Journal of Neuroimmunology 272 (2014) 35–41

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Neonatal *Escherichia coli* K1 meningitis causes learning and memory impairments in adulthood

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ARTICLE INFO

Article history:
Received 18 March 2013
Received in revised form 12 March 2014
Accepted 4 May 2014

Keywords:
Neonatal meningitis
Escherichia coli
Cytokines
Chemokines
Memory

ABSTRACT

Neonatal *Escherichia coli* meningitis continues to be an important cause of mortality and morbidity in newborns worldwide. The aim of this study was to investigate the cytokines/chemokines, brain-derived neurotrophic factor (BDNF) levels, blood–brain barrier integrity in neonatal rats following *E. coli* K1 experimental meningitis infection and subsequent behavioural parameters in adulthood. In the hippocampus, interleukin increased at 96 h, IL-6 at 12, 48 and 96 h, IL-10 at 96 h, cytokine-induced neutrophil chemoattractant-1 at 6, 12, 24, 48 and 96 h, and BDNF at 48 and 96 h. In the cerebrospinal fluid, tumour necrosis factor alpha levels increased at 6, 12, 24, 48 and 96 h. The BBB breakdown occurred at 12 h in the hippocampus, and at 6 h in the cortex. We evaluated behavioural parameters in adulthood: habituation to the open-field, step-down inhibitory avoidance, object recognition, continuous multiple-trials step-down inhibitory avoidance and forced swimming tasks. In adulthood, the animals showed habituation and aversive memory impairment. The animals needed a significant increase in the number of training periods to learn and not had depressive-like symptoms.

1. Introduction

Neonatal *Escherichia coli* K1 meningitis continues to be an important cause of mortality and morbidity in newborns worldwide (Kim, 2012). The prevalence of serious sequelae among a national cohort of 5-year old children, born in England and Wales in 1996–7, who had neonatal meningitis the overall incidence of serious disability was high, 25.5% in 1985 (De Louvois et al., 1991) compared to 23.5% in 1996 (De Louvois et al., 2005). Moderate/severe disability was reported in 34% of children who had *Streptococcus agalactiae* meningitis, 30% who had meningitis due to *E. coli* or other gram-negative bacilli and 35% where meningitis was due to other bacteria (De Louvois et al., 2005). Furthermore, *E. coli* is the second-most common cause of neonatal meningitis after *S. agalactiae* in France (Gaschignard et al., 2012) and other industrialised countries (Bonacorsi et al., 2003). The inflammatory reaction is characterised by an acute purulent infection of the meninges affecting the pia mater, the arachnoid, and the subarachnoid space (Sellner et al., 2010; Zhu et al., 2012). The microorganisms can replicate within the subarachnoid space concomitantly with the release of the bacterial products that are highly immunogenic and can lead to an increased inflammatory response in the host (Barichello et al., 2013a; Sellner et al., 2010). Many brain cells, including glial cells, endothelial cells, ependymal cells, and resident macrophages, can produce cytokines and pro-inflammatory molecules in response to microorganisms (Kronfol and Remick, 2000). Pro-inflammatory mediators such as tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8) and the anti-inflammatory mediator interleukin-10 (IL-10) are involved in the pathophysiology of bacterial meningitis (van Furth et al., 1996). In previous studies in neonatal

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http://dx.doi.org/10.1016/j.jneuroim.2014.05.003
0165-5728/

rats, we have shown an increase in cytokine-induced neutrophil chemoattractant-1 (CINC-1), IL-1β and IL-6 starting at 6 h and in TNF-α and IL-10 starting at 24 h in the hippocampus after meningitis was induced by *S. agalactiae* (Barichello et al., 2011). In neonatal pneumococcal meningitis, the levels of CINC-1, IL-1β and TNF-α are increased in the hippocampus after 6 h (Barichello et al., 2012a). Thus, due to the production of cytokines, polymorphonuclear leukocytes are released from the blood and become activated, resulting in the production of high amounts of reactive oxygen and nitrogen species (Kastenbauer et al., 2002). This cascade leads to lipid peroxidation, DNA single-strand breaks, mitochondrial damage and a breakdown of the blood–brain barrier (BBB) (Klein et al., 2006), thus contributing to brain injury during neonatal meningitis. Therefore, the aim of this study was to investigate the levels of inflammatory mediators, BDNF at different time points, and BBB integrity in neonatal Wistar rats subjected to *E. coli* K1 meningitis and subsequent behavioural parameters in adulthood.

2. Experimental procedures

2.1. Infecting organisms

The *E. coli* K1 strain was cultured overnight in 10 ml of Todd Hewitt Broth, Himedia® and then diluted in fresh medium and grown to logarithmic phase. The culture was centrifuged for 10 min at 5000 *g* and re-suspended in sterile saline to a concentration of 1×10^6 colony-forming units (CFU). The size of the inoculum was confirmed by quantitative cultures (Grandgirard et al., 2007; Barichello et al., 2010a).

2.2. Meningitis animal model

Neonatal male Wistar rats (15–20 g body weight) aged 3–4 postnatal days from our breeding colony were used for the experiments. All procedures were approved by the Animal Care and Experimentation Committee of UNESC 165/2008, Brazil and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996. All surgical procedures and bacterial inoculations were performed under anaesthesia consisting of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg) (Barichello et al., 2010b; Grandgirard et al., 2007). The animals received an intracisternal (i.c.) injection of 10 μl of sterile saline as a placebo or an equivalent volume of *E. coli* K1 suspension. At the time of the inoculation, the animals received fluid replacement and were subsequently returned to their cages (Barichello et al., 2010b; Irazuzta et al., 2008). Following their recovery from anaesthesia, the animals were fed by their mothers. Meningitis was documented from a quantitative culture of 5 μl of cerebrospinal fluid (CSF) obtained by puncturing the cisterna magna (Barichello et al., 2010b).

2.3. Assessment of TNF-α, IL-6, IL-4, IL-10, CINC-1 and BDNF levels

Neonatal rats were randomised and subjected to meningitis by *E. coli* and were killed by decapitation at 6, 12, 24, 48 and 96 h (n = 6 per group). The hippocampus was immediately isolated on dry ice and stored at −80 °C for the analysis of TNF-α, IL-6, IL-4, IL-10, CINC-1 and BDNF levels. The CSF was immediately isolated on dry ice and stored at −80 °C for the analysis of TNF-α levels (n = 4 to 6 animals per group).

2.3.1. Assessment of TNF-α, IL-4, IL-6, IL-10 and CINC-1 concentrations

The CSF and hippocampus were immediately isolated on dry ice and stored at −80 °C for analyses of the cytokine levels. Brain tissue was homogenised in extraction solution (100 mg of tissue per 1 ml) containing: 0.4 mol/l NaCl, 0.05% Tween 20, 0.5% 7 BSA, 0.1 mmol/l Phenyl methyl sulfonil fluoride, 0.1 mmol/l benzethonium chloride, 10 mmol/l EDTA and 20 KI aprotinin, using Ultra-Turrax (Fisher Scientific, Pittsburgh, PA). Brain homogenate was 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 cytokine was determined using ELISA. The supernatants of the brain tissue and CSF were assayed in an ELISA setup using commercially available antibodies, according to the procedures provided by the manufacturer (R&D Systems, Minneapolis, MN). The results are expressed in pg/100 mg of hippocampal tissue. The limit of detection was 20 pg/ml.

2.3.2. Assessment of BDNF expression

The BDNF levels in the hippocampus were measured using an anti-BDNF sandwich-ELISA according to the manufacturer's instructions (Chemicon, USA). Briefly, the hippocampus and frontal cortex were homogenised in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EGTA. Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in a sample diluent, and the standard curve ranged from 7.8 to 500 pg/ml of BNDF. The plates were then washed four times with sample diluent, and a monoclonal anti-BNDF rabbit antibody diluted 1:1000 in sample diluent was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase-conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After the addition of streptavidin-enzyme, substrate and stop solution, the amount of BDNF was determined by measuring the absorbance at 450 nm and was expressed as pg per mg of wet tissue protein. The standard curve demonstrates a direct relationship between Optical Density (OD) and BDNF concentration. Total protein was measured by the method of Lowry et al. (1951) (Lowry et al., 1951) using bovine serum albumin as a standard, as previously described by Frey et al. (2006).

2.4. Blood–brain barrier permeability to Evan's blue

To evaluate the BBB integrity, the animals were separated into two groups: control and meningitis and were killed at different times at 6, 12, 18, 24 and 30 h after induction of *E. coli* meningitis (Smith and Hall, 1996). The animals were injected with 1 ml of Evan's blue at 1% intraperitoneal (i.p.) 1 h before being killed. The anaesthesia consisted of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg) (Barichello et al., 2012b). The chest was subsequently opened, and the brain was transcardially perfused with 200 ml of saline through the left ventricle at a pressure of 100 mm Hg until colourless perfusion fluid was obtained from the right atrium. The samples were weighed and placed in a 50% trichloroacetic solution. Following homogenization and centrifugation, the extracted dye was diluted with ethanol (1:3), and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer (Hitachi 650-40, Tokyo, Japan). Calculations were based on the external standard (62.5–500 ng/ml) in the same solvent. The Evan's blue tissue content was quantified from a linear standard line derived from known amounts of the dye, and it was expressed per gramme of tissue (Smith and Hall, 1996).

2.5. Behavioural tasks

To evaluate the behavioural responses, the animals were separated into two groups: control and meningitis (n = 10 per group and 20 per each behavioural task; n = 100). Eighteen hours after induction, the animals received ceftriaxone (100 mg/kg body weight given s.c./7 days) (Barichello et al., 2013b). Sixty days after inoculation, the animals were free from infection. All blood cultures that were performed during this period were negative. The animals recovered their weight and grooming habits; their blood counts returned to control levels, and the reactive protein C values were negative (data not shown). Finally, the