Intrauterine infection promotes brain region specific cytokine activation and hyperactivity in developing rat

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Intrauterine infection during pregnancy is associated with premature birth, periventricular leukomalacia, early activation of the fetal immune system and poor neurodevelopmental outcomes. Previous clinical studies and studies with animal models have shown increased activation of the immune system evidenced by increased levels of inflammatory cytokines, white matter damage and delays in behavioral development. Animal models of intrauterine infection with consistent behavioral results, white matter damage and immune activation have been difficult to validate. Using a rodent model of intrauterine infection, we examined neurobehavioral development and locomotor development in the resulting pups, and measured inflammatory cytokines in the striatum, frontal cortex and cerebellum. Pregnant rats at gestational day 17 were inoculated with 1 × 10⁵ colony forming units of Escherichia coli or 0.1 ml of saline. Intrauterine infection led to a significant increase in the expression of interleukin 1 β, interleukin-6 and tumor necrosis factor-α. E. coli injection increased walking, turning and overall motor activity in rats. In summary, the results of this study indicate that E. coli induced intrauterine infection resulted in neuroinflammation and led to hyperactivity in basic locomotion.

Key words: Intrauterine infection, E. coli, neurodevelopment behavior, immune activation.

INTRODUCTION

Intrauterine infection is a major risk factor for the development of neurodevelopmental brain damage (Dammann et al., 2002; Dammann and Leviton, 1998; Nelson and Willoughby, 2000; Yoon et al., 2000) and is associated with an increased risk of the development of motor impairments similar to those seen in cerebral palsy and periventricular leukomalacia (Bell and Hallenbeck, 2002; Dammann and Leviton, 1998; Wu and Colford, 2000; Yoon et al., 2003). White matter damage, astrocytosis and cytokine activation have been demonstrated in experimental models of intrauterine infection, all of which are capable of leading to delays in brain development (Bell and Hallenbeck, 2002; Cai et al., 2000; Debillon et al., 2000). Animal models of intrauterine infection have white matter lesions similar to those seen in children with cerebral palsy, and have delays in basic neurodevelopmental task (Poggi et al., 2005; Tosó et al., 2005). These experimental models show that maternal or postnatal endotoxin exposure sensitizes the immature brain and increases cytokine levels, both of which can be detrimental to posture and motor development.

Endotoxin exposure is a common and powerful stimuli for inflammatory cytokine activation in the setting of intrauterine infection (Kadhim et al., 2001). Several studies looking at the incidence of in utero bacterial infections have also found an increased incidence in neurodevelopmental problems in these exposed infants (Schendel, 2001; Vigneswaran et al., 2004). In addition to these clinical studies, animal studies have shown that bacterial/endotoxin-induced intrauterine infection leads to an increase in inflammatory cytokines in both the uterine tissues and the fetal brain (Bell and Hallenbeck, 2002; Cai et al., 2000; Urakubo et al., 2001). Escherichia coli is a gram-negative bacterium, which is often found in the urinary tract leading to urinary tract infections. In the

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setting of pregnancy, the bacteria can colonize in the uterine cavity and potentially the uterine tissues. Recent studies have shown that *E. coli* is one of the most common uropathogens, up to an incidence of 62.9% in pregnant women with infection, and that *E. coli* infection is strongly correlated with neonatal sepsis (Guiral et al., 2011; Kuhn et al., 2010; Rafal'skii et al., 2009). Even though *E. coli* is not the only bacteria associated with prenatal infection in humans, it has been isolated in 20 to 40% of prenatal infection related cerebral palsy cases (Mittendorf et al., 2001; Vigneswaran et al., 2004).

We recently reported that in a rodent model of intrauterine infection, developmental, cognitive and motor deficits are present in juvenile and adult offspring (Wallace et al., 2010a; Wallace et al., 2010b). In addition, white matter damage similar to that seen in periventricular leukomalacia is present, including astrocytosis, ventriculomegaly and changes in oligodendrocyte precursors and decreases in Purkinje cell density (Pang et al., 2005; Rodts-Palenik et al., 2004). Our goal in this study is to determine if intrauterine injection of *E. coli* alters postural and motor development in this rodent model of intrauterine infection, and if any changes are accompanied by increased cytokine production in the brain.

**MATERIALS AND METHODS**

**Animal model**

Twelve timed-pregnant Sprague Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were obtained at gestational day (GD) 13. Food and water were available *ad libitum* and the colony was maintained in a 12:12 h light/dark schedule. On GD 17 dams were randomly assigned to either saline (n=6), or *E. coli* (ATCC #25922, Manassas, VA) (n=6). Animals were anesthetized and inoculated with 100 µl of sterile saline or 100 µl of 1 × 10^7 colony forming units of *E. coli* at the bifurcation of the uterine horns (Pang et al., 2005; Rodts-Palenik et al., 2004; Wallace et al., 2010a; Wallace et al., 2010b). Dams were maintained on a standard maintenance 8640 Teklad 22/5 rodent diet from Harlan for the length of the study. All animals were observed daily and allowed to deliver without any additional experimental manipulation and experiments were carried out with the approval of the institutional animal care and use committee of the University of MS Medical Center.

**Behavioral testing**

After delivery, rat pups (*E. coli* = 30; control = 30) were weighed and remained with dams for the length of the study. Locomotor testing (Altman and Sudarshan, 1975; Clarac et al., 2004) began at postnatal day (PND) PND 2 and ended at PND 12. Throughout this time rat pups were separated from dams for a period not exceeding one hour a day. All testing was done between 08:00 and 11:00 h in a room specialized for behavioral testing.

Locomotor testing was analyzed under the following parameters: (1) Crawling was indicated by paddling movements of the paws which results in the pup dragging or pulling itself. (2) Walking was defined as an advanced form of crawling in which the pup can move forward or backward without its’ pelvis touching the floor. (3) Pivoting was indicated by the pup making broad swipes with the paws, producing a paddling motion which results in a turn. (4) Turning was defined as an advanced form of pivoting in which the pup can turn without its’ pelvis touching the floor. (5) Head raise was indicated by the pup raising its head in a vertical motion with the nose pointing up. (6) Grooming was indicated by the pup licking its’ paws or pawing its head.

For locomotor testing animals were placed individually in a clear cage and their movements were video-taped for 5 min. All cages were kept under an infrared light to insure that the animals would not lose any body heat while separated from their litter mates. Video tapes were viewed after testing, using a time-sampling method in which every 10 s the pups’ activity was scored, for a total of 300 sc. Tape raters were blind to treatment and inter-rater reliability was r²=0.91.

**Homogenate preparation and ELISA**

At PND 16 pups were sacrificed and the brains were extracted for cytokine analysis. The frontal cortex, striatum and cerebellum, were dissected, weighed and fast-froze with dry ice and stored at -80°C. To prepare tissue for biochemical analysis, tissue was crushed under liquid nitrogen using a mortar and pestle, followed by homogenization in ice-cold phosphate buffered saline containing a commercial mix of protease inhibitors (Roche Diagnostics, Indianapolis, IN). The homogenate was centrifuged at 4°C for 10 minutes at 1600 RPM. The supernatant was extracted and used for ELISA testing. Commercially available ELISA kits for tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin 1β (IL-1β) (RnD Systems, Minneapolis, MN) were used to analyze the amount of cytokine expression for each region of interest and was carried out according to manufacturer’s instructions. The immunoassay intra-assay coefficient of variation was less than 5% and the detection limits were 5 pg/ml, <5 pg/ml and 21 pg/ml, respectively. A commercially available BCA kit for total protein concentration (Pierce Chemical, Rockford, IL) was used to determine the total amount of protein in each sample. The total protein concentration was normalized for each brain region to the corresponding cytokine value.

**Statistical analysis**

Behavioral data was analyzed by RM-ANOVA using a general linear model created in SPSS version 14.0 for PC. Postnatal day was the repeated measure and prenatal treatment a fixed factor. Post-hoc analysis was done using Bonferroni method of analysis. To determine if intrauterine *E. coli* injection altered the appearance of any of the above behaviors (crawling, walking, etc.) Fisher’s exact test using a two-tailed p value was used. Cytokine protein production in specific brain regions as measured by ELISAs were tested by ANOVA. Post-hoc analysis was performed by Bonferroni correction. P values < 0.05 were considered significant.

**RESULTS**

**General newborn characteristics**

All dams delivered between GD 21 to 22 and there were no statistically significant differences between survival rates in the *E. coli* group versus control (p = 0.087) or in birth weights (p=0.149) between the two groups. Surrogate dams were not used in this study, but dams in the saline groups had 1 to 2 pups taken away per litter to
equalize litter sizes among treatment groups. There were no significant differences in pup weights (p = 0.45) among males or females within and between the treatment groups during the study (Figure 1).

**Intrauterine infection leads to hyperactivity**

Intrauterine infection did not have a gender specific effect on the development of locomotion, so all pups are reported together (saline males [n=16], saline females [n=14], E. coli males [n=13], E. coli females [n=17]; data not shown).

Intrauterine infection significantly reduced the total incidence of crawling (p = 0.05) and pivoting (p = 0.05; Figure 2A and B) while significantly increasing the incidence of walking (p = 0.05) and turning (p = 0.05) (Figure 2C and D) as compared to rats in the control group.

There was no significant difference in the incidence of head raises (p = 0.319) or grooming behavior (p = 0.497; data not shown) between the two groups.

There were no significant differences in the appearance of any locomotor behaviors between the two groups (Table 1).

To determine if the overall activity level of pups changed due to intrauterine infection, the total time spent moving was calculated. Intrauterine infection, significantly decreased the time spent active as compared to pups in the saline group on PND 2 (p = 0.05; Figure 3), while causing hyperactivity between PNDs 6, 9 to 11 as compared to pups in the saline group (p < 0.01; Figure 3).

**Intrauterine infection leads to region specific increases in cytokine expression**

As endotoxin administration increases the immune response, we set out to determine if fetal immune activation still persisted after the in utero insult. Intrauterine infection significantly increased IL-1β (15.86 ± 1.34 versus 7.26 ± 1.4 pg/ml; p = 0.001), TNF-α (40.94 ± 3.81 versus 17.38 ± 3.18 pg/ml; p < 0.001) and IL-6 (11.98 ± 2.27 versus 4.75 ± 1.15 pg/ml; p = 0.008) in the frontal cortex at PND 16 (Figure 4A) as compared to the saline group.

In the striatum intrauterine infection significantly increased IL-1β (6.48 ± 1.09 versus 3.36 ± 0.65 pg/ml; p = 0.021), TNF-α (10.18 ± 2.07 versus 3.62 ± 1.51 pg/ml; p = 0.016) and IL-6 (4.27 ± 0.19 versus 1.54 ± 0.63 pg/ml; p = 0.001) as compared to the saline group (Figure 4B). Intrauterine infection also significantly increased IL-1β (16.51 ± 2.56 versus 7.8 ± 1.19 pg/ml; p = 0.005), TNF-α (33.07 ± 3.95 versus 21.51 ± 1.34 pg/ml; p = 0.010) and IL-6 (10.14 ± 0.94 versus 3.31 ± 0.54 pg/ml; p < 0.001) in the cerebellum as compared to the saline group (Figure 4C).
**DISCUSSION**

It is now well recognized that intrauterine infection can potentially lead to neurodevelopmental and neurological disorders through activation of the fetal immune system (Bell and Hallenbeck, 2002; Bell et al., 2004; Chew et al., 2006; Yoon et al., 2003). We have previously shown that *E. coli* induced intrauterine infection leads to white matter damage, astrogliosis, and increased IL-1β expression in the hippocampus and cerebellum (Rodts-Palenik et al., 2004; Wallace et al., 2010a; Wallace et al., 2010b). Furthermore, rat pups exposed *in utero* to *E. coli* have long-term sensorimotor, motor and cognitive deficits compared to rat pups from non-infected dams (Wallace,
Intrauterine infection increases the total time spent active. E. coli induced-intrauterine infection increased the time spent active after PND 5, indicating that these pups were more hyperactive in comparison to pups in the saline group. Each pup spent 5 min in a clear cage from PN 2 to 12, in which their activity was recorded and scored at a later date. Every 10 s the mode of locomotion was recorded, for a total recording of 30 incidents of movement. The overall activity was assessed as the time spent motionless. Data is represented as average incidence of a motionless score + S.E.M. Significance by two way RM-ANOVA was set to p < 0.05. Post-hoc analysis with Bonferroni, corrected t-tests set to p < 0.01. * denotes days in which E. coli spent significantly less time motionless as compared to pups in the saline group. # denotes days in which E.coli spent significantly more time motionless as compared to pups in the saline group. At each time point, saline n = 30 and E.coli n = 30.

Figure 3. Intrauterine infection increases the total time spent active. E. coli induced-intrauterine infection increased the time spent active after PND 5, indicating that these pups were more hyperactive in comparison to pups in the saline group. Each pup spent 5 min in a clear cage from PN 2 to 12, in which their activity was recorded and scored at a later date. Every 10 s the mode of locomotion was recorded, for a total recording of 30 incidents of movement. The overall activity was assessed as the time spent motionless. Data is represented as average incidence of a motionless score + S.E.M. Significance by two way RM-ANOVA was set to p < 0.05. Post-hoc analysis with Bonferroni, corrected t-tests set to p < 0.01. * denotes days in which E. coli spent significantly less time motionless as compared to pups in the saline group. # denotes days in which E.coli spent significantly more time motionless as compared to pups in the saline group. At each time point, saline n = 30 and E.coli n = 30.
the pups' coordination or timing sequence of ambulatory skills. This would suggest that inflammation alone is not a causative factor for delays in basic ambulation.

Previous studies investigating the role of inflammatory cytokines on rodent behavior have shown that increased mRNA, plasma and neuronal levels of IL-6 are associated with animal models of autism and maternal immune infection (Pang et al., 2006; Parker-Athill and Tan, 2010; Wei et al., 2011). IL-6 plays a role in normal brain development, learning and memory through activation of the STAT (signal transducers and activators of transcription) pathway (Bauer et al., 2007; He et al., 2005). Through mechanisms that are not well understood, IL-6 can trigger the inflammatory cascade and lead to neuronal degeneration and lymphocyte migration thus leading to the development of behavioral abnormalities. IL-1β is capable of activating the hypothalamic-pituitary-adrenal axis and also works through activation of the STAT pathway. IL-1β is similar to IL-6 in that it also has a role in the normal development of the CNS, but can have long-term effects on developmental skills and cognition when activated above physiological levels (Giulian et al., 1988; Wallace et al., 2010a). TNF-α mediates and increases inflammatory cytokine activity similar to IL-1β. Several studies have also shown an immediate increase in TNF-α following intracerebral injections of lipopolysaccharide (LPS) (Cai et al., 2003; Gatti and Bartfai, 1993; Siren et al., 1992), which indicates that LPS is capable of increasing TNF-α production in the brain in response to either direct or indirect endotoxin exposure.

While there is converging evidence that there is a relationship between endotoxin infections during pregnancy and alterations in offspring behavior, the exact physiological mechanism linking the immune response to behavior is still unclear. Proinflammatory cytokines such as TNF-α, IL-1β and IL-6 act in a cascade-like fashion to induce each other and potentially affect the fetal brain when activated in utero (Dammann and Leviton, 1998; Luheshi, 1998), it is probable that the increase in inflammatory cytokines in the perinatal brain, is not the only factor leading to the altered behaviors in the offspring.

The peripheral immune system may also be activated, leading to further increases and activation of the inflammatory cascade. However, if this is possible it

**Figure 4.** The effects of intrauterine infection on the inflammatory cytokine expression in the brain. IL-1Beta, IL-6 and TNF-alpha were significantly increased due to intrauterine injection of *E. coli* in all brain regions. Data is represented as mean ± S.E.M. Data was analyzed using a student’s t-test. *p < 0.05 significantly different as compared to corresponding saline group. Saline n = 15 and *E. coli* n = 15.
should be noted that the increase in inflammatory cytokines did not lead to sickness behavior, which is characteristic of high levels of inflammatory cytokines. Elevations in inflammatory cytokines in rodents can lead to a set of behaviors, primarily weight loss and lethargy, termed sickness behavior (Konsman et al., 2002). In this study we did not use surrogate dams or monitor mothers for sickness behavior, but used the ability of the pup to gain weight as a main index of health (Jen et al., 1978). Although pup weight in the E. coli group was not significantly less than pups in the saline group, it is possible that there were some effects of sickness behavior among the dams (Figure 1). However due to the hyperactivity seen in the E. coli group it would appear as if these animals did not experience any malnourishment.

E. coli did not delay the development of walking but instead increased the activity of walking and turning. The lack of impairments in the development of locomotion as determined by postural assessments indicates that intrauterine infection did not appear to delay or impair any of the neuromuscular networks needed to have successful locomotion (Clarac et al., 2004). This is despite increasing cortical, striatal and cerebellar levels of inflammatory cytokines. Our group and others have shown that endotoxin and cytokine administration does impair cognitive and sensorimotor tasks, which suggest that more complex forms of behavior may be more sensitive to inflammation (Pang et al., 2006; Toslo et al., 2005; Wallace et al., 2010a; Wallace et al., 2010b). Further studies are needed to fully elucidate the mechanism(s) by which neurochemical alterations and neuroinflammation may result in behavioral deficits.

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REFERENCES


