Wat je zoekt is niet te vinden,

Wat je vindt niet wat je zocht.

(De Dijk)

4 A new murine model to study the pathogenesis of tuberculous meningitis

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ABSTRACT

Tuberculous meningitis (TBM) 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 TBM.

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 chemoattractants 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.

BACKGROUND

Tuberculosis (TB) remains a global threat to mankind. One third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tub*), and 1.7 million people die each year as a consequence of TB [1]. Tuberculous meningitis (TBM), 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 TBM 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. tub*, was grown for 4 days in liquid Dubos medium containing 0.01% Tween 80. A replicate culture was incubated at 37 degrees Celsius, harvested at mid-log phase, and stored in aliquots at -70 degrees Celsius. For the experiment, a vial was thawed and washed twice with sterile 0.9% NaCl.

Mice were anesthesized by inhalation with isoflurane (Abbott Laboratories) and infected intracerebrally with 1 x 10e5 colony forming units (CFU) of *M. tub* in 50 mL 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. Brain, lungs, and spleen were removed. Half of each organ was fixed in 10% buffered formaline for 24 h; after paraffin embedding, 4-mm sections were stained with hematoxylin-eosin. The other half was homogenized in 5 volumes of sterile 0.9% NaCl by use of a tissue homogenizer (Biospec Products), and

10-fold serial dilutions were plated on M7H11 agar plates to determine the numbers of CFU. CSF was also plated on M7H11 agar plates. CFU were counted after 21 days of incubation at 37 degrees Celsius and are provided as the total number per microliters of organ homogenate or of CSF.

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 MgCl, 1 mmol/L CaCl, 1% Triton, and 100 mg/mL pepstatin A, leupeptin, and aprotinin) and incubated on ice for 30 min. Supernatants were sterilized using 0.22-mmfilters (Corning) and stored at -20 degrees Celsius for later analysis.

Cytokine concentrations were measured using cytometric bead array (CBA) 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 commercially available ELISA kits in accordance with the recommendations of the manufacturer (R&D Systems). Concentrations are given as mean ± standard error (SE) values.

The experiments were repeated using 5 x 10e5 CFU of *M. tub* 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 and no growth retardation after infection. 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. tub* 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 CFU of *M. tub* 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 ± 1.5 x 10e4 CFU/mL of brain homogenate.

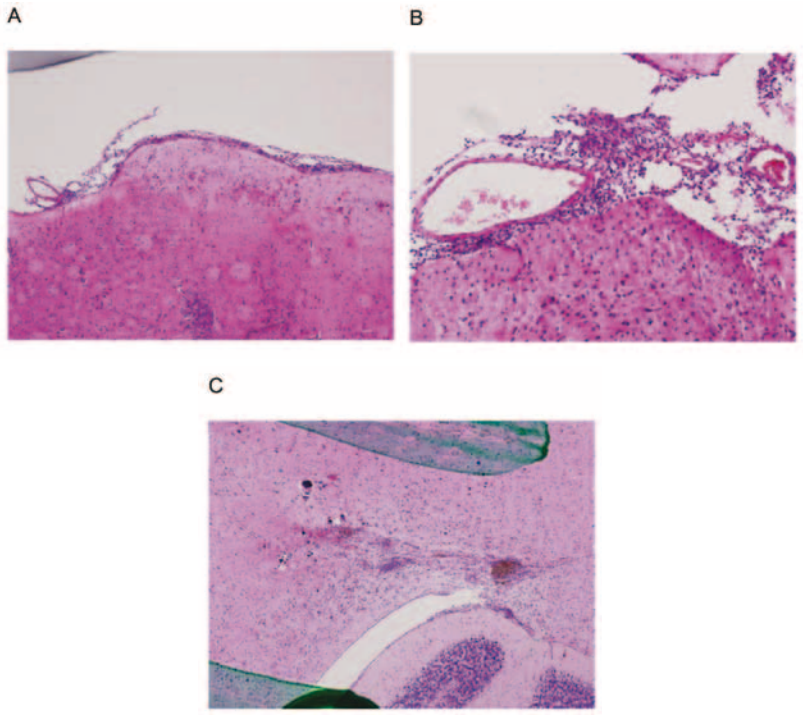

Figure 1. Hematotoxylin-eosin-stained slides of murine brain with meningitis after infection with 1 x 10 cfu of *Mycobacterium tuberculosis*.
Shown are the infiltration of meninges with leukocytes (*A;* original magnification, 4 x 3.3) and perivasculair infiltration (*B;* original magnification, 10 x 3.3). Also shown is the site of bacterial inoculation, with iron deposits due to small hemorrhage (*C;* original magnification, 4 x 3.3)

This decreased to a minimum of 5.2 ± 1.5 x 10e3 CFU/mL of brain homogenate at 12 weeks. Interestingly, after 24 weeks, the bacterial load increased to 1.0 ± 0.6 x 10e5 CFU/mL, implicating bacterial reproduction in the mouse brain. Bacterial counts in lung homogenates gradually increased from 2.6 ± 1.5 x 10e5 CFU/mL 3 weeks after infection to a maximum of 3.0 ± 1.5 x 10e6 CFU/mL 24 weeks after infection. Bacterial counts in spleen homogenates increased from 5.0 ± 0.8 x 10e4 CFU/mL 7 weeks after infection to 2.7 ± 0.8 x 10e5 CFU/mL 24 weeks after infection.

In a second experiment, with 5 x 10e5 CFU of *M. tub*, we were able to reproduce these results: all mice developed meningitis with histopathological findings similar to those of the first experiment. *M. tub* could be cultured from all organs throughout the experiments. After 24 weeks, we cultured 2.2 ± 0.8 x 10e4 CFU/mL from brain homogenates, 4.6 ± 1.8 x 10e5 CFU/mL from lung homogenates, and 2.7 ± 0.5 x 10e5 CFU/mL from spleen homogenates. No growth of *M. tub* was found in CSF.

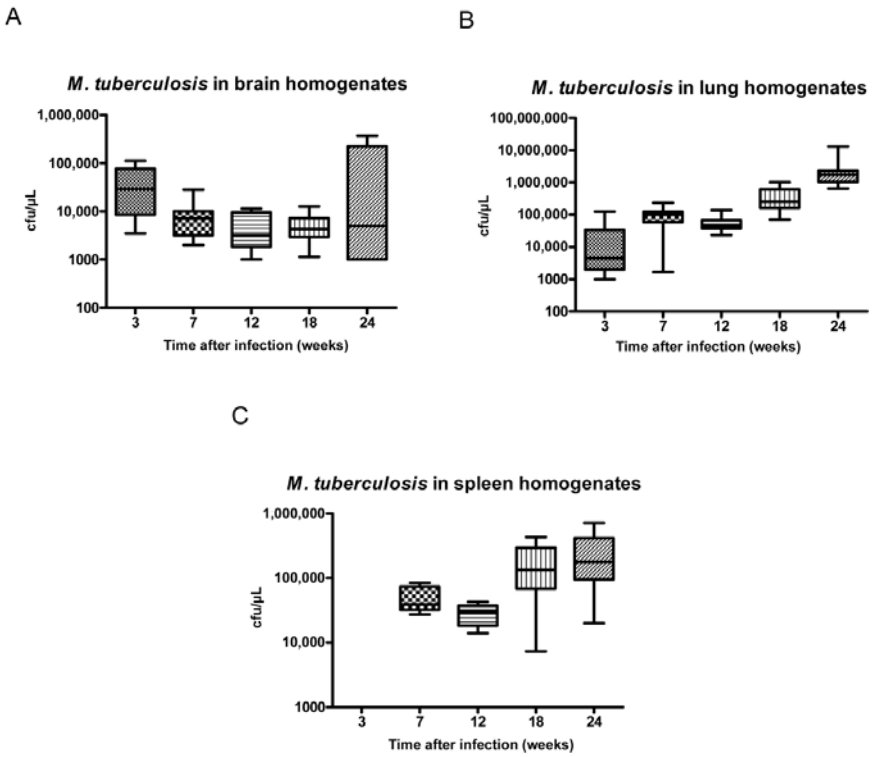

Figure 2. No. of colony-forming units per microliter (on a logarithmic scale) cultured from brain homogenates *(A)*, lung homogenates *(B)*, and spleen homogenates *(C)* 3, 7, 12, 18, and 24 weeks after intracerebral infection with 1 x 10 cfu of *Mycobacterium tuberculosis*. Data are mean ± SE values

Tissue homogenates and CSF of control mice injected with saline and of non-injected 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 x 10e5 CFU of *M. tub*, 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. tub*, compared with those in non-infected mice. Mean concentrations for MIP-2 after infection with 1 x 10e5 CFU were 591 ± 84 pg/mL after 3 weeks, 112 ± 32 pg/mL after 7 weeks, 368 ± 112 pg/mL after 12 weeks, 608 ± 113 pg/mL after 18 weeks, and 695 ± 97 pg/mL after 24 weeks. Mean concentrations for KC were 123 ± 16 pg/mL after 3 weeks, 37 ± 4 pg/mL after 7 weeks, 86 ± 15 pg/mL after 12 weeks, 61 ± 11 pg/mL after 18 weeks, and 72 ± 6 pg/mL after 24 weeks. Mean concentrations of MCP-1 were 237 ± 19 pg/

mL after 3 weeks, 73 ± 8 pg/mL after 7 weeks, 80 ± 7 pg/mL after 12 weeks, 52 ± 7 pg/mL after 18 weeks, and 43 ± 5 pg/mL after 24 weeks. Mice infected with 5 x 10e5 CFU of *M. tub* 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 to study TBM 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 TBM in vivo, focusing on the role played by inflammatory mediators in the central nervous system (CNS). Knowledge of the pathogenesis of TBM originates from experiments in guinea pigs and rabbits and postmortem observations in children by Rich and McCordock in 1933 [5]. They found that TBM results from hematogenous dissemination of *M. tub* to the CNS after primary pulmonary infection. However, TBM develops not directly by hematogenous spread of bacilli to the meninges but by release of bacilli from focal lesions, called *Rich foci*, which are typically located subpial or subependymal. Infection of mononuclear phagocytes is a critical step in the immunopathogenesis of TBM. Microglial cells, the macrophages of the brain, are the preferential cells in the CNS to be infected with *M. tub*. 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 TBM, 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 TBM [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 TBM, TNF-α in CSF also peaks after 2 h and then decreases to 10-fold-lower levels 8 days after infection [8]. In human TBM, 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 upregulated on activation by stimuli such as bacteria or proinflammatory mediators (e.g. TNF-α) [14]. Human TBM 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 TBM 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.

CONCLUSIONS

In conclusion, we were able to develop a reproducible in vivo model to study the inflammatory response in TBM. However, this model has limitations in terms of translation to human disease: although the route of infection mimics the natural way TBM is acquired, mice did not show clinical signs of disease, contrary to human disease, and the cytokine profile was different from that of human disease. On the other hand, we did 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 TBM, especially the role played by Toll-like receptors and other signaling pathways that orchestrate an immunological response.

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REFERENCES

1. World Health Organization. Global tuberculosis control: surveillance, planning, financing. Document WHO/HTM/TB/2005.349. Geneva: World Health Organization, 2005. Available at: http://www.who.int/tb/publications/2005/en/index.html. Accessed 26 August 2005.
2. Farinha NJ, Razali KA, Holzel H, Morgan G, Novelli VM. Tuberculosis of the central nervous system in children: a 20-year survey. J Infect 2000; 41:61–8.
3. Hosoglu S, Geyik MF, Balik I, *et al.* Predictors of outcome in patients with tuberculous meningitis. Int J Tuberc Lung Dis 2002; 6:64–70.
4. Rock RB, Gekker G, Hu S, *et al.* Role of microglia in the central nervous system. Clin Microbiol Rev 2004; 17:942–64.
5. Rich AR, McCordock HA. The pathogenesis of tuberculous meningitis. Bull Johns Hopkins Hosp 1933; 52:5–37.
6. Curto M, Reali C, Palmieri G, *et al.* Inhibition of cytokine expression in human microglia infected by virulent and non-virulent mycobacteria. Neurochem Int 2004; 44:381–92.
7. Rock RB, Hu S, Gekker G, *et al. Mycobacterium tuberculosis*–induced cytokine and chemokine expression by human microglia and astrocytes: effects of dexamethasone. J Infect Dis 2005; 192:2054–8.
8. Tsenova L, Sokol K, Freedman VH, Kaplan G. A combination of thalidomide plus antibiotics protects rabbits from mycobacterial meningitis–associated death. J Infect Dis 1998; 177:1563–72.
9. Tsenova L, Bergtold A, Freedman VH, Young RA, Kaplan G. Tumour necrosis factor alpha is a determinant of pathogenesis and disease progression in mycobaterial infection in the central nervous system. Proc Natl Acad Sci USA 1999; 96:5657–62.
10. Mastroianni CM, Paoletti F, Lichtner M, D'Agostino C, Vullo V, Delia S. Cerebrospinal fluid cytokines in patients with tuberculous meningitis. Clin Immunol Immunopathol 1997; 84:171–6.
11. Thwaites GE, Simmons CP, Than Ha Quyen N, *et al.* Pathophysiology and prognosis in Vietnamese adults with tuberculous meningitis. J Infect Dis 2003; 188:1105–15.
12. Saez-Llorens X, Ramilo O, Mustafa MM, Mertsola J, McCracken GH Jr. Molecular pathophysiology of bacterial meningitis: current concepts and therapeutic implications. J Pediatr 1990; 116:671–84.
13. Mustafa MM, Lebel MH, Ramilo O, *et al.* Correlation of interleukin-1 beta and cachectin concentrations in cerebrospinal fluid and outcome from bacterial meningitis. J Pediatr 1989; 115:208–13.
14. Zwijnenburg PJ, van der Poll T, Roord JJ, van Furth AM. Chemotactic factors in cerebrospinal fluid during bacterial meningitis. Infect Immun 2006; 74:1445–51.