therapeutic substance preceded the use of chemical as drug. Ayurvedha, Siddha, which date back to more than two thousand years, seemed to have observed the usefulness of the plant extracts.

Medicinal plants, as natural industries for the synthesis of variety of chemicals either as primary or secondary metabolites, owe their healing power to one or more bioactive compounds. Unraveling these compounds for their therapeutic utilization is one of the thrust areas of research throughout the world. The annual global-trade on herbal preparations accounts for about 30,000 billion dollars per Year. There are 121 therapeutic agents of known structure from 90 species of plants. Yet there are few compounds like Chloroquinone from Cinchona bark which could not be synthesized but to be obtained only from plants (2).

The first record on the efficacy of phytochemical as an antimicrobial agent was of quinine form the bark of Cinchona species for *Plasmodium vivax*, a protozoan, by Joseph Pelletier & Joseph Caventou in 1820. The Chinese emperor, in his pharmacopoeia 2700 BC had mentioned on the use of Ephedra in a variety of diseases other than syphilis (5).

Since then, greater attention has been given to the study on efficacy of plant extracts against microbial pathogens of human namely bacteria, fungi, protozoan and viruses. Whenever an extract was found to be efficacious, the follow- up action was to isolate its antimicrobial principles and later to understand its pharmacokinetics.

The first chemist to investigate on plant products in India was W.O Shangnessy in1840 of Kolkata Medical college. Later his associate Warder & Hooper, isolated Abrin, a toxin protein form the seeds of *Abrus precatorius*. Later several phytochemicals have been studied extensively for their pure active constituents and these have been included in Modern pharmacopoeia Morphine form *Papaver* sp., Ephedrine from *Ephedra* sp., Emetine from *Ipecae*, Senna glycosides from *Cassia* sp., and Reserpine from *Rawoolfia serpentina*.

During the later part of this century collaborative investigations among Ethnobotanists, Biochemists, Microbiologists and Pharmacologists have resulted in unraveling the efficacy of substantial number of compounds. This gave greater impetus to the development of Phytochemistry and phytochemical pharmacology.

Scientific investigations of a medicinal plant is extremely essential because contrary to the belief, a newer and unrelated activities was found in plants. For instance leaves of well known medicinal plant *Catharanthus roseus* (Vinca rosea) had been

Table: 1 - *In Vitro* Minimum bactericidal value in mg/ml of the Seitz filtered aqueous extracts

S. No	Bacteria	Extracts						
		Terminalia chebula		Ocimum canum	Cassia auriculata	Plectranthus ambonicus		Lawsonia inermis
		UR	R	L	L	Non vareigated	Variegated	
1	Strep pyogenes	13.5	24	-	-	-	-	18.5
2	Staph aureus ATCC 25923	13.5	24	-	-	-	-	18.5
3	Staph aureus	13.5	24	-	-	-	-	18.5
4	Staph epidermidisl	-	-	-	-	-	-	-
5	E coli ATTC 25922	13.5	24	-	5	-	-	-
6	Escherichia coli	13.5	24	-	-	-	-	-
7	Shigella sonnei	13.5	24	-	-	-	-	-
8	Salmonella typhi	13.5	24	10	20	-	-	-
9	S paratyphi A	13.5	24	-	10	-	-	-
10	S. paratyphi B	13.5		10	20	-	-	-
11	Vibrio cholerae	13.5	24	10	2.5	27.5	17.5	-

L – Leaf, UR – Unripe Fruit, R – Ripe Fruit

used for the treatment of diabetes in Mexico but a detailed investigation of the plants led to the isolation of alkaloid leurosine and P 1534 leukoblastine which have antitumor activity on lymphocytes leukaemia , inhibiting the growth of human choriocarcinoma transplanted into hamster cheek pouch (6) but found to lack any chemicals that is related to Diabetes mellitus.

It is estimated that now more than two thirds of the world populations rely on plant based drugs About 7,000 medicinal compounds used in the western pharmacopoeias are derived from plants (7). In the USA, approximately 25% of all prescribed drugs contain one or more bioactive compounds derived form vascular plants (8). The bioactive compounds are not only derived form higher plants, but are also derived form lower plants like fungi.

Need for Newer Drugs from Plants

A worldwide resurgence of interest on the exploration of newer drugs form the plants and their utilization for human health care has come for various reasons namely :

1. Reports of acquiring resistance by disease causing organisms to the existing antibiotics as in the case of chloroquinone.
2. Plants are seen as goldmines by industrialists in their attempt to exploit a variety of phytochemicals as drugs.

Table: 2 - *In Vitro* Minimum fungicidal value in mg /ml of the Seitz filtered Aqueous extracts

S. No	Fungi	Extracts					
		Lawsonia inermis	Terminalia catappa		Decalepis hamiltonii RB	Terminalia chebula	
			Ripe FR	Unripe FR		Unripe FR	Ripe FR
1	Epidermophyton floccosum	2.31	1.5	0.4	-	0.67	0.6
2	Microsporum gypseum	2.31	0.375	0.4	0.537	0.67	0.6
3	Trichophyton mentagrophytes	4.62	1.5	0.4	-	0.67	0.6
4	Candida albicans	18.5	-	-	-	0.67	0.6
5	Mucor	-	-	-	2.15	0.67	0.6
6	Rhizopus	-	-	-	2.15	0.67	0.6
7	Penicillium	-	-	-	-	0.67	0.6
8	Aspergillus flavus	-	—	-	-	-	-
9	Aspergillus niger	-	—	-	-	-	-

- lack of fungicidal properties

3. Need for an honest attempt to evaluate the usefulness of plant drugs due to the existing threat to the precious flora as realized by the international organization like IUCN (International Union for Conservation of Nature) had given necessary impetus for aggressive research on medicinal plants. IUCN had estimated that 10-20% of the plant species would have become extinct.

A review of the reference on the past investigations on the isolation, characterization of active principles form the medicinal plants clearly reveal us that:

1. more than 90% of the plants remain to be analyzed for bioactive components.

2. even in respect to the chemically well studied plants, not all the isolated and identified biochemicals were tested for their biological, pharmacological and toxicological effects.

In vitro standard protocols have been evolved to obtain uniformity and reproducibility and consistency. *In vitro* antimicrobial Assay of National committee for Clinical Laboratory Standards (NCCLS) prescribe protocols for the *in vitro* antibacterial(9) and antifungal (10) evaluation. Standardization of inoculum for *in vitro* antibacterial assay is also available (11). Standard *in vitro* antiviral (12) and *in vitro* safety (13) protocols. Also standard protocols have been evolved for isolation and characterization of compounds (14) and estimation of anti-inflammatory property and other specific physiological activities.

Another visible lacuna in the traditional systems and a stumbling block in utilization of the time honoured medicinal plants is the lack of knowledge about the possible toxic effects of these plants on the human organ and tissues. It is interesting to note that some of the plants like *Tribulus terrestris (Puncture vine)*, mentioned in the traditional systems of medicine and described in Indian Materia Medica (15) for its diuretic property, has been found to possess profound toxic effect on liver due to possible nitrate poisoning (16). Hence screening of every potential medicinal/ health plants to observe the presence or absence of toxicity in their useful parts by suitable safety study experiments in suitable animal model, is a prerequisite for making it as a potential drug for human use. Hence it is imperative to evaluate the medicinal properties of Indian medicinal plants to facilitate discovery of newer cost effective medicines for human and veterinary beings by utilizing Drug Development Programme of DST, Government of India.

Evaluation on the safety of the plant extracts and on isolated active principle by *in vitro (13)* assay are also available. More than these - the conduct of clinical trials in volunteers have been standardized in n order to identify the safety of the drug during long period of usage. Statistical analysis of the experimental data is uniformly suggested to justify the significance of the results by adopting suitable statistical softwares like ANOVA.

RESULTS

Intensive studies on *Phyllanthus amarus* had shown the efficacy of the whole plant in the control of Hepatitis – B and its safety had been ensured through safety studies in animal models and human volunteers. Clearance of HBsAg had been carried out and the clearance rate was highest from a species collected from Chennai. *Phyllanthus amarus* collected from other than Tamil

Nadu state did not show appreciable HBs Ag clearance (18,19). All these results obtained form 28 National and International centres had resulted in development of the drug named as "VIROHEP" by Prof. S.P. Thyagarajan and a patent had been obtained in the name of University of Madras. Similarly detailed study on an another plant *Pongamia pinnata* (20) resulted in the observation of *in vitro* antiviral activity for HSV and clinical trials (21) suggested its usefulness in clearance of Herpes infections. Seitz filtered extract was adopted as suitable method for *in vitro* estimation of antimicrobial properties of aqueous extract by a modified serial tube two fold dilution method and had reported that Seitz filtration did not affect antimicrobial activity of Henna leaf extract (22). Since then Seitz filtered aqueous extract of useful plant parts have been tested for the bactericidal and fungicidal properties. The impressive antibacterial activity for 14 gastroenteritis bacteria have been reported .The flower buds and seed extract of *Cassia auriculata* (Tanners Cassia) and the ripe fruit of *Terminalia chebula* (Chebulic myrobalan) (23), were found to posses antibacterial activity on gastroenteritis bacteria. (Tables 1&2). Varietal variation had been reported in the fruits of Bael tree (24). However, there was no difference in the antibacterial activity between the leaf extracts of *Plectranthes ambonicus, namely,* of the variegated and non variegated varieties. Extracts of both varieties did have marked inhibitory activity on *Vibrio cholerae* and lack of activity on other nine gastroenteritis bacteria (25). However, a plant of high esteem, *Ocimum teniflorum* (Holy basil) was found to lack bactericidal activity while Hoary basil (*Ocimum canum)* was found to possess appreciable antibacterial activity (26). It is interesting to note that fruit rind extracts of *Terminalia catappa* (Indian almond) were found to exhibit remarkable antifungal activity on dermatophytic fungi (27). Likewise, highly appreciable antifungal property was found in the fruit rind extracts of ripe and unripe fruits of Chebulic myrobalan (*Terminalia chebula*) and were also found to be fungicidal to all opportunistic and dermatophytic fungi (28).

MBC (Minimum Bactericidal concentration) of the seitz filtered extracts of the useful plant part for the susceptible bacteria and its Minimum Fungicidal Concentration (MFC) for susceptible fungi had been estimated. (Tables 1 & 2)

CONCLUSION

Modern science has helped us to get a clear and better understanding of the "Herbal Splendour". Improvements in the evaluation techniques both *in vitro* and *in vivo* enable us to understand the antimicrobial efficiacy of the traditional medicinal plants. Completion of i*n vivo* antibacterial studies are confined to handful plants like *Phyllanthus amarus* and *Pongamia pinnata.* Safety studies had been carried out only for few plants/plant parts.namely *Phyllanthus amarus, Pongamia pinnata, Terminalia chebula* and *Aegle marmelos.* In the Indian context, only few plants like *Phyllanthus amarus* has crossed through all scientific investigations and developed into a drug with a patent. Studies on other plants have helped to understand their specific profile on their efficacy to clinically important bacteria, fungi and few viruses. Plant parts of Chebulic myrobalan have very high antibacterial and antifungal activities while certain plants have narrow activity as in *Aegle marmelos.*

Similar estimations on *in vitro* profile for plant ingredients of traditional medicines in relation to infectious diseases

would create a useful database that would facilitate *in vivo* safety study followed by clinical trial and ultimately help in the development of many newer drugs of plant origin. India, being basically an agricultural country and major section of the people are in the rural areas, the evaluated herbal traditional medicine by modern science is the solution for the cost – effective management and treatment of diseases and maintenance of good health.

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