Chorioamnionitis induced by intraamniotic lipopolysaccharide resulted in an interval-dependent increase in central nervous system injury in the fetal sheep

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OBJECTIVE: We quantified the impact of chorioamnionitis on both the white and gray matter structures of the preterm ovine central nervous system (CNS).

STUDY DESIGN: The CNS was studied at 125 days of gestation, either 2 or 14 days after the intraamniotic administration of 10 mg of lipopolysaccharide (LPS) (Escherichia coli) or saline. Apoptotic cells and cell types were analyzed in the brain, cerebellum, and spinal cord using flow cytometry.

RESULTS: Apoptosis and microglial activation increased in all regions with prolonged exposure to LPS-induced chorioamnionitis. Astrocytes were increased in the brain and cerebellum of LPS-exposed fetuses but not in the spinal cord. Mature oligodendrocytes decreased in the cerebral and cerebellar white matter, the cerebral cortex, caudate putamen, and hippocampus 14 days after LPS. Neurons in the cerebral cortex, hippocampus, and substantia nigra were reduced 14 days after LPS.

CONCLUSION: Fetal inflammation globally but differentially affected the CNS depending on the maturational stage of the brain region.

Key words: central nervous system, chorioamnionitis, fetal inflammation, lipopolysaccharide, oligodendrocytes


Chorioamnionitis (CA) and the corresponding fetal inflammatory response are both inversely related to gestational age in preterm infants, affecting mainly the lungs and the central nervous system (CNS). The predominantly studied CNS pathology is cerebral periventricular white matter (WM) disease (WMD), which results in permanent structural brain damage and severe long-lasting neurodevelopmental impairment, such as periventricular leukomalacia. Cortical and subcortical gray matter (GM) and cerebellum are affected as well, contributing to a complex long-term neurologic morbidity. This study aimed to overcome 2 general drawbacks from the most commonly used experimental paradigms. First, lipopolysaccharide (LPS) is usually given intravenously (IV) to induce fetal inflammation. In addition to fetal inflammation, however, this approach also induces important superimposed hypoxia ischemia. Second, little attention has been paid to other CNS regions besides the cerebral WM. To overcome the first drawback, we studied the sheep CNS pathology after CA by LPS administration into the amniotic fluid. This intraamniotic administration avoids the hemodynamic changes and the secondary postasphyctic encephalopathy induced by fetal IV LPS. In addition, this experimental time window corresponds to approximately 28 weeks of human CNS maturation, which is the most vulnerable period for the human brain to develop WMD. To overcome the second drawback, we studied the regional impact on the developing CNS in different regions of the WM and GM using flow cytometry. We hypothesize both a global CNS impact and a time-related effect after LPS-induced CA.

MATERIALS AND METHODS

Animals and surgical procedures

All experimental procedures were approved by our animal ethics board accord-
Bonferroni testing; *P < .05 and **P < .001.

Effect of fetal endotoxin exposure on body and brain weight. A, No significant differences in bodyweight between groups. B, Brain weight was significantly lower in lipopolysaccharide (LPS) 2-day (2D) and 14-day (14D) groups than in control group. C, Endotoxin exposure did not cause any changes in brain to body ratio among 3 groups. Data are represented as mean ± SEM. Black bars = control (n = 7); gray bars = LPS 2D (n = 5); and white bars = LPS 14D (n = 6) groups. Multivariate analysis of variance + Bonferroni testing.

Flow cytometric analysis

AnnexinV/propium iodide staining

Annexin V conjugated to fluorescein (FITC) and propidium iodide (PI) were used to detect annexin V and propidium iodide stained cells. Apoptotic and necrotic cells were detected with annexin V conjugated to fluorescein (FITC) and propidium iodide (PI) (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences Pharmingen, Breda, The Netherlands). The cells were washed twice with phosphate buffered saline (PBS), followed by a wash in Annexin V-binding buffer. Then, all samples were run on an FACScalibur (BD Biosciences), equipped with an argon ion laser (488 nm). Analysis was done using the Cell Quest Pro software (BD Biosciences). Forward and sideward light angle scatters were collected from all samples. Using those plots, samples were gated (region 1; R1) to exclude cell debris and cellular aggregates for further analysis. Intracellular staining (glial fibrillary acidic protein, 2',3'-cyclic nucleotide 3'-phosphodiesterase, and antineurofilament 200-kd)

Antiglial fibrillary acidic protein antibodies were used to identify activated astrocytes. Anti-2',3'-cyclic nucleotide 3'-phosphodiesterase antibodies were used to detect mature oligodendrocytes, whereas antineurofilament 200-kd antibodies were used to detect neurons. Cells were washed twice with staining buffer by centrifugation at 1200 rpm at 4°C. After washing, cells were fixed with 4% paraformaldehyde in staining buffer (2% final concentration) for 20 minutes at RT. Permeabilization was done after 2 more washing steps with permeabilization buffer (0.05% saponin + 2% FBS + PBS) for 10 minutes at RT. The cell suspension was then incubated with the primary antibody for 30 or 45 minutes at RT. The antibodies were mouse antiovine 2',3'-cyclic nucleotide phosphodiesterase (clone 11-5B; Sigma, Munich, Germany); monoclonal antibody 1:200 in permeabilization buffer/rabbit antiovine fibrillary acidic protein (Dako, Glostrup, Denmark); and polyclonal antibody 1:200 in permeabilization buffer/mouse antineurofilament 200-kd (Abcam, Cambridge, UK). Flow cytometry

All samples were run on an FACScalibur flow cytometry system (BD Biosciences, San Jose, CA), equipped with an argon ion laser (488 nm). Analysis was done using the Cell Quest Pro software (BD Biosciences). Forward and sideward light angle scatters were collected from all samples. Using those plots, samples were gated (region 1; R1) to exclude cell debris and cellular aggregates for further analysis. For each marker, the mean fluorescence intensity and the percentage of positive cells stained above background were measured for a total of 10,000 cells per sample within the gate (R1). The cut-
off was defined using control tissue negative for the different markers processed and stained alongside the experimental samples. The mean fluorescence intensity was corrected for autofluorescence using the values from cells only incubated with secondary antibody.

Statistical analysis
Data were analyzed using a multivariate analysis of variance (mean ± SEM). Significant effects were analyzed by post hoc Bonferroni corrections. The accepted level of significance was \( P < .05 \). All calculations were done using software (SPSS 12.0; SPSS, Inc, Chicago, IL).

Representative flow cytograms of control and lipopolysaccharide (LPS) 14-day (14D) animals. Flow cytograms of AnnexinV (AnnV) binding vs propidium iodide (PI) uptake of A, control and B, LPS 14D animals. Four populations of cells are visualized. Viable cells, in lower left quadrant (Q3), are double negative. Apoptotic cells in lower right quadrant (Q4) are AnnV\(^+\) and PI\(^-\). Double-positive cells (AnnV\(^+\)/PI\(^+\)) are in upper right quadrant (Q2). They are thought to be necrotic or advanced apoptotic. Cells in last quadrant (upper left, Q1) are isolated nuclei or cellular debris. There was significant decrease in number of viable cells in LPS 14D animal as compared with control animal. Further, significant increase in apoptotic (Q4) and necrotic index (Q2) was observed in LPS 14D animal compared with control animal. Flow cytograms of C and D, OX42, E and F, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), G and H glial fibrillary acidic protein (GFAP), and I and J, antineurofilament 200-kd (NF-200) staining of C, E, G, and I, control and D, F, H, and J, LPS 14D animals. Data were plotted as fluorescence (FL\(_1\)) in function of number of counts. Percentage of positive cells in histograms is indicated by line. There was significant increase in percentage of activated microglia and astrocytes in LPS 14D compared with control animal. Moreover, proportion of mature oligodendrocytes and neurons was lower in LPS 14D animal than in control animal.

**RESULTS**

**Brain and bodyweight**

The mean body and brain weights of the LPS 2-day (n = 5), LPS 14-day (n = 6), and control (n = 7) groups are shown in Figure 1. There were no significant differences in body weight among the 3 groups (Figure 1, A). The mean brain weight of both the LPS 2-day (P = .031) and LPS 14-day (P < .001) group was significantly lower than the mean brain weight of the control group (Figure 1, B). A significant difference between the LPS 2-day and the LPS 14-day group was shown, with the last group being most affected (P = .048 vs control). The percentage brain of total body weight did not differ significantly between the different groups (Figure 1, C).

**Apoptotic and necrotic cell death**

Figure 2, A and B, shows representative examples of the histograms of a control animal and LPS 14-day animal. Percentages of living (AnnexinV<sup>-</sup> and PI<sup>-</sup>), apoptotic (AnnexinV<sup>+</sup> and PI<sup>-</sup>), and necrotic (AnnexinV<sup>+</sup> and PI<sup>+</sup>) cells are given in Figure 3. Double-negative (AnnexinV<sup>-/PI</sup>⁻) cells are viable cells (Figure 3, G-I). Values of living cells were significantly lower in the LPS 14-day group (33-43%) in all CNS areas investigated compared with controls (57-75%). Only the cortex, hippocampus, hypothalamus, and the T11 level of the SC showed a significant decrease in the LPS 2-day group. AnnexinV<sup>+/PI</sup>⁺ cells are considered to be apoptotic cells (Figure 3, A-C). The percentage of apoptotic cells increased significantly in the LPS 14-day group (28-46%) compared with the saline group (12-23%) in all regions of interest. In the cortex, hippocampus, hypothalamus, cerebellar cortex, cerebellar WM, and T11 level of the SC, the percentage of apoptotic cells in the LPS 2-day group was higher than in controls. Double-positive cells (AnnexinV<sup>+/PI</sup>⁺) are considered to undergo necrosis or advanced apoptosis (Figure 3, D-F). A significant increase in the necrotic index in the LPS 14-day group (14-30%) in comparison with the saline group (5-22%) was shown in the cerebral cortex, CPU, hippocampus and periventricular WM in the brain, T11 and L1 level of SC, and in the cerebellar WM. In addition, there was also a significant increase in the necrotic in-
dex in the cortex, CPU, hippocampus, T11 level of the SC, and cerebellar cortex in the LPS 14-day group compared with the LPS 2-day group. The necrotic index was not different in the SN, hypothalamus, C1 of the SC, or the cerebellar cortex.

**Cell populations**

**Activated microglia**

The representative histograms are shown in Figure 2, C and D. The number of activated microglia (OX42⁺) was very low in the saline group (0.8-1.6%). In almost all regions (CPU, hippocampus, SN, hypothalamus, L1 level of SC, the cerebellar cortex, and cerebellar WM), the percentage of activated microglia was significantly higher in the LPS 2-day group (2.5-5%) than in the saline group. There was an even higher increase in the proportion of activated microglia in the LPS 14-day group (3.2-6.8%), affecting all areas investigated (Figure 4, A-C).

**Mature oligodendrocytes**

The representative histograms are shown in Figure 2, E and F. The values of the mature oligodendrocytes observed were similar for the saline (9-52%) and the LPS 2-day (8-57%) groups for all areas investigated (Figure 4, D-F). In the cortex, CPU, hippocampus, periventricular WM, and cerebellar WM, the proportion of mature oligodendrocytes was much lower in the LPS 14-day group (3-35%) than in the saline group (9-45%). All other regions investigated were not affected in the LPS 14-day group.

**Reactive astrocytes**

The representative plots are shown in Figure 2, G and H. No significant differences were found in the percentage of astrocytes in the LPS 2-day group (12-27%) compared with the saline group (10-22%) (Figure 4, G-I). However, in the LPS 14-day group (14-36%) a
significant increase in the proportion of astrocytes was observed in all areas of the brain and cerebellum. The SC, in contrast, was not affected.

**Neurons**

No significant differences were found in the proportion of neurons between the control (41-82%) and the LPS 2-day (43-82%) groups for any of the areas investigated (Figure 4, J-L); representative histogram is in Figure 2, I and J. In the LPS 14-day group (41-73%), however, the percentages of neurons were significantly lower in the cortex, hippocampus, and SN compared with the control group. No differences were detected in the cerebellum or SC.

There was no influence of sex, singleton/twin, or weight on any of the analyzed variables.

**COMMENT**

CA resulted in an interval-dependent increase in CNS injury and different regional injury patterns. Microglial activation and apoptotic cell death were increased in all brain, cerebellum, and SC regions in an interval-dependent manner. In addition, most brain regions showed reductions in oligodendrocytes and neurons and an increase in astrocytes, whereas the cerebellum and especially the SC seemed to be less affected. This study is the first to show a global inflammatory response to acute CA in the brain, cerebellum, and SC and a selective vulnerability of the developing regions.

These changes might be explained by the state of CNS maturation at that given gestational age. In the fetal brain, the WM is predominantly affected by systemic inflammation/infection as highlighted by fetal animal experiments in rabbits, guinea pigs, mice, rats, and sheep. Furthermore, CA is associated with both cerebral palsy and WMD seen with neonatal imaging. More recent evidence points toward a relationship between WMD and GM abnormalities. This could explain the permanent neurodevelopmental and intellectual deficits in ex-preterm infants, which are not limited to cerebral palsy. The use of this model of CA has several advantages, making it clinically more relevant than others. First, the CA-associated systemic inflammation has been previously characterized in this model by Kramer et al. Second, the IA LPS strategy avoids the superimposed hypoxia ischemia of the IV strategy and the local processes caused by an intracerebral route. Third, species with a long gestation duration, such as human beings and sheep, share several aspects of development and function. In addition, the current study not only focused on brain WMD, but it also analyzed the effect of developmental inflammation on both the cerebral cortical and subcortical GM structures and the cerebellar and SC regions. Experimental data combining all these regions are not available.

The loss of oligodendrocytes, especially in the periventricular and the subcortical WM, has been reported before in intracerebral, IV, intrauterine, and maternal LPS models in different animal species. In addition, a previous study using a similar ovine intraamniotic LPS model also demonstrated a decrease in oligodendrocytes in the areas of extensive focal damage. In contrast to our study, no global effects were previously found on the density and/or number of mature oligodendrocytes throughout the whole brain. Moreover, our study showed a decreased number of neurons in the cortex, hippocampus, and SN in the LPS 14-day group compared with the control group, whereas neither the cerebellum nor the SC differed from the control animals. GM and/or neuron-specific damage have not been identified before in intracerebral or IV LPS models. Maternal administration of LPS in rats, however, led to a loss of tyrosine hydroxylase-positive neurons in the SN of 16-month-old offspring. In addition, maternal LPS caused the loss of pyramidal cells in the hippocampus at the age of 8 months.

Apoptotic cell death was increased in some of the regions in the LPS 2-day group (cortex, hippocampus, hypothalamus, cerebellar cortex, cerebellar WM, and T11) and in all areas studied in the LPS 14-day group. Necrotic cells were also higher in most areas of the LPS 14-day group compared with the control group (cortex, CPU, hippocampus, periventricular WM, and cerebellar cortex T11 and L1). In a human postmortem study, apoptosis has already been reported as a contributing mechanism for cell death in infants with WMD. Garnier et al also found evidence of apoptotic cell death, especially in the periventricular WM, in a sheep model for IV LPS. Moreover, an intracervical LPS injection of pregnant rats led to the activation of both the intrinsic and the extrinsic pathway of apoptosis. One possible explanation for LPS-induced apoptotic cell death could be that LPS causes the up-regulation of the systemic production of tumor necrosis factor (TNF)-α, activating the TNF-α-caspase pathway and an increased production by microglia and astrocytes in the brain. Apoptosis has been reported to affect mainly oligodendrocytes and/or their precursors, but our study suggests that neurons are affected as well.

Microglial activation was increased in all regions of both LPS groups with the exception of cerebral cortex and WM, and the C1 and T11 level of SC in the LPS 2-day group. Increased astrocytic activation was observed in all areas of the brain and cerebellum in the LPS 14-day group, whereas the SC was not affected. Similar increases were found in almost all other LPS models, in particular in the cerebral WM. Our study shows for the first time general microglial activation or astrogliosis throughout the whole brain. The activation of microglia and astrocytes in the brain may be caused by the entry of systemic inflammatory cells and cytokines through a permeable blood-brain barrier. Activated microglia and astrocytes respond to an inflammatory insult, which may affect the WM and, second, explain the concomitant selective GM compromise. First, neurons destined for the frontal cerebral cortex migrate through the developing WM and form the subplate during late gestation. These subplate neurons are the only neuronal element of developing cerebral WM and persist up to 6 months postnatally. They may guide thalamocortical and cortical axons. In patients with WMD, apoptotic changes are seen in subplate neurons, WM, and cor-
global deficits at 2 years of corrected age and imposed GM abnormalities and genetic resonance imaging analysis in ex-preterm infants, where WMD was associated with both a reduced cerebral cortical and basal ganglia volume and with a reduced thalamic volume. In addition, WM and cortical GM volume reductions are correlated with working memory deficits at 2 years of corrected age and hippocampal volume reductions are correlated with a memory impairment in ex-preterm infants at 13 years. Further, qualitative term magnetic resonance imaging analysis with WMD is positively correlated with superimposed GM abnormalities and global deficits at 2 years of corrected age in ex-preterm infants.

Myelination proceeds from caudal in the first trimester of gestation to rostral up until infancy. This maturational hierarchy is a plausible explanation for the lesser general impact of CA on the SC as compared with the cerebellum and brain, and the unchanged cerebellar neuronal counts in LPS-treated animals. The developing cerebellum is of particular importance because it might not only influence motor control and posture, but also the more complex cognitive processes.

In summary, the CNS inflammatory impact was universal, because all subjects of a given group were almost equally affected. The impact was also interval dependent, because damage was more prominent in the 14-day CA group than in the 2-day group. Moreover, the impact showed selective regional differences, meaning that neither astrocytic and neuronal SC changes nor neuronal cerebellar changes were found in either CA group. Whether the differences between groups are caused by: (1) length of intraamniotic LPS exposure (2- vs 14-day interval); (2) stage of fetal neurodevelopment at the time of LPS exposure (day 111 of gestation vs day 123 of gestation); or (3) both cannot be differenti-ated on the basis of the experimental design of this study.

These findings are relevant because this is the first study showing both global WM and GM impact of an acute CA in association with regional differences.  

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REFERENCES


