Antenatal Bacterial Endotoxin Sensitizes the Immature Rat Brain to Postnatal Excitotoxic Injury

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Abstract
Intracerebral injection of ibotenate in newborn rodents produces brain damage that mimics that of infants with cerebral palsy. Because maternal infection may contribute to brain injury in preterm infants, we investigated brain damage after maternal inflammation and postnatal ibotenate treatment in a rat model of cerebral palsy. Pregnant rats were injected intraperitoneally with lipopolysaccharide at Days 19 and 20 of gestation. Neonates were given intracerebral injections of ibotenate at postnatal Day 4 and were then killed at Day 9. Lesion sizes were measured by cresyl violet staining, and microglial activation, astrogliosis, and myelination were evaluated by immunohistochemistry. The lipopolysaccharide groups had larger cortical and white matter lesions than the control group; they also had significantly greater microglial activation and astrogliosis and less white matter myelination in the lesioned hemispheres compared with the controls. Thus, maternal endotoxin exposure may affect prenatal development of the offspring and modulate the subsequent development of excitotoxic brain lesions. These results demonstrate the critical influence of prenatal immune events on neonatal central nervous system vulnerability and provide a model for studying the pathophysiology of cerebral damage in preterm infants and, specifically, the interplay between brain inflammation and excitotoxicity.

Key Words: Brain development, Cerebral palsy, Excitotoxicity, Ibotenate, Lipopolysaccharide, Periventricular leukomalacia, White matter injury.

INTRODUCTION
Prenatal events have an important influence on neonatal outcome. Among these events, intrauterine infections seem to predispose the developing brain to neonatal white matter injury, the major cause of cerebral palsy. Several clinical studies support the hypothesis that an immune-mediated inflammatory process could be a critical pathogenetic factor. For example, large concentrations of proinflammatory cytokines in the amniotic fluid and in fetal plasma have been observed during perinatal infection (1, 2), and cytokine immunoreactivity is also increased in the white matter, mainly in astrocytes and in microglia (3). In animal models, intracerebral, intravenous, or intrauterine administration of lipopolysaccharide (LPS) is clearly associated with white matter damage in the neonatal brain (4–6). Moreover, we recently demonstrated that maternal intraperitoneal administration of LPS at Days 19 and 20 of gestation (E19 and E20) results in lower brain weight in rat pups at postnatal Days 1 and 7 (P1 and P7), higher interleukin 1β mRNA expression in the neonatal brain, and a higher rate of cell death in the periventricular area and in white and grey matter than in control animals (7). When the reaction of parenchymal cells in the brain was investigated, it seemed that maternal LPS exposure induced hypomyelination and astrogliosis in the white matter, both at short and long terms, in the offspring (7, 8).

In full-term and preterm newborns, the glutamate excitotoxic cascade is often considered to represent the final pathway of numerous risk factors that lead to neuronal cell death. Intracerebral injection of glutamate analogs in newborn rodents produces striatal, cortical plate, and cystic periventricular white matter lesions that mimic those observed in human neonates (9). Furthermore, in a fetal sheep model of ischemia, umbilical cord occlusion-induced brain damage in the cerebral white matter is associated with increased glutamate (10). Proinflammatory cytokines affect glutamate metabolism and can potentiate excitotoxic neuronal death in vitro (11). A more recent in vivo study using a neonatal murine model further demonstrated that proinflammatory cytokines administered systemically exacerbate brain excitotoxic lesions (12). N-methyl-D-aspartate receptors are likely involved in these effects because MK-801, their specific antagonist, diminished the lesion size. Little is known, however, about the cerebral effects of the combination of antenatal inflammation (i.e. immune challenges) and a second excitotoxic insult after birth. We hypothesize that antenatal maternal immune challenge causes an inflammatory reaction that renders the fetal central nervous system vulnerable to secondary insults later in life. Therefore, the purpose of this study was to investigate a “double hit” rat model in which there will be combined effects on the developing brain of maternal inflammation induced by antenatal intraperitoneal LPS administration and a second excitotoxic insult induced by postnatal intracerebral injection of ibotenate, a glutamate antagonist, diminished the lesion size. Little is known, however, about the cerebral effects of the combination of antenatal inflammation (i.e. immune challenges) and a second excitotoxic insult after birth. We hypothesize that antenatal maternal immune challenge causes an inflammatory reaction that renders the fetal central nervous system vulnerable to secondary insults later in life. Therefore, the purpose of this study was to investigate a “double hit” rat model in which there will be combined effects on the developing brain of maternal inflammation induced by antenatal intraperitoneal LPS administration and a second excitotoxic insult induced by postnatal intracerebral injection of ibotenate, a glutamate antagonist, diminished the lesion size. Little is known, however, about the cerebral effects of the combination of antenatal inflammation (i.e. immune challenges) and a second excitotoxic insult after birth. We hypothesize that antenatal maternal immune challenge causes an inflammatory reaction that renders the fetal central nervous system vulnerable to secondary insults later in life. Therefore, the purpose of this study was to investigate a “double hit” rat model in which there will be combined effects on the developing brain of maternal inflammation induced by antenatal intraperitoneal LPS administration and a second excitotoxic insult induced by postnatal intracerebral injection of ibotenate, a glutamate
analogue that acts on N-methyl-D-aspartate and metabotropic receptors.

MATERIALS AND METHODS

Animals and Drugs

Experiments on rats were carried out in compliance with appropriate European Community guidelines (86/609/EEC). Time-pregnant Wistar rats were purchased from CERJ (Le Genest, France). Rats had free access to food and water and were kept at 22°C with normal light cycle (12-hour/12-hour). Lipopolysaccharide (Escherichia coli, serotype 055:B5; Sigma, St Louis, MO) diluted in saline was injected intraperitoneally into pregnant rats on gestational Days 19 and 20. To determine the effect of LPS dose, 2 doses were used: 300 μg/kg [as previously reported (7)] (LPS 300 group) and 400 μg/kg (LPS 400 group). The control group consisted of pregnant rats injected with saline on the same days.

Twelve litters were used; they were culled to 8 or 10 pups/litter, according to the numbers of animals needed (13). Five animals from each of 6 dams (2 controls, 2 LPS 300, and 2 LPS 400) were used for histological studies of lesion size in paraffin sections, and 4 animals from each of the other dams (2 controls, 2 LPS 300, and 2 LPS 400) were used for immunohistochemistry on frozen sections. There was no maternal mortality after LPS injections, and there was no mortality in the pups after ibotenate injections.

Ibotenate-Induced Excitotoxic Lesion

Intracerebral injections were performed under isoflurane anesthesia at P4 as previously described (14). Using a Hamilton syringe mounted on a calibrated microdispenser, a 26-gauge needle was inserted 2 mm under the external surface of the scalp skin in the frontoparietal area of the right hemisphere, 2 mm from the midline in the lateral-medial plane and 3 mm from the junction between the sagittal and lambdoid sutures in the rostrocaudal plane. Two 1-μL boluses of ibotenate (Sigma; 5 μg/μL diluted in phosphate-buffered saline [PBS] with 0.02% acetic acid) were injected at a 30-second interval. In all cases, the tip of the needle reached the periventricular white matter. After the injections, the pups were returned to their dams.

Determination of Lesion Size

Five days after intracerebral injection of ibotenate (P9), the pups were killed by decapitation, and their brains were fixed in 4% paraformaldehyde for 5 days. P9 was selected on the basis of previous studies showing that the ibotenate-induced lesion has reached its final size by 5 days after injection (14). Serial paraffin sections, 16 μm thick, were cut in the coronal plane, from the pial surface to the lateral ventricle, and 3) the fronto-occipital axis (in a sagittal plane). In previous studies, we showed an excellent correlation between the maximal radial and fronto-occipital diameters of ibotenic acid-induced lesions (9, 14). Therefore, sectioning the entire brain in the coronal plane provided an accurate and reproducible determination of the maximal fronto-occipital diameter of the lesion that can then be used as an index of lesion size. All microscope examinations were performed by an observer who was blinded to the group assignment of the animals.

Immunohistochemical Studies

After the newborn rats were killed at P9 by decapitation, the brains were dissected on ice and immersed in 4% paraformaldehyde for 48 hours and a bath of 10% cacodylate for 24 hours. Brains were frozen in isopentane cooled at −35°C using dry ice and stored at −80°C. Frozen coronal sections (16 μm thick) were cut on a cryostat (Jung CM3000; Leica, Wetzlar, Germany), mounted on Superfrost slides at −20°C, and stored at −80°C for further use. Sections were taken from brain regions corresponding to plates 13 and 31 of the atlas of Paxinos and Watson (15).

FIGURE 1. (A) Cresyl violet-stained section showing lesions in the cortical plate (arrow) and the white matter (asterisk) induced by ibotenate. Scale bar = 60 μm. (B) Quantitative analysis of white matter and cortical plate lesions produced by intracerebral ibotenate injections. Columns represent the mean length of the lesion along the sagittal fronto-occipital axis. Control, n = 10; lipopolysaccharide (LPS) 300, n = 10; and LPS 400, n = 10. Results are expressed as mean ± SEM. Comparisons with controls were performed using the Mann Whitney U test and the Kruskal-Wallis test (***, p < 0.001). LV indicates lateral ventricle.
Anti-mouse myelin basic protein (MBP), anti-mouse glial fibrillary acidic protein (GFAP), and anti-mouse CD11b mouse monoclonal antibodies (Sigma and Serotec, Raleigh, NC) were used as primary antibodies for detection of myelin, astrocytes, and microglia, respectively. Sections were fixed for 3 minutes in methanol, 4 minutes in acetone, and then air dried for 30 minutes. After rinsing the sections twice with PBS (without calcium and magnesium 1X [Gibco] with gelatin 2% and Triton 0.25%), brain sections were incubated with the primary antibody diluted at 1:1000 in PBS at room temperature overnight. Sections were rinsed 4 times in PBS and incubated with biotinylated anti-mouse immunoglobulin G diluted at 1:400 in PBS for 90 minutes for MBP staining with 3,3'-diaminobenzidine (Sigma) for at least 10 minutes and mounted with counterstaining. To avoid regional and experimental variations in labeling intensity, sections from different experimental groups (including comparable anatomical regions) were stained simultaneously.

### Counting GFAP and CD11b-Labeled Cells

Two investigators, both blinded to group assignment, conducted the quantitative analysis. Glial fibrillary acidic protein- and CD11b-positive cells were counted in the ibotenate-injected hemispheres (ipsilateral hemispheres) at the level of the cingulate region (at the site of ibotenate-induced periventricular white matter lesion) and in the following neuroanatomic locations: cortical plate (at the level of ibotenate-induced lesion), hippocampus, striatum, and internal capsule. Labeled cells were also counted in the contralateral untreated hemispheres at the same anatomic level. For each animal, cell counts were performed in a 0.036-mm² area of the most affected section (determined at 40× magnification) with a Leica microscope coupled to a computer-assisted image analyzer (Histolab; Microvision Instrument, Evry, France). At least 4 matched sections from each animal were used for quantification. Five to 7 animals were included in each experimental group.

### MBP Quantitation

The optical density of MBP-stained fibers was measured in the cingulum and the internal capsule of coronal sections. At least 4 sections per brain of 6 animals per group (i.e., controls, LPS 300, LPS 400) were examined. Optical density was measured at 200× magnification using a computerized image-analysis system (Mercator; Explora Nova, La Rochelle, France), which reads optical density as gray levels. Nonspecific background densities were measured at each brain level in a region devoid of MBP immunostaining and were subtracted from the cingulum and internal capsule values (16).

### Statistical Analysis

Quantitative data were expressed as mean values ± SEM for each group, and results were analyzed using the Mann-Whitney U test or the Kruskal-Wallis test. The significance level was set at p < 0.05.

## Results

### Lesion Size After Ibotenate Injection

Pups injected intracranially with ibotenate on P4 developed cortical lesions and periventricular white matter cysts (Fig. 1A). Pups whose mothers had received LPS had

| TABLE 1. Activated Microglia in Different Brain Regions at P9* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Cingulum        |                 | Cortical Plate  |                 | Hippocampus     |                 | Striatum        |
|                 | Ipsilateral     | Contralateral   | Ipsilateral     | Contralateral   | Ipsilateral     | Contralateral   | Ipsilateral     |
| Control         | 24 ± 5†         | 8 ± 2           | 40 ± 6‡         | 27 ± 3          | 31 ± 2          | 26 ± 5          | 18 ± 6          |
| LPS 300         | 33 ± 4†         | 18 ± 3          | 44 ± 5†         | 32 ± 4          | 30 ± 2          | 29 ± 5          | 25 ± 8          |
| LPS 400         | 38 ± 4†         | 20 ± 4          | 48 ± 4†         | 28 ± 3          | 32 ± 3          | 27 ± 5          | 15 ± 6          |

*CD11b immunostaining was used to examine activated microglia. Data are presented as means ± SEM per field of 0.036 mm² at 40× magnification. Bold font indicates significant differences between the LPS and control group (p < 0.05). Daggers indicate significant differences between ipsilateral and contralateral hemispheres.

†p < 0.01.

‡p < 0.05.

LPS indicates lipopolysaccharide.
Effect of LPS and Ibotenate on Microglial Activation

CD11b immunodetection was used to examine activated microglia. Intracerebral injection of ibotenate induced more CD11b-positive microglia in the ipsilateral than in the contralateral cingulum in all 3 groups (Figs. 2A–C). Higher numbers of activated microglia were also observed in the contralateral cingulum of animals in the LPS groups compared with the controls; there was, however, no dose effect of maternal LPS treatment. When the data from the 2 LPS groups were combined, there was a significant effect of LPS in both the ipsilateral hemisphere (p < 0.01) and the contralateral hemisphere (p < 0.01) compared with controls (Table 1).

In the internal capsule of LPS-treated pups, immunostaining with CD11b showed a significantly higher cell density in the ipsilateral and contralateral hemispheres compared with control pups. In the LPS groups, the numbers of activated microglia were significantly higher in the ipsilateral hemispheres than in the contralateral hemispheres (Table 1).

In the cortical plate, the numbers of activated microglial cells were higher in the ibotenate-treated ipsilateral hemisphere compared with the untreated contralateral hemispheres in all 3 groups. In the LPS groups, the numbers of activated microglia were significantly higher in the ipsilateral hemispheres than in the contralateral hemispheres. No significant differences were found between groups in the hippocampus and the striatum (Table 1).

Effect of LPS and Ibotenate on Astroglisis

In pups prenatally exposed to LPS, there was a higher cell density in the ibotenate-injected hemispheres at the

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<th>TABLE 2. Astroglisis in Different Brain Regions at P9*</th>
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<td><strong>Cingulum</strong></td>
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<td><strong>Internal Capsule</strong></td>
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<td>Control</td>
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<td><strong>Cortical Plate</strong></td>
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<td><strong>Striatum</strong></td>
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*Glial fibrillary acidic protein immunostaining was used to assess astroglisis. Data are presented as means ± SEM per field of 0.036 mm² at 40× magnification. Bold font indicates significant differences between the LPS and control groups (p < 0.05). Daggers indicate the significant difference between ipsilateral and contralateral hemispheres.

†p < 0.01.
‡p < 0.05.
LPS indicates lipopolysaccharide.
level of the cingulum and the internal capsule compared with controls, as revealed by immunostaining for GFAP (Figs. 3A–F). Significantly higher numbers of astrocytes were observed in the contralateral hemispheres of the LPS groups in the cingulum and the internal capsule compared with the contralateral hemispheres of controls. In the cortical plate, there was a significantly higher number of astrocytes in the ibotenate-treated ipsilateral hemisphere compared with the contralateral hemispheres of all 3 groups. No significant differences were found between groups in the hippocampus and the striatum (Table 2).

Effect of LPS and Ibotenate on Myelination

There was less MBP immunostaining in the ibotenate-treated ipsilateral cingulum compared with the untreated contralateral cingulum in all 3 groups (Figs. 4A–C). There was also a lower level of MBP immunostaining in the contralateral cingulum of the LPS groups compared with controls. The optical density of MBP-stained fibers was significantly lower in the ipsilateral and the contralateral internal capsule of LPS-treated pups compared with controls (Table 3).

DISCUSSION

The central finding of this study is that prenatal exposure to LPS enhances the susceptibility to subsequent excitotoxic insult induced at P4 in neonatal rats, that is, the stage of brain maturity corresponding to that of preterm human infants. This was accompanied by a significant increase in microglial activation in the ipsilateral hemisphere upon ibotenate injection compared with the contralateral hemisphere in LPS-treated animals. Although neuronal death is the ultimate consequence of developmental brain lesions, it is now widely accepted that alterations in the function of surrounding glial cells are key features in the progression of these lesions. In response to alteration in their local environment, the microglial cells become activated and release a variety of soluble factors. Among these, proinflammatory cytokines and free radicals actively participate in the pathogenesis of perinatal brain lesions (17, 18).

The inflammatory response seems to play an important role in enhancing the vulnerability of the immature brain to excitotoxic insults. Experimental neuroinflammation is commonly induced after direct exposure to bacterial LPS both in vivo and in vitro, causing robust microglial activation. Activation of toll-like receptor 4 by LPS in the microglia induces the release of major proinflammatory mediators such as tumor necrosis factor, interleukin 1β, and interferon γ; the effects of these cytokines on glutamatergic receptors are well established (19, 20). A synergistic effect of LPS and hypoxia-ischemia has been demonstrated in neonatal mice subjected to LPS 1 hour before the hypoxic injury. The sensitizing effect is dependent on toll-like receptor 4, because mice deficient in this receptor fail to develop brain injury (21). The LPS-mediated increase in microglial susceptibility to ibotenate could also be due to an increase in microglial glutamate transporter-1 expression and glutamate uptake (22).

In addition to inflammation, excitotoxicity is also a common hallmark of perinatal brain lesions (23, 24). Several arguments support the hypothesis that microglial cells are involved in the pathophysiology of in vivo excitotoxic brain lesions: 1) numerous microglial cells became activated at the site of the excitotoxic lesion within the first hours after ibotenate injection (25); 2) these early activated microglial cells were derived chiefly from resident microglial cells and

FIGURE 4. Myelin basic protein (MBP) expression in the cingulate white matter. Control (A, B), lipopolysaccharide (LPS) 300 (C, D), and LPS 400 (E, F). Sections are contralateral (A, C, E, G) and ipsilateral (B, D, F, H) to the unilateral ibotenate injection. (G, H) Higher magnification from a rat in the LPS 400 group highlights the differences in extent of myelination. Scale bar = (A–F) 200 μm; (G, H) 40 μm.

TABLE 3. Densitometric Analysis of MBP Immunostaining in the White Matter at P9

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<th>Cingulum</th>
<th>Internal Capsule</th>
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<tr>
<td></td>
<td>Ipsilateral</td>
<td>Contralateral</td>
</tr>
<tr>
<td>Control</td>
<td>5.8 ± 0.5†</td>
<td>8.3 ± 0.3</td>
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<tr>
<td>LPS 300</td>
<td>3.1 ± 0.3‡</td>
<td>6.2 ± 0.3</td>
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<tr>
<td>LPS 400</td>
<td>3.2 ± 0.4‡</td>
<td>5.8 ± 0.5</td>
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*Myelin basic protein (MBP) immunostaining in the cingulum and the internal capsule was estimated by computerized densitometry. Optical density (mean ± SD) of MBP-positive fibers was significantly lower in the ipsilateral and the contralateral cingulum and internal capsule of lipopolysaccharide (LPS)-treated pups compared with controls. Bold font indicates significant differences between the LPS groups and the control group (p < 0.05). Daggers indicate a significant difference between ipsilateral and contralateral hemispheres.

†p < 0.05.
‡p < 0.01.
not from circulating monocytes; protection against ibotenate-induced lesions was obtained when the number of activated microglial cells was reduced by drugs that kill macrophages/microglia or that modulate their activation status (26); 3) activated microglial cells are known to release several cytotoxic agents capable of inducing cell death (27, 28); and 4) pretreatment of mouse pups with proinflammatory cytokines before ibotenate injection was accompanied by an increase in microglial density and by exacerbation of the excitotoxic lesions (12).

In the present model, combining antenatal LPS and postnatal excitotoxicity also resulted in astrogliosis and white matter hypomyelination. There is increasing awareness that the astrocytes in the immature periventricular white matter are vulnerable to ischemia and respond to inflammation (29). In the white matter of preterm human infants who have sustained perinatal cerebral injury, astrocytes are more abundant, strongly immunoreactive to GFAP, and have more highly ramified, beaded processes (30, 31). We have previously demonstrated that maternal exposure to LPS induces astrogliosis, with hypertrophic astrocytes in the external and internal capsule at P7 (7). Hyperplastic and hypertrophic astrocytes were also observed in the white matter subsequent to systemic injection of LPS in kittens (31) and fetal sheep (6), and vulnerability of immature astrocytes to ischemia has also been recently documented (32, 33). Interestingly, Fern (34) has established that the vulnerability to ischemia of immature astrocytes, like that of oligodendrocyte precursors, changes as they mature; they demonstrated that immature white matter astrocytes are easily injured precisely at the same time of development when oligodendrocyte precursors are most vulnerable.

Within the injured diffuse periventricular white matter in preterm infants, the primary populations of degenerating cells are the premyelinating oligodendroglia and the immature oligodendrocytes (35). Both neuroinflammation and excitotoxicity may interact to promote oligodendrocyte injury. Activated microglia release interleukin 1, tumor necrosis factor, and interferon γ, which are directly toxic to preoligodendrocytes and mature oligodendrocytes (36, 37). The oligodendrocyte progenitors and immature oligodendrocytes that predominate in the developing periventricular white matter are particularly susceptible in vitro to both receptor-independent and receptor-dependent glutamate-mediated toxicity. Receptor-independent glutamate-induced oligodendrocyte death is mediated by glutathione depletion, which results in free radical generation (38). The timing of glutamate receptor expression during human and rat white matter development coincides with the window of cerebral white matter vulnerability. Glutamate-induced injury to preoligodendrocytes is mediated by ionotropic glutamate receptors of the AMPA/kainate type (39). Recent studies have shown that the expression of N-methyl-D-aspartate receptors is concentrated on the processes of oligodendrocyte progenitors and mature oligodendrocytes. These receptors mediate the rapid Ca ++-dependent disintegration of the oligodendrocyte processes under ischemic conditions (40).

Taken together, the present results illustrate the critical influence of prenatal immune events on neonatal central nervous system vulnerability and susceptibility to a second insult and provide evidence that maternal endotoxin exposure may have effects in prenatal development of the offspring that modulate the subsequent development of excitotoxic brain injury.

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