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A kinetic study of the cytokine/chemokines levels and disruption of blood-brain barrier in infant rats after pneumococcal meningitis

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ABSTRACT

Bacterial meningitis is an inflammation of the meninges and subarachnoid space that occurs in response of bacteria. Young children are particularly vulnerable to bacterial meningitis, two thirds of meningitis deaths in low-income countries occur among children under the age of fifteen. The main bacterial pathogens causing meningitis beyond the neonatal period are *Streptococcus pneumoniae*, *Haemophilus influenza* type b and *Neisseria meningitidis*. Therefore, the aim of this study is to evaluate the kinetic and the levels of TNF-α, IL-1β, IL-6, IL-10 and CINC-1 in different brain regions as well as the blood-brain barrier permeability after meningitis induced by *S. pneumoniae* in infant Wistar rats. The animals underwent a magna cistern tap receiving either 10 μL sterile saline as a placebo or an equivalent volume of a *S. pneumoniae* suspension at the concentration 1×10^6 CFU/mL. The animals were killed at different times after induction. The brain was removed and the hippocampus and the cortex were isolated and used for the determination of cytokine/chemokine levels and blood-brain barrier permeability. The cerebrospinal fluid was obtained by puncture of the cisterna magna to TNF-α and IL-1β analysis. In the hippocampus, the CINC-1 and IL-1β levels were found increased at 6 h, 12 h and 24 h after pneumococcal meningitis induction. In the cortex the levels of the CINC-1 were increased at 6 h, 12 h and 24 h. The IL-1β and TNF-α were increased at 12 h and 24 h. The level of IL-6 was increased only after 24 h after pneumococcal meningitis induction. In cerebrospinal fluid, the TNF-α was increased at 12 h, 24 h and IL-1 was increased at 24 h after *S. pneumoniae* induction. The blood-brain barrier breakdown in hippocampus and cortex were observed at 12 h until 24 h during meningitis. In conclusion, a peak of pro-inflammatory cytokine/chemokine is associated with disruption of the blood-brain barrier in infants with pneumococcal meningitis.

1. Introduction

Bacterial meningitis is an inflammation of the meninges and subarachnoid space that occurs in response to bacteria (Kim, 2010). Young children are particularly vulnerable to bacterial meningitis, two thirds of meningitis deaths in low-income countries occur among children under the age of fifteen. The main bacterial pathogens causing meningitis beyond the neonatal period are *Streptococcus pneumoniae*, *Haemophilus influenza* type b and *Neisseria meningitidis* (Ramakrishnan et al., 2009). The mortality from pneumococcal meningitis ranges from 15% in industrialized to 40% in developing countries (Brandt, 2010). About 50% of survivors present sequelae including learning impairment, deafness, mental retardation and hydrocephalus (Merkelbach et al., 2000). The multiplication of bacteria within the subarachnoid and ventricular space compartments triggers an intense inflammatory host response at killing the invading microorganism (Yadav et al., 2009). Many cells in the central nervous system can produce cytokines and pro-inflammatory molecules in response to bacteria (Sellner et al., 2010). Pro-inflammatory mediators released in the process include tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6 (Waage et al., 1989; van de Beek, 2009) matrix metalloproteinases (Leib et al., 2000), cytokine-induced neutrophil chemoattractant (CINC-1) (Rosenthal et al, 2009), all of which have been shown to contribute to the development of brain injury in bacterial meningitis (Waage et al., 1989; Leib et al., 2000; Leppert et al., 2000).

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Therefore, the aim of this study was to evaluate the inflammatory process in meningitis induced by *S. pneumoniae* in infant Wistar rats. For this purpose, we measured the levels of TNF-α, IL-1β, IL-6, IL-10, and CINC-1 and evaluated the blood-brain barrier permeability in those rats.

2. Materials and methods

2.1. Infecting organism

S. pneumoniae (serotype 3) was cultured overnight in 10 mL of Todd Hewitt broth, diluted in fresh medium and grown to logarithmic phase. The culture was centrifuged for 10 min at (5000 × *g*) and resuspended in sterile saline to the concentration of 1×10^6 CFU/mL. The size of the inoculum was confirmed by quantitative cultures (Irazuzta et al., 2002; Grandgirard et al., 2007a,b).

2.2. Animal model of meningitis

Infant male Wistar rats (15–20 g body weight), postnatal day 11, from our breeding colony were used for the experiments. All procedures were approved by the Animal Care and Experimentation Committee of UNESC, Brazil, and followed 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 anesthesia, consisting of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg) (Hoogman et al., 2007; Grandgirard et al., 2007a,b; Barichello et al., 2009). Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 μL of sterile saline as a placebo or an equivalent volume of *S. pneumoniae* suspension. At the time of inoculation, animals received fluid replacement (10 mL of saline subcutaneously) and were subsequently returned to their cages (Irazuzta et al., 2002, 2008).

Following their recovery from anesthesia, animals were fed by their progenitor. Meningitis was documented by a quantitative culture of 5 μL of CSF obtained by puncture of the cisterna magna (Barichello et al., 2010a).

2.3. Assessment of TNF-α, IL-1β, IL-6, IL-10 and CINC-1 concentrations

Animals were killed by decapitation at different times after the meningitis induction: 0, 6, 12, 24, 48 and 96 h. The brain structures hippocampus, cortex and cerebrospinal fluid were immediately isolated on dry ice and stored at −80 °C for analyses of the TNF-α, IL-1β, IL-6, IL-10 and CINC-1 levels. Briefly, hippocampus and cortex were homogenized in extraction solution containing aprotinin (100 mg of tissue per 1 mL). The CSF volume from each animal was adjusted to 100 μL with extraction solution. The concentration of cytokines/chemokines was determined in hippocampus and cortex using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet kits, R&D Systems; Minneapolis). The results are shown in pg/100 μL of CSF and pg/100 mg of tissue in cortex and in hippocampus.

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

The blood-brain barrier integrity was investigated using Evan's blue dye extravasations (Smith and Hall, 1996). The animals were injected with 1 mL of Evan's blue at 1% (ip) 1 h before being killed (Coimbra et al., 2007). The anesthesia consisted of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg) (Hoogman et al., 2007; Grandgirard et al., 2007a,b). The chest was subsequently opened 30 min later and the brain was transcardially perfused with 200 mL of saline through the left ventricle at 100 mm Hg pressured until colorless perfusion fluid was obtained from the right atrium. Samples were weighed and placed in 50% of 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 tissue content of EB was quantified from a linear standard line derived from known amounts of the dye and was expressed per gram of tissue (Smith and Hall, 1996). The animals were killed at different times: 3, 6, 12, 18, 24 and 30 h after pneumococcal meningitis induction.

2.5. Statistics

The variables were shown by mean ± S.E.M. of 4–6 animals in each group. Differences among groups were evaluated by using analysis of variance (ANOVA) followed by Student-Newman–Keuls post-hoc test. For cytokine and chemokine analyses, Student's t test was used among the different hours post-infection and sham group. p values < 0.05 were considered statistically significant.

3. Results

The mortality of animals in the sham group was 16.6% and 47.3% in the meningitis group. In hippocampus, the levels of CINC-1 and IL-1β were increased at 6 h ($p<0.01$; $p<0.05$, respectively), 12 h ($p<0.05$) and 24 h ($p<0.001$) after pneumococcal meningitis induction. The cytokines IL-6, IL-10 and TNF-α were not altered when compared with the control group (Fig. 1). In the cortex, the levels of CINC-1 were increased at 6 h ($p<0.05$), 12 h ($p<0.05$) and 24 h ($p<0.001$). The IL-1β was increased 12 h ($p<0.05$) and 24 h ($p<0.001$). The IL-6 was increased after 24 h ($p<0.001$). IL-10 was not altered and TNF-α was increased 12 h ($p<0.05$) and 24 h ($p<0.001$) after pneumococcal meningitis induction (Fig. 2). In cerebrospinal fluid, the cytokine TNF-α was increased at 12 h and 24 h and IL-1β was increased at 24 h after *S. pneumoniae* induction (Fig. 3).

The blood-brain barrier integrity of the hippocampus (Fig. 4A) and cortex (Fig. 4B), was investigated using Evan's blue dye extravasation. We observed the blood-brain barrier breakdown between 12 h and 24 h ($p<0.05$) after meningitis induction.

4. Discussion

Bacterial invasion of meninges induces a complex immune response (Coimbra et al., 2006). Glia cells, endothelial cells and macrophages can produce cytokines and other pro-inflammatory molecules in response to *S. pneumoniae* and the large amounts of subcapsular bacterial components that are released, which include lipopolysaccharide, lipoteichoic acid, pneumolysin and bacterial DNA (Hirst et al., 2004).

We showed that the kinetic production of chemokine CINC-1 and pro-inflammatory cytokine IL-1 was similar in hippocampus and cortex of infant Wistar rats with pneumococcal meningitis. CINC-1 is a CXC chemokine implicated in the infiltration of inflammatory cells into the brain parenchyma (Katayama et al., 2009). CXCL8 (Spanaus et al., 1997) and the cytokine IL-1β are found early in CSF samples of patients with bacterial meningitis, and their concentration is significantly correlated with inflammatory parameters and adverse disease outcomes (Rusconi et al., 1991; Koedel et al., 2002).

IL-1β, IL-6 and TNF-α are considered as major early-response cytokines, which trigger a cascade of inflammatory mediators (Dinarello, 2000). As a consequence, polymorphonuclear leukocytes are attracted and activated. In this work, the cytokines IL-6 and TNF-α levels showed a different profile in these brain anatomic regions of infant rats. The cortex, but not hippocampus of infant rats was able to produce IL-6 and TNF-α. The levels of IL-6 and TNF-α in the cortex