A New Murine Model to Study the Pathogenesis of Tuberculous Meningitis

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Tuberculous meningitis (TM) is a severe complication of tuberculosis that mainly occurs during childhood. No murine models are available to study this disease. The purpose of the present study was to develop a murine model to investigate the pathogenesis of TM. Mice were intracerebrally injected with Mycobacterium tuberculosis. Bacilli could be cultured from brain homogenates, and, on histopathological examination, all mice were found to have meningeal cellular infiltration. We found elevated levels of chemoattracants for mononuclear phagocytes and neutrophilic granulocytes. This is the first murine model for TM that can be used for research on the host response to TM, in particular the innate immune response.

Tuberculosis (TB) remains a global threat to humankind. One-third of the world’s population is infected with Mycobacterium tuberculosis, and 1.7 million people die each year as a consequence of TB [1]. Tuberculous meningitis (TM), which mainly occurs during childhood, is considered to be the most severe extrapulmonary complication of TB [2]. Its fatality rate is ∼30%, and 25% of survivors develop severe neurological sequelae [3].

The aim of the present study was to develop an in vivo model for TM to investigate the inflammatory response. We examined brain tissue samples and measured local cytokine and chemokine production. These mediators orchestrate the host defense to TB in the brain but are also responsible for neuronal damage [4].

Materials and methods. Pathogen-free 6-week-old female C57Bl/6 mice were obtained from Harlan Sprague Dawley and were maintained in biosafety level 3 facilities. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

H37Rv (American Type Culture Collection), a virulent laboratory strain of M. tuberculosis, was grown for 4 days in liquid Dubos medium containing 0.01% Tween 80. A replicate culture was incubated at 37°C, harvested at mid-log phase, and stored in aliquots at −70°C. For the experiment, a vial was thawed and washed twice with sterile 0.9% NaCl.

Mice were anesthetized by inhalation with isoflurane (Abbott Laboratories) and infected intracerebrally with 1 × 10⁷ cfu of M. tuberculosis in 50 μL of saline, as determined by viable counts on Middlebrook 7H11 (M7H11) agar plates. To inoculate bacteria in or close to the cisterna magna, we used a mouse cadaver to determine a standardized puncture location. We also determined the standard length of the needle to reach the subarachnoidal space and used a stopping mechanism to prevent overshoot of the needle. Afterward, we histologically checked the site of inoculation.

Groups of 8 mice were killed 3, 7, 12, 18, and 24 weeks after infection. Mice were anesthetized with Hypnorm (Janssen Pharmaceuticals) and midazolam (Roche). Blood was obtained by cardiac puncture, and cerebrospinal fluid (CSF) was collected by puncture of the cisterna magna. We used a mouse cadaver to determine a standardized puncture location. We also determined the standard length of the needle to reach the subarachnoidal space and used a stopping mechanism to prevent overshoot of the needle. Afterward, we histologically checked the site of inoculation.

For cytokine and chemokine measurements, organ homogenates were diluted 1:1 in lysis buffer (150 mmol/L NaCl, 15 mmol/L Tris, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 1% Triton, and 100 μg/mL pepstatin A, leupeptin, and aprotinin) and incubated on ice for 30 min. Supernatants were sterilized using 0.22-μm filters (Corning) and stored at −20°C for later analysis.
Cytokine concentrations were measured using a cytometric bead array in accordance with the recommendations of the manufacturer (BD Biosciences Pharmingen). The mouse inflammation kit contains antibodies for tumor necrosis factor (TNF)–α, interleukin (IL)-6, IL-10, IL-12p70, and monocyte chemoattractant protein (MCP)–1, and the mouse Th1/Th2 kit contains antibodies for interferon (IFN)–γ, IL-2, IL-4, and IL-5. The chemokines cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein (MIP)–2 were measured using a commercially available ELISA in accordance with the recommendations of the manufacturer (R&D Systems). Concentrations are given as mean ± SE values.

The experiments were repeated using $5 \times 10^5$ cfu of $M. tuberculosis$ H37Rv. Mice intracerebrally injected with saline ($n = 2$) and mice not injected at all ($n = 2$) were used as controls.

**Results.** The experimental mice did not show physical signs of being unwell or of growth retardation. Gross pathological analysis of the brains did not show exudate or signs of infarction. Tuberculomas, however, were present. We also found granulomatous inflammation of the lungs and spleen.

Meningitis was defined as positive CSF or brain tissue culture for $M. tuberculosis$ and histopathological evidence of meningitis consisting of infiltration of leukocytes in the meninges. All mice developed meningitis. Cellular infiltration of the meninges is shown in figure 1A and was predominantly perivascular, as shown in figure 1B. The site of inoculation could be identified in 36 of 40 mice and was in or very close to the subarachnoidal space, as shown in figure 1C. This mimics the rupture of a tuberculoma to the CSF.

The number of colony-forming units of $M. tuberculosis$ cultured from tissue homogenates at different time points after infection is shown in figure 2. CSF cultures were negative. After culturing, no CSF was left for further biochemical analysis or cytokine measurements. Three weeks after infection, we counted $4.3 \pm 1.5 \times 10^4$ cfu/μL of brain homogenate. This decreased to a minimum of $5.2 \pm 1.5 \times 10^3$ cfu/μL of brain homogenate at 12 weeks. Interestingly, after 24 weeks, the bacterial load increased to $1.0 \pm 0.6 \times 10^5$ cfu/μL, implicating bacterial reproduction in the mouse brain. Bacterial counts in lung homogenates gradually increased from $2.6 \pm 1.5 \times 10^3$ cfu/μL 3 weeks after infection to a maximum of $3.0 \pm 1.5 \times 10^4$ cfu/μL 24 weeks after infection. Bacterial counts in spleen homogenates increased from $5.0 \pm 0.8 \times 10^3$ cfu/μL 7 weeks after infection to $2.7 \pm 0.8 \times 10^3$ cfu/μL 24 weeks after infection.

In a second experiment, with $5 \times 10^5$ cfu of $M. tuberculosis$, we were able to reproduce these results: all mice developed meningitis, with histopathological findings similar to those of the first experiment. $M. tuberculosis$ could be cultured from all organs throughout the experiments. After 24 weeks, we cultured $2.2 \pm 0.8 \times 10^4$ cfu/μL from brain homogenates, $4.6 \pm 1.8$
Figure 2. No. of colony-forming units per microliter (on a logarithmic scale) cultured from brain homogenates (A), lung homogenates (B), and spleen homogenates (C) 3, 7, 12, 18, and 24 weeks after intracerebral infection with $1 \times 10^5$ cfu of *Mycobacterium tuberculosis*. Data are mean $\pm$ SE values.

$\times 10^3$ cfu/µL from lung homogenates, and $2.7 \pm 0.5 \times 10^4$ cfu/µL from spleen homogenates. No growth of *M. tuberculosis* was found in CSF. Tissue homogenates and CSF of control mice injected with saline and of noninjected control mice did not contain bacilli.

We measured concentrations of cytokines and chemokines in serum and brain homogenates. In both compartments, levels of TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 were $<10$ pg/mL and were comparable with those of uninfected mice. After infection with $5 \times 10^5$ cfu of *M. tuberculosis*, levels of these inflammatory cytokines were also $<10$ pg/mL. In contrast, chemokine concentrations in brain homogenates and serum were highly elevated in mice infected with *M. tuberculosis*, compared with those in noninfected mice. Mean concentrations for MIP-2 after infection with $1 \times 10^5$ cfu were $591 \pm 84$ pg/mL after 3 weeks, $112 \pm 32$ pg/mL after 7 weeks, $368 \pm 112$ pg/mL after 12 weeks, $608 \pm 113$ pg/mL after 18 weeks, and $695 \pm 97$ pg/mL after 24 weeks. Mean concentrations for KC were $123 \pm 16$ pg/mL after 3 weeks, $37 \pm 4$ pg/mL after 7 weeks, $86 \pm 15$ pg/mL after 12 weeks, $61 \pm 11$ pg/mL after 18 weeks, and $72 \pm 6$ pg/mL after 24 weeks. Mean concentrations of MCP-1 were $237 \pm 19$ pg/mL after 3 weeks, $73 \pm 8$ pg/mL after 7 weeks, $80 \pm 7$ pg/mL after 12 weeks, $52 \pm 7$ pg/mL after 18 weeks, and $43 \pm 5$ pg/mL after 24 weeks. Mice infected with $5 \times 10^5$ cfu of *M. tuberculosis* had chemokine concentrations comparable with those observed in the first experiment (data not shown). Control mice injected intracerebrally with saline and naive mice had concentrations $<10$ pg/mL of all measured cytokines and chemokines.

**Discussion.** In this report, we describe a new model of TM in mice. In our experiments, all mice developed meningitis, characterized by a positive brain tissue culture and meningeal inflammation. Meningitis could be diagnosed up to 24 weeks after infection. The histopathological findings in combination with elevated levels of chemokines in the brain homogenates mimic the progression of this disease in untreated human patients.

We designed our model to study the pathogenesis of TM in vivo, focusing on the role played by inflammatory mediators in the central nervous system (CNS). Knowledge of the pathogenesis of TM originates from experiments in rabbits and children postmortem by Rich and McCordock in 1933 [5]. They found that TM results from hematogenous dissemination of *M. tuberculosis* to the CNS after primary pulmonary infection. However, TM develops not directly by hematogenous spread of bacilli to the meninges but by release of bacilli from focal lesions, called “Rich foci,” that are typically located subpial or subependymal.

Infection of mononuclear phagocytes is a critical step in the immunopathogenesis of TM. Microglial cells, the macrophages of the brain, are the preferential cells in the CNS to be infected...
by *M. tuberculosis*. They also regulate the immune response against mycobacteria by secretion of cytokines and chemokines [4, 6, 7]. TNF-α is a crucial cytokine of mycobacterial pathogenicity in the CNS [6, 8, 9, 10, 11]. In human bacterial meningitis other than TM, TNF-α levels in CSF correlate with the clinical course of meningitis: high levels are associated with more-severe disease [12, 13]. This correlation could not be proven in human TM [11]. In vitro data from Curto et al. show production of TNF-α by microglia that peaks 2 h after infection and lasts up to 48 h. Later, a mechanism of inhibition is triggered, leading to the return to basic concentrations of TNF-α [6]. In the rabbit model of TM, TNF-α in CSF also peaks after 2 h and then decreases to 10-fold-lower levels 8 days after infection [8]. In human TM, TNF-α in CSF is also shown to be increased after infection: Mastroianni et al. reported moderately elevated levels detectable for several weeks after infection [10]; Thwaites et al., however, found elevated levels of TNF-α up to 7 days after infection only [11]. In our model, we did not find elevated levels of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-6 in serum or brain homogenates, but we killed the first mice 3 weeks after infection. However, we did find elevated levels of chemokines lasting until up to 24 weeks after infection. Chemokines are small chemotactic proteins that contribute to leukocyte recruitment. In microglial cells in the CNS, chemokine production is up-regulated on activation by stimuli such as bacteria or proinflammatory mediators (such as TNF-α) [14]. Human TM is characterized by increased CSF expression of IL-8, a chemoattractant for neutrophils and a subset of T lymphocytes [10]. Rodent analogues for this chemokine are MIP-2 and KC [14]. In our model, concentrations of MIP-2 and KC were increased in infected mice, explaining the leukocyte influx in the CNS we found on histologic examination. We also found elevated concentrations of MCP-1, a chemokine that attracts mononuclear phagocytes, leading to macrophage activation in general or activation of microglial cells in the CNS.

Histopathological changes in human TM consist of cellular response in the CSF as well as protein influx (cytokines and chemokines), causing perivascular inflammation and occlusion of small vessels, which leads to infarcts and cranial nerve palsies [12]. The infected mice in the present study did have infiltration of leukocytes in their meninges—especially perivascular—comparable with that observed in human infection.

In conclusion, we were able to develop a reproducible in vivo model to study the inflammatory response in TM. However, this model has limitations in terms of translation to human disease: the route of infection mimics only the natural way TM is acquired; mice do not show clinical signs of disease, contrary to human disease; and the cytokine profile is different from that of human disease. On the other hand, we do find bacterial growth of TB in the CNS that leads to a chronic inflammatory response in terms of chemokine production. We intend to use this model to further analyze the role played by the innate immunity in TM, especially the role played by Toll-like receptors and other signaling pathways that orchestrate an immunological response.

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References