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Modelling of cerebral tuberculosis in BALB/c mice using clinical strain from patients with CNS tuberculosis infection

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Background & objectives: Central nervous system (CNS) infection caused by *Mycobacterium tuberculosis* (MTB) is the most severe form of extrapulmonary tuberculosis (EPTB) due to a high level of mortality and morbidity. Limited studies are available on CNS-TB animal model development. The present study describes the development of a murine model of CNS-TB using a clinical strain (C3) isolated from the cerebrospinal fluid (CSF) of CNS-TB patients.

Methods: Groups of mice were infected by the intravenous route with MTB C3 strain isolated from the CSF of CNS-TB patients. Brain and lung tissue were evaluated for bacterial burden, histopathology and surrogate markers of TB infection at 30 and 50 days post-infection.

Results: Mice infected intravenously with MTB C3 strains showed progressive development of CNS disease with high bacillary burden in lungs at the initial stage (30 days), which eventually disseminated to the brain at a later stage (50 days). Similarly, high mortality (60%) was associated in mice infected with C3 strain compared to control.

Interpretation & conclusions: The study showed development of a novel murine model of CNS-TB using the C3 strain of MTB that replicated events of extrapulmonary dissemination. The developed model would be helpful in understanding the pathogenesis of CNS-TB infection for the development of improved therapeutic interventions in future.

Key words Adenosine deaminase activity - Ag85B - BALB/c mice - central nervous system tuberculosis - clinical strain

Central nervous system (CNS) infection caused by *Mycobacterium tuberculosis* (MTB) is among the most devastating forms of extrapulmonary tuberculosis (EPTB), accounting for 70-80 per cent of all neurological TB cases[1,2]. In India, the estimated mortality due to CNS-TB is 1.5/100,000 population[3]. The diagnosis of CNS-TB remains a formidable challenge owing to non-specific clinical presentation, poor diagnostic tools and limited clinical resources often leading to high mortality and morbidity[4,5]. There is a need for the development of CNS-TB animal models to enhance our understanding of CNS invasion by MTB for the development of improved therapeutic interventions against CNS infection.

There are only a few *in vitro* and animal models reported in the literature for replicating events of CNS infection, including breaching of the blood-brain barrier (BBB) and induction of identified surrogate markers of protection[6-8]. In many studies the investigators have used invasive procedures which involved direct inoculation of bacteria in the cerebellum[8,9]. Although such procedures may induce infection, these do not replicate actual events of BBB invasion and CNS dissemination leading to disease development.

The present study describes the development of a murine CNS-TB model of extrapulmonary dissemination using a strain of MTB (C3) isolated from the cerebrospinal fluid (CSF) of clinically confirmed CNS-TB patients.

Material & Methods

Isolation and culture of clinical strain of MTB: The MTB strain C3 was earlier isolated from the CSF of clinically confirmed CNS-TB patients admitted to the Neurology ward of the Central India Institute of Medical Sciences (CIIMS), Nagpur, India. Briefly, diagnosis of CNS-TB infection was based on clinical features, which included signs of meningeal irritation and sub-acute or chronic fever with or without other features of CNS abnormality. CSF profile of these patients included increased protein levels, decreased CSF/blood glucose ratio (<0.5) and pleocytosis with lymphocyte predominance. All recruited patients were culture negative for other microorganisms. All confirmed cases with normal chest X-rays were included to rule out pulmonary TB infection. The study was carried out for one year from July 2014 to July 2015. The study protocol was approved by the ethics committee of CIIMS, Nagpur.

For culture and isolation, CSF samples (2-3 ml) were cultured in Middlebrook 7H9 liquid medium containing oleic acid, albumin, dextrose and catalase (OADC) enrichment and antibiotic supplements in BacT/Alert culture bottles (Biomeriux, France) and were incubated at 37°C in BacT/Alert 3D system (Biomerieux, France) for 28-35 days. Before infection, the isolated strain was subjected to drug sensitivity testing using automated BACTEC MGIT 960 system (Becton Dickinson, USA) and was found sensitive to all standard first-line drugs for TB. For development of CNS-TB infection, MTB strain C3 was further incubated in Middlebrook 7H9 broth containing 0.2 per cent glycerol and 0.05 per cent Tween-80 supplemented with OADC and allowed to grow to log phase. The viability of the bacteria was determined by plating on Middlebrook 7H11 medium and counting the number of colony-forming units (cfu).

MTB antigens and antibodies: A panel of MTB $H_{37}Rv$ antigens; Ag85B (Rv1886c), ESAT-6 (Rv3875), CFP-10 (Rv3874), Gro-ES (Rv3418c) and Hsp16 (alpha-crystallin/Hsp-X, Rv2031c) along with monoclonal antibodies against MTB antigens Ag85B and Rv2623 were obtained from Colorado State University, USA [under the TB research materials and vaccine testing contract (NO1-AI-75320)]. The secondary antibody rabbit anti-mouse IgG-HRP was obtained from Genei, Bengaluru, India.

Experimental mice: Female BALB/c mice aged 6-10 wk were purchased from Central Drug Research Institute (CDRI), Lucknow, India, and housed in a Biosafety Level (BSL) 3 facility at National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJILOMD), Agra. All protocols for animal experiments were approved by the Institutional Animal Ethics Committee of the NJILOMD.

Development of CNS infection in mice, and estimation of mycobacterial burden: The experimental mice (n=12) were infected intravenously through the tail vein with 2×10^7 cfu of MTB C3 strain. A control group of mice (n=10) was separately maintained and sham-infected using sterile saline. Mice (n=3) were sacrificed at 30 and 50 days after infection, for estimating MTB colonizing the brain (CNS dissemination) and lungs and for evaluating histopathological changes in these organs. Organs were also collected from mice which underwent mortality during experimental duration for counting cfu and histopathology studies. Organs were also used for estimation of MTB antigens and antibodies. The mycobacterial burden was determined by plating and counting the number of cfu. Briefly, the lungs (lower and middle left lobe) and brains were homogenized using ultrasonic homogenizer (Sigma, USA) in 10 ml of phosphate buffered saline (PBS). Ten-fold dilution of organ homogenates was prepared and plated separately on Middlebrook 7H11 medium with 0.05 per cent Tween-80 and glycerol. The colony forming units were counted after 3-4 wk of infection at 37°C.

Histopathological analysis: Organs were fixed in 10 per cent buffered formalin. Histological sections were cut using a microtome (Leica Biosystems, USA) and stained using haematoxylin and eosin.
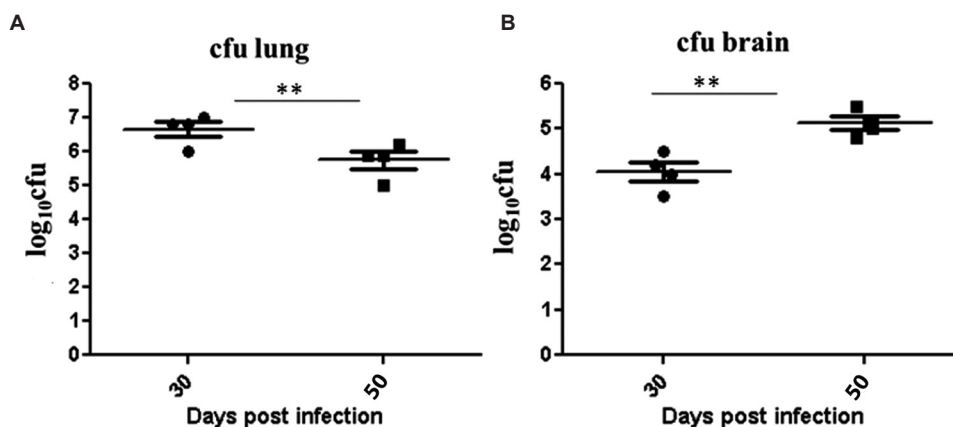
Fig. 1. (A) Bacillary load in lung and brain tissues of mice infected with *Mycobacterium tuberculosis* C3 strain at 30 and 50 days post-infection (n=3, per group). **(B)** Bacillary burden in both organs are represented as $\log_{10}$ cfu [**]*P*<0.01, comparison between days 30 and 50.

Theses sections were scored for the percentage of the section occupied by granulomatous inflammation and necrosis using visualization at ×100 magnification. Images were captured using high-resolution camera (Nikon 50i, Japan). The presence of epithelioid cells, the extent of fibrosis, oedematous lesions and lymphocytic infiltration (whether around vessels or in lesions) in lungs and brain sections were also assessed.

Analysis of immune markers: The organ homogenates prepared were centrifuged at 5000 ×g for 15 min. The supernatant was collected and stored at 4°C until use. Antigens Ag85B and Rv2623 were estimated as per in-house ELISA protocols described elsewhere[1,6]. The antibody response against mycobacterial antigens (Ag85B, ESAT-6, CFP-10 and Hsp16) was evaluated using in-house ELISA methods[7]. Adenosine deaminase (ADA) activity in the supernatant was determined at 37°C according to the method of Guisti and Galanti[10] and quantified spectrophotometrically (UV-Visible spectrophotometer, Systronic, India). All assays were performed in triplicates.

Statistical analysis: All data in results are expressed as mean±standard deviation and presented on a linear scale except for cfu, where a logarithmic scale is used. All statistical analyses were performed using Prism 6 version 6.01, GraphPad Software, (San Diego, CA, USA). Differences in the immune markers and cfu between experimental and control groups were tested by *t* test. Kaplan–Meier analysis was used for survival analysis in the experimental and control groups. A test for proportion was used for comparison of total mortality in the experimental and control groups.

Results

Mycobacterial load in CNS with progress in infection: Fig. 1 shows the mycobacterial burden in the lungs and brain of infected mice (n=4). The analysis of cfu at 30 days post-infection revealed significantly (*P*<0.05) high bacterial load in lungs (6.80±0.1 $\log_{10}$ cfu) of infected mice. The lung burden was gradually found to decrease to 5.8±0.2 $\log_{10}$ cfu at 50 days. In contrast, significantly high (*P*<0.05) mycobacterial load was observed in the brains of these mice at 50 days (5.0±0.2 $\log_{10}$ cfu) compared to infection at 30 days (4.0±0.1 $\log_{10}$ cfu). No mycobacterial load was observed in brains and lungs of control mice (data not shown).

MTB antigens, specific-IgG response and T-cell adenosine deaminase (ADA) activity in infected mice: At 50 days post-MTB infection, mice were sacrificed and homogenates of brains were used for detection of MTB antigens and antibodies. Mice infected with C3 strain showed increased (*P*<0.001) Ag85B and Rv2623 antigens levels compared to control groups (Fig. 2A). This significance was also evident in MTB antibodies evaluated against panel of MTB antigens, all of which were considerably elevated (*P*<0.001) in brain homogenates of infected mice (Fig. 2B). Similarly, high ADA levels (*P*<0.001) were observed in experimental mice suggesting development of infection (Fig. 2C).

Survival and mortality in infected mice: To associate pathogenesis of C3 infection with survival of mice post-infection, survival analysis was carried out. Mice infected with C3 strain showed considerable decrease in survival percentage with progress in infection,
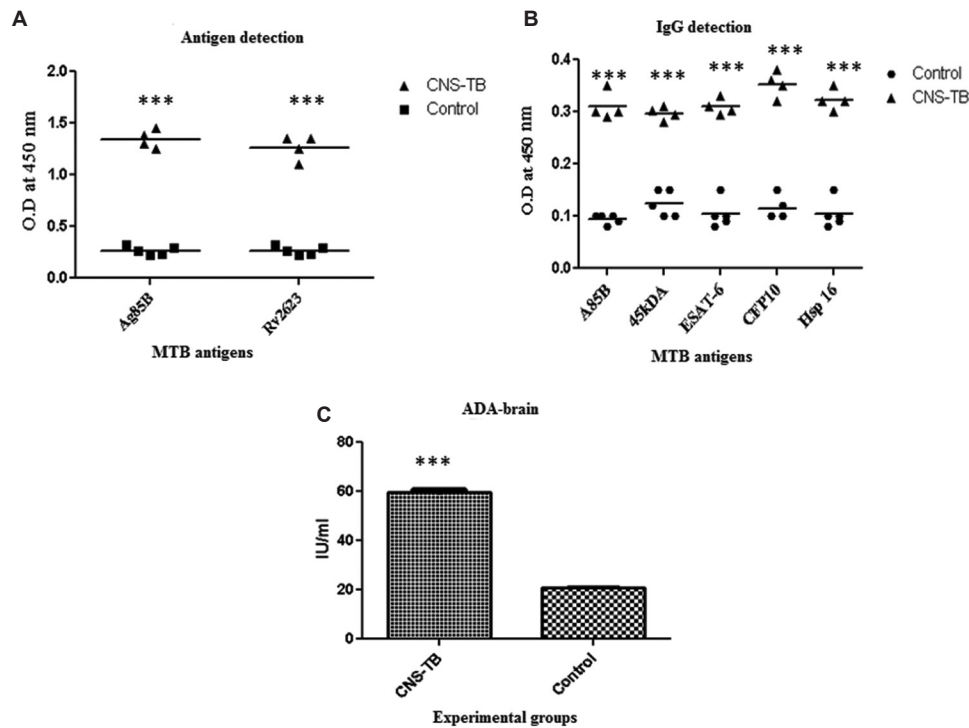
Fig. 2. Scatter plots of (**A**) antigen response (Ag85B, Rv2623), (**B**) IgG response against panel of *Mycobacterium tuberculosis* H37Rv antigens in brain homogenates (n=4 of BALB/c mice at 50 days post-infection. (**C**) Shows mean±SEM adenosine deaminase (ADA) (n=4) levels in brain homogenates of CNS-TB and control mice. ***$P<0.001$ compared to control.
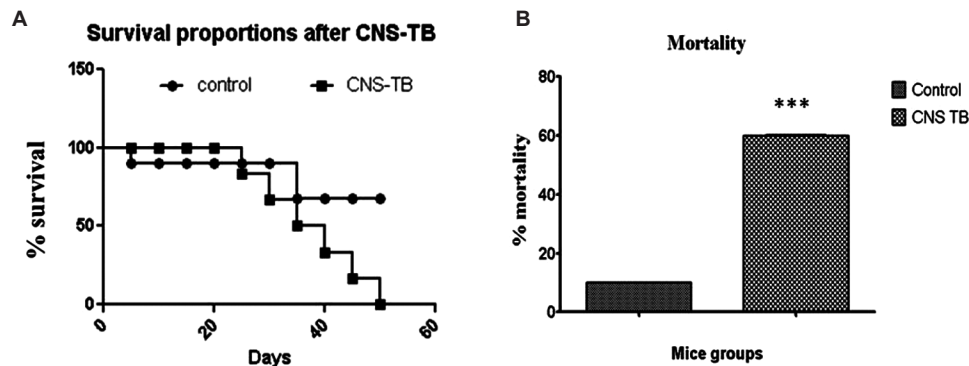

Fig. 3. (**A**) Kaplan Meier survival analysis and (**B**) percentage mortality in control and CNS-TB infected mice. Mice infected with C3 strain showed reduced survival rates with progressively high mortality rates than control with progress in infection. ***$P<0.001$ compared to control.

compared to control (Fig. 3A). Comparison of overall mortality between the two groups showed significantly ($P<0.001$) high (60%) mortality in C3-infected mice compared to 10 per cent reported in control mice (Fig. 3B).

MTB C3 strain induced brain oedema and lung pathology: To study pathogenesis of C3 strain, organs from mice (n=4) were isolated at 30 and 50 days post-infection and examined for lung and brain pathology. After isolation, organs were first visually examined for

observation of amount of oedema/swelling in the brain and granulomatous lesions in lungs and brains. Mice infected with C3 strain showed prominent swelling/oedema of the brain in the left hemisphere at 30 days, which was further increased with progress in infection at 50 days compared to control mice (Fig. 4A). Examination of lungs showed chronic pathology at 30 days which progressively reduced at 50 days post-infection (Fig. 4B). Histopathological examination of the brains of infected mice showed swelling of neurons along with lymphocytic infiltration which
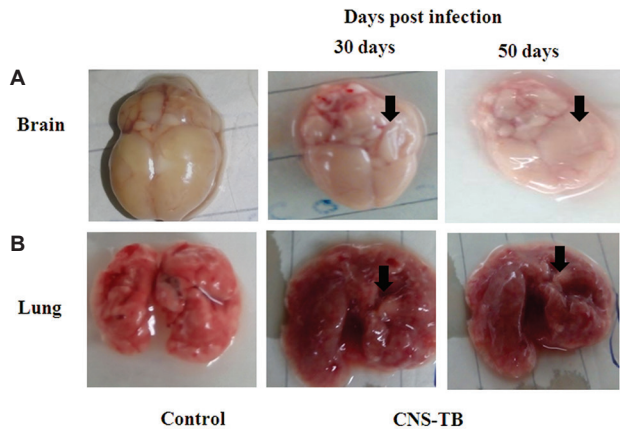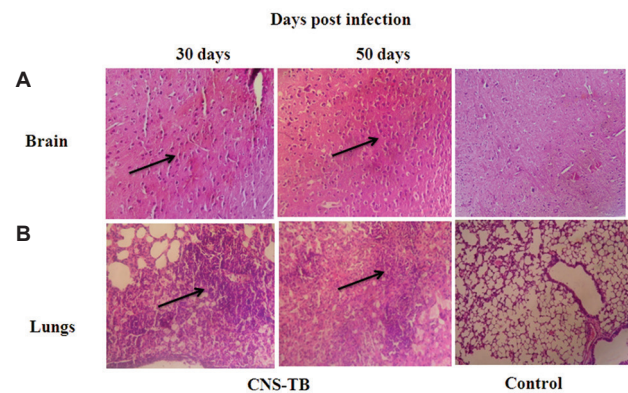
Fig. 4. Representative photographs of (**A**) brains and (**B**) lung tissue of BALB/c mice with CNS-TB along with control at 30 and 50 days post-infection. Lungs and brains of both mice groups were visually examined for pathological changes compared to control. Lungs of mice with CNS-TB showed gross reduction in pathology at 50 days with progress in infection. Brains of infected mice showed swelling on left hemisphere which progressively increased with development of infection at 50 days. Arrows indicate gross lung and brain pathology in mice with CNS-TB.

Fig. 5. Representative photomicrographs of histopathological examination of (**A**) brain and (**B**) lungs section (5 µm) of control and CNS-TB mice stained with haematoxylin and eosin. Mice were sacrificed 30 and 50 days after intravenous infection with *Mycobacterium tuberculosis* C3 strain. Organs (n=4) were used for histological examination. Gross pathological examination of brain sections of CNS-TB mice showed lymphocytic proliferation (arrows) with development of oedema of brain parenchyma which was more prominent at 50 days post-infection. Analysis of lung section at 30 days revealed high lymphocytic infiltarion, with alveolar space mostly filled with inflammatory cells (arrows) and oedematous fluid. This gross lung pathology was considerably less on examination at 50 days post-infection (×100).

was progressively more at 50 days. Oedema of brain parenchyma was also evident in brains of infected mice at both time points after infection (Fig. 5A). The analysis of lung sections at 30 days revealed >40 lesions in lung parenchyma, with high lymphocytic infiltration, and alveolar space mostly filled with inflammatory cells and oedematous fluid. This lung pathology improved at 50 days, with lesions reduced to <20 along with less lymphocytic infiltration. Alveolar space was largely preserved as clear air space. A few cells appeared filled with oedematous fluid (Fig. 5B). Brains and lungs of control mice showed preserved histology with no infiltration and oedema.

Discussion

In the present study, mice infected intravenously with the C3 strain showed development of slow progressive CNS disease, the severity of which increased with advancement in infection from the 30th to the 50th day. All C3-infected mice showed elevated titres of mycobacterial antigen and antibodies along with increased ADA activity in brain homogenates and increased bacterial load in brains. All infected mice showed chronic brain pathology with increased lymphocytic infiltration and oedema of brain parenchyma which subsequently increased towards later stages of infection.

Intravenous inoculation of free MTB or *Mycobacterium bovis* in guinea pigs and rabbits has been

shown to produce CNS invasion as evidenced by the formation of tuberculomas in their brain parenchyma[11]. In our study the high dose of infection was particularly used to study dissemination post-primary infection since amount of initial load in lungs post-primary infection determines extent of mycobacterial invasion of BBB and causing CNS-TB disease[12,13].

High loads of mycobacteria were found in lungs initially (30 days) which induced chronic pathology in lungs of infected mice. However, there were no evident titres of antigen and antibodies in brain homogenates of mice at this stage (data not shown), despite an initial load of mycobacteria in brain. One reason for this observation could be that a load of mycobacterial antigens and antibodies in brain homogenates at this stage was undetectable by ELISA. Another plausible explanation could be a lack of expression of the selected antigens during the early stage in the CNS, as the clinical strain may have divergent antigen expression compared with the standard strain. An increased mycobacterial load was observed colonizing the brain at 50 days, which correlated with high levels of both antigen and antibodies. This rise in mycobacterial load in brain was associated with decreased cfu counts in lungs and improved lung histology. This was indicative of extrapulmonary dissemination of C3 strain to CNS with progress in infection. Our findings were in agreement

with an earlier report that suggested that dissemination to CNS was dependent on initial load of mycobacteria in lungs[14]. Another reason for this observation may be involvement of specific strains for causing the different manifestation of TB disease. Be *et al*[7] have shown that dissemination to CNS is associated with distinct MTB strains, which may have distinct virulence factors that promote dissemination. In another study, the same investigating group has shown association of five mutant MTB genes (Rv0311, Rv0805, Rv0931c, Rv0986 and MT3280) with CNS-specific phenotypes, absent in lung tissue, suggesting virulence associated with specific CNS-specific MTB strains for extrapulmonary dissemination[15]. Other studies have also shown association of different MTB strains with extrapulmonary dissemination[16-18]. Although such a concept is interesting, but detailed animal studies are needed on pathogenesis of these strains isolated from different pulmonary and extrapulmonary cases.

Earlier many investigators have focused on the development of CNS-TB models through *in vitro* and animal studies. Jain *et al*[6] through their *in vitro* studies using a monolayer of primary human brain microvascular endothelial cells have shown that MTB strains H37Rv and CDC1551 are capable of invading and traversing the BBB with much higher efficiency than the non-pathogenic *Mycobacterium smegmatis*. Tsenova *et al*[19] has used a rabbit model for the development of TBM by intracisternal inoculation of MTB. Other investigators have shown that inoculation by intracranial route leads to progressive development of TBM infection in different animal models[9,20]. Most of these models are based on intracranial route of MTB infection and suffer from the limitations of not replicating the natural mechanism of infection, which involves primary lung infection followed by dissemination to CNS through BBB invasion. Our study although based on the intravenous route provides a better model to study extrapulmonary dissemination to CNS based on infection dynamics observed in lungs and brain at 30 and 50 days post-infection. However, infection using aerosol route will be further helpful to establish and correlate pathogenesis of C3 strain for CNS dissemination. An association of the virulence protein PknD with pathogenesis of CNS-TB and disease development using intravenous models of CNS-TB infection has been shown[21]. The present study was in line with these studies, where we used markers earlier reported in our laboratory for demonstration of TBM infection[22-24].

Our study had some limitations. Inflammatory cytokine response in the brain was not studied due to limited sample size of the study. Another limitation was a lack of evaluation of markers of BBB damage, which would have been more supportive to demonstrate the development of our existing model.

In conclusion our findings demonstrated a novel murine model of CNS-TB using intravenous inoculation of C3 strain which replicated events of extrapulmonary dissemination. The developed model may help in understanding the pathogenesis of CNS-TB infection for the development of effective vaccines and drugs in future.

Conflicts of Interest: None.

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