Klebsiella pneumoniae meningitis induces memory impairment and increases pro-inflammatory host response in the central nervous system of Wistar rats

Tatiana Barichello,1 Lutiana R. Simões,1 Samira S. Valvassori,2 Jaqueline S. Generoso,1 Paulo Eduardo D. V. Aveline,1 Diogo Dominguini,2 Samuel G. Elias,1 Marcia C. Vilela,3 João Quevedo2 and Antonio Lucio Teixeira4

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1Laboratório de Microbiologia Experimental, Programa de Pós-Graduação em Ciências da Saúde, Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
2Laboratório de Neurociências, Programa de Pós-Graduação em Ciências da Saúde, Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
3Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG, Brazil
4Laboratório de Imunofarmacologia, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

INTRODUCTION
Previous studies have demonstrated that Klebsiella pneumoniae and other Gram-negative bacilli have become a common cause of meningitis, mainly in patients with head trauma, neurosurgical operations and impaired host defences (Lu et al., 1999, 2002; Mancebo et al., 1986; Mangi et al., 1975). However, there are few studies concerning K. pneumoniae meningitis in the literature (Wen et al., 2007). Bacterial meningitis is a life-threatening infection with high mortality and neurological sequelae (Sellner et al., 2010). Several studies have reported that cognitive processes, such as learning and memory, are affected after bacterial meningitis (Bedford et al., 2001; Schmand et al., 2010).

Klebsiella pneumoniae meningitis has recently become an increasingly common cause of central nervous system infection. The invasion of bacteria within the subarachnoid space stimulates the release of pro-inflammatory cytokines and chemokines, triggering a host immune response. The aim of the present study was to evaluate memory and pro-inflammatory mediators at different times in the brains of adult Wistar rats with K. pneumoniae meningitis. The animals were sacrificed at 6, 12, 24, 48 and 96 h after meningitis induction. The hippocampus, frontal cortex and cerebrospinal fluid were isolated to determine the cytokine, chemokine and brain-derived neurotrophic factor (BDNF) levels. In the first 6 and 24 h following meningitis induction, there was a significant increase of the TNF-α, IL-1β, IL-6, cytokine-induced neutrophil chemoattractant-1 and BDNF levels in the central nervous system. Ten days after meningitis induction, cognitive memory was evaluated using an open-field task and step-down inhibitory avoidance task. In the control group, significant differences in behaviour were observed between the training and testing sessions for both tasks, demonstrating habitation and aversive memory. However, the meningitis group did not exhibit any difference between the training and testing sessions in either task, demonstrating memory impairment. As a result of these observations, we believe that the meningitis model may be a good research tool to study the biological mechanisms involved in the pathophysiology of this illness, while recognizing that animal models should be interpreted with caution before extrapolation to the clinic.

Abbreviations: CSF, cerebrospinal fluid; BDNF, brain-derived neurotrophic factor; CINC-1, cytokine-induced neutrophil chemoattractant-1.
and memory is well described in the literature (Bekinschtein et al., 2007; Rattiner et al., 2005), suggesting that the cognitive dysfunction observed after bacterial meningitis may be linked to changes in this neurotrophin. Morichi et al. (2013) also demonstrated that alterations in BDNF were correlated with increased cytokine levels in the CSF, blood platelet counts and neurological prognoses in patients with bacterial meningitis.

In addition, recent studies from our laboratory have demonstrated that meningitis caused by *Streptococcus pneumoniae* and *Streptococcus agalactiae* resulted in cognitive impairment, decreased BDNF levels and increased cytokine/chemokine levels in the brains of rats (Barichello et al., 2010, 2012a, b, 2013). Bacterial meningitis induces a distinct systemic inflammatory response that has been well described in clinical and experimental subjects (Barichello et al., 2013; Morichi et al., 2013). Clinical and preclinical studies have demonstrated that bacterial invasion of the subarachnoid space triggers potent host responses with elevated levels of pro-inflammatory cytokines/chemokines, such as TNF-α, IL-1β, IL-6 and cytokine-induced neutrophil chemoattractant-1 (CINC-1) (Barichello et al., 2013; Morichi et al., 2013; Scheld et al., 2002).

However, the host immune inflammatory response and behavioural alteration caused by *K. pneumoniae* meningitis infection remain poorly understood (Wen et al., 2007). The current study examined the effects of acute bacterial meningitis induced by *K. pneumoniae* on memory, and the levels of pro-inflammatory cytokines, chemokines and BDNF in the frontal cortex, hippocampus and CSF of rats.

**METHODS**

**Infecting organism.** *K. pneumoniae* was cultured overnight in 10 ml Todd–Hewitt broth, diluted in fresh medium and grown to the exponential phase. The culture was centrifuged for 10 min at 5000 g and resuspended in sterile saline to a concentration of 1 c.f.u. ml⁻¹. The size of the inoculum was confirmed by quantitative culture of 5⁰ C for BDNF concentration. The animals were sacrificed by decapitation at 6, 12, 24, 48 or 96 h after meningitis induction. The hippocampus, frontal cortex and CSF were immediately isolated on dry ice and stored at −80 °C to stabilise the cytokine, chemokine and BDNF levels. Brain tissue was homogenized in extraction solution (100 mg ml⁻¹) containing 0.4 M NaCl, 0.05 % Tween 20, 0.5 % BSA, 0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 kallikrein inhibitor units aprotinin, using an Ultra-Turrax homogeniser (Fisher Scientific). Brain homogenates were centrifuged at 3000 g for 10 min at 4 °C, and the supernatants were collected and stored at −20 °C. The concentration of chemokines and cytokines were determined using ELISA and commercial available antibodies according to the procedures provided by the manufacturer (R&D Systems). BDNF levels were measured as described previously (Frey et al., 2006), using commercial sandwich ELISA kits according to the manufacturer’s instructions (Chemicon). The results were expressed as pg (100 μl CSF)⁻¹ and pg (100 mg cerebral tissue)⁻¹.

**Open-field task.** Eighteen hours after meningitis induction, the animals received ceftriaxone [100 mg (kg⁻¹ body weight)⁻¹] given subcutaneously, over the course of 7 days. Ten days after meningitis induction, the animals were free from infection. Blood cultures performed after the course of treatment were negative (data not shown). The animals recovered their weight and grooming habits, and blood counts returned to control levels. Habituation to an open field was performed in a 40 × 60 cm open field surrounded by 50 cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear quadrant and allowed to explore the arena for 5 min (training session). Immediately following training, the animals were taken back to their home cage and were submitted to a similar open-field session (test session) 24 h later. The number of line crossings and rearings were recorded for both sessions. The decrease in the number of crossings and rearings between the two sessions was used to measure retention of habituation (Vianna et al., 2000). After the animals were sacrificed by decapitation, the hippocampus and frontal cortex were immediately isolated on dry ice and stored at −80 °C for BDNF analysis.

**Step-down inhibitory avoidance task.** The apparatus and procedures have been described in previous reports (Quevedo et al., 1997; Roesler et al., 2003). Briefly, the training apparatus was a 50 × 25 × 25 cm acrylic box (Albarsch) with a floor consisting of parallel 1 mm calibre stainless steel bars spaced 1 cm apart. A 7 cm wide, 2.5 cm high platform was placed on the floor of the box against the left wall. In the training session, the animals were placed on the platform, and the latency before they stepped down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, the animals received a 0.4 mA, 2.0 s foot shock and were returned to their home cage. A retention test trial was performed 24 h after training (long-term memory). The retention test trial was procedurally identical to the training, except that no foot shock was presented. The retention test step-down latency (maximum, 180 s) was used as a measure of inhibitory avoidance retention. Reactivity to the foot shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed. Each animal was placed on the grid and allowed a 1 min habituation period prior to the start of a series of 0.5 s shocks delivered at 10 s intervals. The shock intensities ranged from 0.1 to 0.5 mA in 0.1 mA increments. The adjustments in shock intensity were made in accordance to each animal’s response. The intensity was raised by one increment when no response occurred and was lowered by one increment when a response was observed. A ‘clinical’ response was defined as the...
withdrawal of one paw from the grid floor, and a 'jump' response was defined as rapid withdrawal of three or four paws. Two measurements of the 'flinch' threshold were made, and then two measurements of the 'jump' threshold were made. For each animal, the means of the two scores for the flinch and the jump thresholds were calculated (Bevilaqua et al., 2003; Izquierdo et al., 1998).

**Statistics.** For the cytokine, chemokine and BDNF level analyses, the variables were reported as the means ± SEM of the five to six animals in each group. Differences among groups were evaluated using ANOVA followed by a Student–Newman–Keuls post-hoc test. *P*, 0.05, **P*, 0.01, ***P*, 0.001; statistical significance compared with the control group.

![Graphs of cytokine, chemokine, and BDNF levels](http://jmm.sgmjournals.org)

**Fig. 1.** Kinetics of BDNF, TNF-α, IL-1β, IL-6 and CINC-1 expression in the hippocampus determined at 6, 12, 24, 48 and 96 h after induction of K. pneumoniae meningitis. The concentrations of the cytokines, chemokine and BDNF were assessed by ELISA, and the results are presented as pg (100 mg tissue)⁻¹. The results are the means ± SEM of five to six animals in each group. Differences among groups were evaluated using ANOVA followed by a Student–Newman–Keuls post-hoc test. *P*, 0.05, **P*, 0.01, ***P*, 0.001; statistical significance compared with the control group.
RESULTS

Fig. 1 shows the effects of *K. pneumoniae* meningitis on the TNF-α, IL-1β, IL-6, CINC-1 and BDNF levels in the hippocampus at 6, 12, 24, 48 and 96 h after meningitis induction. In the hippocampus, TNF-α was increased at 6 and 12 h (P<0.05 and P<0.001, respectively); IL-1β and IL-6 were increased at 6, 12 and 24 h (P<0.05); CINC-1 was elevated at 6, 12, 24, 48 and 96 h (P<0.05) and BDNF levels were increased at 24 h and decreased at 96 h (P<0.01 and P<0.05, respectively) after *K. pneumoniae* meningitis induction.

Fig. 2 shows the effects of *K. pneumoniae* meningitis on the TNF-α, IL-1β, IL-6, CINC-1 and BDNF levels in the frontal cortex at 6, 12, 24, 48 and 96 h after meningitis induction. In the frontal cortex, IL-1β levels were increased at 6 (P<0.001), 12 (P<0.001) and 24 h (P<0.01); IL-6 levels were increased at 6, 12, 24, 48 and 96 h (P<0.05, respectively) after *K. pneumoniae* meningitis induction.
DISCUSSION

Several published studies suggest that cytokines and chemokines are the predominant inflammatory mediators in cases of meningitis (Pfister & Scheld, 1997; Scheld et al., 2002; Tunkel & Scheld, 1993). However, the host immune inflammatory response caused by K. pneumoniae meningitis infection remains poorly understood (Wen et al., 2007). In this study, we investigated the effects of K. pneumoniae meningitis on the inflammatory response, BDNF levels and cognition of Wistar rats. The extent of the inflammatory response was monitored by measuring the production levels of the inflammatory mediators TNF-α, IL-1β and IL-6 in the CSF. Additionally, we evaluated habituation memory. Ten days after meningitis induction, there were significant reductions in the number of crossings and rearings in the training sessions of all animals, demonstrating a lack of difference in motor and exploratory activity between the control and meningitis groups. In the control group, there were significant reductions in both crossings (P<0.05) and rearings (P<0.05) in the test session compared with the training session, indicating habituation to the environmental task. Conversely, the meningitis group did not show any significant difference in crossings and rearings between the training and testing sessions, demonstrating memory impairment in that group (P>0.05).

Fig. 3 shows the effects of K. pneumoniae meningitis on the inflammatory response caused by K. pneumoniae meningitis induction. In the same brain structure, the BDNF levels were increased at 24 h (P<0.001) and 96 h (P<0.001) after K. pneumoniae meningitis induction. In the control group, there were significant reductions in the number of crossings and rearings between the training and testing sessions, demonstrating a lack of difference in motor and exploratory activity between the control and meningitis groups. Conversely, the meningitis group compared with the training session, indicating both crossings (P<0.01) and rearings (P<0.001) after K. pneumoniae meningitis induction.

Fig. 4 shows the effects of K. pneumoniae meningitis on the number of crossings and rearings of survivor rats. Data are expressed as the means ± SEM of five to six animals in each group. Differences among groups were evaluated using ANOVA followed by a Student–Newman–Keuls post-hoc test. *P<0.05, **P<0.01, ***P<0.001; statistical significance compared with the control group.

Fig. 5 shows the effects of K. pneumoniae meningitis on the step-down inhibitory avoidance task. A difference was observed between the training and testing sessions in the control group, demonstrating aversive memory (P<0.05). In the meningitis group, there was no difference between the training and testing sessions, demonstrating an impairment of aversive memory in that group (P>0.05).
Pneumoniae led to elevated levels of TNF-α in the control group. Whitney and comparisons among groups were performed using a Mann–Whitney U test. *P<0.05, statistical significance compared with the control group.

In conclusion, cognitive performance was impaired after K. pneumoniae-induced bacterial meningitis in Wistar rats and could be associated with alterations in the levels of cytokines, chemokines and BDNF in the central nervous system.

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