Neuropathogenesis Induced by Rhesus Cytomegalovirus in Fetal Rhesus Monkeys (Macaca mulatta)

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Rhesus cytomegalovirus (RhCMV) infection of rhesus macaques offers opportunities to analyze mechanisms of CMV pathogenesis in a primate species. Four fetal rhesus monkeys were inoculated intraperitoneally with RhCMV early in the second trimester, and pregnancies were terminated by hysterotomy during the third trimester. Three fetuses had evidence of severe CMV disease, including intrauterine growth restriction, ventriculomegaly, microcephaly, lissencephaly, and extensive degenerative changes of the cerebral parenchyma. Histopathologic examination revealed polymicrogyria, gliosis, leptomeningitis, periventricular calcifications, and inclusion-bearing cells. These results demonstrate that the developing macaque brain is susceptible to infection with RhCMV early in the second trimester and that intrauterine infection results in neuropathologic outcomes similar to those observed in humans congenitally infected with CMV.

Human cytomegalovirus (HCMV) is a common cause of neurologic disease in congenitally infected children. The incidence of congenital HCMV infection has been estimated to be 1% in the United States [1]. Approximately 10% of infected infants will have clinical symptoms at birth or in early childhood, including multiple organ involvement, mild to severe central nervous system (CNS) deficits, hearing loss, and death [2]. The frequency of intrauterine infection and HCMV-induced sequelae emphasizes the importance of designing strategies that limit or prevent damaging congenital disease.

The goal of our studies was to develop a monkey model of HCMV infection that could address intrauterine CMV disease. The rhesus monkey is developmentally, reproductively, and evolutionarily similar to the human and has been used extensively as a model for human pediatric diseases [3–5]. Additionally, rhesus CMV (RhCMV) is highly related to HCMV in terms of genetic sequence, virology, and pathogenesis ([6, 7], unpublished results). Thus, studies were designed to focus on the pathogenesis of direct intrauterine infection of fetal monkeys with RhCMV at a defined gestational time point.

Methods

Animals. Rhesus monkeys were time-mated according to established criteria, and once pregnancy was confirmed [4], gestational day (GD) 0 was assigned to the last day of mating (term = 165 ± 10 days [3]). All 4 dams were seropositive for RhCMV at the time of fetal inoculation [7].

Inoculations and virus stocks. Ultrasound-guided fetal intraperitoneal inoculations with RhCMV (0.4 mL) were performed in 4 rhesus macaques on GD 60 (early second trimester) as previously described [8]. The inoculum consisted of either the 68-1 isolate of RhCMV [9] (fetus 1), isolate 21252 of RhCMV (isolated from a healthy rhesus monkey at the primate center; fetuses 2 and 3), or a combination of both viruses (fetus 4) (table 1). Fetus 1 was inoculated with $1 \times 10^5$ TCID50 of 68-1, fetuses 2 and 3 were inoculated with 8 $\times 10^5$ TCID50 of isolate 21252, and fetus 4 was inoculated with a mixture of 68-1 (1 $\times 10^5$ TCID50) and 21252 (6 $\times 10^5$ TCID50). RhCMV was propagated on human diploid fibroblasts, and inoculation stocks were prepared from a 3-fold freeze-thaw lysate of infected cells exhibiting 100% cytopathic effect.

Maternal and fetal monitoring. Health and physical signs of the dam were monitored daily. All fetuses were sonographically assessed prior to study assignment and weekly after inoculation using standardized techniques [4]. Established sonographic measures of the head, abdomen, and limbs, in addition to gross anatomic evaluations, were incorporated at each evaluation [4].

Fetal necropsy. Fetuses were removed aseptically by hysterotomy on GD 130 (mid–third trimester), and fetal morphometrics were performed using standardized methods [5]. The carcass and all organs were grossly examined, and multiple tissues were collected for histopathology. The brain was initially fixed with formalin in situ and then removed intact after 48 h of fixation for further evaluation. All formalin-fixed specimens were paraffin-embedded, sectioned, and stained with hematoxylin-eosin for histopathologic evaluation.
Table 1. Outcome for fetal monkeys (Macaca mulatta) inoculated intraperitoneal on gestational day (GD) 60 with rhesus CMV in utero and harvested for tissues on GD 130.

<table>
<thead>
<tr>
<th>Fetal no.</th>
<th>Virus isolate (inoculum titer)</th>
<th>Sonographic findings (GD)</th>
<th>Gross findings at necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68-1 (3 \times 10^8 TCID_{50})</td>
<td>Increased AF flocculence (75–130)</td>
<td>IUGR, Ventriculomegaly, lissencephaly, Hepatic adhesions, Focal hepatic mineralization</td>
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<td></td>
<td></td>
<td>Echogenic hepatic foci (95–130)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IUGR (95–130)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Ventriculomegaly (95–130)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Echogenic bowel (95–110)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mild oligohydramnios (80–130)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21252 (8 \times 10^7 TCID_{50})</td>
<td>Increased AF flocculence (75)</td>
<td>Lymphadenopathy, Splenomegaly, Hepatic adhesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple echogenic hepatic foci (95–130)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21252 (8 \times 10^7 TCID_{50})</td>
<td>Increased AF flocculence (75)</td>
<td>Underossified cranial vault, Discoloration lateral border, spleen, Hepatic adhesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Echogenic kidneys (95)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>68-1 + 21252 (1 \times 10^7 + 6 \times 10^7 TCID_{50})</td>
<td>Increased AF flocculence (85)</td>
<td>Microcephaly, Ventriculomegaly, lissencephaly, Discoloration lateral border, spleen, Hepatic adhesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Microcephaly (105–130)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Ventriculomegaly (130)</td>
<td></td>
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</tbody>
</table>

NOTE. IUGR, intrauterine growth restriction; AF, amniotic fluid.

Results

Fetal monitoring. All fetuses had sonographic evidence consistent with CMV infection and end-organ damage, ranging from mild (hepatic echogenic foci, amniotic fluid flocculence) to severe manifestations, including intrauterine growth restriction, ventriculomegaly, and microcephaly (table 1), similar to that observed for congenital CMV infection in humans [2].

Fetus 1 received the highest virus dose and had the most significant disease (table 1). Intrauterine growth restriction was detected within 2 weeks of intraperitoneal inoculation (GD 80). All head, abdominal, and limb parameters assessed were significantly less than age-matched controls (data not shown). Ventriculomegaly was also detected 4 weeks after inoculation (GD 95), with an increase in severity with advancing gestation. All findings persisted until fetal tissue harvest on GD 130.

Fetus 4 (inoculated with isolates 68-1 and 21252) exhibited microcephaly roughly 5 weeks after inoculation that persisted until hysterotomy. Acute-onset ventriculomegaly was documented immediately prior to tissue harvest.

Fetuses 2 and 3 (both inoculated with isolate 21252) had normal growth and development of the head, abdomen, and limbs.

Histologic analysis. The most significant histologic findings were within the brains of 3 of 4 fetuses. The brains of fetuses 1 and 4 both revealed mildly opacified basal leptomeninges and significant changes in the posterior cerebral hemispheres (figure 1). Developmental abnormalities included attenuation of the cerebral mantle to the point of symmetric translucency in the occipital, posterior parietal, and posterior temporal lobes, with findings more severe in fetus 1 than in fetus 4. For fetus 4, the occipital, posterior parietal, and posterior temporal lobes were partially to completely agyric and of reduced sulcal formation. Fetus 1 showed only a shallow lateral sulcus and, with the exception of the frontal lobes, the cerebral hemispheres were agyric. Significant parenchymal necrosis and ventriculomegaly were confirmed in both fetuses with a greater severity for fetus 1 (figure 1). These changes were essentially symmetrical and most severe in the posterior cerebral hemispheres; dilation of the anterior horns of the lateral ventricles was also present in fetus 1 where mantle attenuation was 0.5 cm in the occipital lobes. Multiple periventricular and white-matter calcific plaques were also observed. Microscopically, chronic leptomeningitis (fetuses 1 and 4) was accompanied by varying fibrosis, parenchymal necrosis, severe cystic changes, calcification, and abundance of cytoplasmic and nuclear viral inclusions (figure 1). Gliosis, reactive blood vessels, inflammation, and denudement of ependyma were noted most severely in the posterior telencephalon. Neither of these fetuses revealed any ectopic gray matter or significant neuronal heterotopia.

No cerebral or cerebellar gross abnormalities were detected in fetuses 2 and 3; all findings were comparable to those for age-matched controls (data not shown). Although there was no ventriculomegaly, cystic degenerative changes, calcification, or white-matter attenuation detected in the brains of these fetuses, there were histologic changes consistent with CMV disease. These included foci of intracytoplasmic and intranuclear inclusions, focal degeneration, microcalcification, gliosis, and microcystic changes (figure 1). Inclusion-bearing cells in fetuses 1, 2, and 4 were positive for RhCMV immediate-early 1 (IE1).
A. Lateral view of cerebral hemisphere of age-matched control (Control), fetus 4, and fetus 1. Note almost complete lack of sulci and gyri (lissencephaly), particularly in occipital, temporal, and parietal lobes for fetus 1. Posteriorly, cerebral mantle was extremely thin (~2 mm).

B. Hematoxylin-eosin-stained brain sections of fetuses 2, 4, and 1. Lower magnifications (1×) are at bottom, and higher magnifications (10, 20, and 40×, respectively) are at top. Illustrated are examples of focal areas of rhesus CMV inclusions (fetus 2); white-matter pallor and degeneration (fetus 4); and polymicrogyria and almost complete absence of white matter (fetus 1).

expression by immunohistochemistry and in situ hybridization (data not shown). For fetus 3, microscopic analysis of serial coronal sections indicated lymphocytic leptomeningeal infiltration only. Occasional microscopic cellular nodules composed primarily of mononuclear cells were found in the cerebral cortex and white matter of this fetus. No obvious intracytoplasmic or intranuclear inclusions were noted in fetus 3, although occasional RhCMV IE1-expressing cells were detected (data not shown).

Discussion

The results of these studies indicate that the developing monkey brain is sensitive to the pathogenic consequences of intrauterine RhCMV infection. Two of 4 rhesus monkey fetuses directly inoculated with RhCMV (1 and 4) developed severe changes in CNS architecture, the third (fetus 2) exhibited focal, histologic abnormalities associated with RhCMV infection, and the fourth (fetus 3) did not develop any overt virally induced...
neurologic lesions. The neuropathology observed in RhCMV-infected rhesus fetuses parallels that observed in congenitally infected humans. Reported neuropathologic features of congenital HCMV infection include lissencephaly-pachygyria, polymicrogyria, ventriculomegaly, periventricular and white-matter calcifications, focal necroses, and microcephaly [10, 11].

The pattern of neuropathology in RhCMV-infected fetuses (1, 2, and 4) has not been previously reported for monkey fetuses either spontaneously (Tarantal A, unpublished results) or as a result of experimental infection with other infectious agents such as simian immunodeficiency virus [5, 12]. The normal incidence of congenital RhCMV infection in the primate center breeding colony is also most likely extremely low. A seroepidemiologic study of infants at the primate center has demonstrated that <4% have developed anti-RhCMV IgM antibodies by 2 months of age [7]. Thus, the cranial abnormalities observed in 3 of 4 fetuses in this study were clearly the result of direct RhCMV inoculation.

The severity of neuropathology observed in humans congenitally infected with HCMV may be associated with both the status of maternal antiviral antibodies and the stage of CNS development when infection occurs. In women with anti-HCMV seroimmunity prior to conception, intrauterine HCMV infection is <0.5% [1] and is only rarely associated with clinical evidence of infection at birth [13]. In contrast, 40%–50% of pregnant women who undergo a primary infection transmit virus to the fetus; ~10%–15% of infected fetuses have evidence of HCMV disease [14]. These observations suggest that a threshold level of protective maternal anti-HCMV immunity can reduce the potential for HCMV-induced developmental abnormalities. Also, infection early in gestation may result in more severe CNS abnormalities than infections later in gestation [10, 11].

In this study, sonographic and histopathologic findings suggested differences in outcome after fetal CNS infection even though all animals were inoculated at the same gestational age. Fetus 1 exhibited the most rapid onset of intracranial abnormalities (<1 month), whereas microcephaly and ventriculomegaly was not observed in fetus 4 until 1 and 2 months after inoculation, respectively. In contrast, fetuses 2 and 3 did not show any gross abnormalities. These differences in the onset of clinically apparent CNS damage could be explained by the larger inoculum used in fetuses 1 and 4 than in fetuses 2 and 3 (table 1). However, we have preliminary data that suggest that fetuses inoculated with virus titers similar to those used in fetus 1, but at later stages of gestation, do not develop the gross developmental abnormalities observed in fetuses 1 and 4 (unpublished results).

Alternatively, it could be argued that the course of CNS disease was influenced by differences in the composition of the inoculum. The experimental design of this pilot study included different titers and strains of RhCMV to optimize the chances of observing pathologic outcomes. Polymerase chain reaction analysis of multiple tissues from all 4 fetuses for RhCMV DNA demonstrated that both strains of RhCMV disseminated throughout the fetuses with apparently equal efficiency (not shown). Preliminary quantitative polymerase chain reaction analysis indicated that comparable titers of RhCMV genome equivalents of 68-1 and 21252 isolates were observed in different fetal tissues (not shown). Focal areas of white-matter degeneration and inclusion-bearing cells in fetus 2 demonstrated that the 21252 isolate was capable of inducing CNS disease (figure 1B). Thus, it is unlikely that differences in neuropathologic outcome were related to different replicative capacities or pathogenic potential of the two viral strains.

On the basis of the results of these studies, we conclude that the potential for RhCMV to induce developmental abnormalities in experimentally inoculated fetal rhesus macaques is a function of host factors, perhaps similar to those postulated to account for differences in the outcome of human fetuses congenitally infected with HCMV [15]. These factors may include the type and specificity of transplacentally acquired antiviral antibodies and the developmental state of the fetus at the time of virus infection. The transplacental transfer of antibodies in humans begins during the first trimester, and the concentrations of IgG within fetal and maternal sera are nearly equivalent by 26 weeks gestation [16]. On the basis of our findings, the transfer of maternal IgG across the rhesus placenta appears to follow similar kinetics (Barry PA and Tarantal AF, unpublished results). The dams in these studies were seropositive for anti-RhCMV IgG antibodies at the time of fetal inoculation because RhCMV is ubiquitous in the rhesus monkey colony [7]. The titers of protective maternal antibodies in the fetal circulation may have been very low at the time of fetal inoculation compared with titers in the maternal circulation. There would have been increasing titers of maternal IgG in the fetal circulation during the time frame of this experiment (GD 60–130), coincident with viral dissemination in the infected fetuses. Increasing concentrations of antiviral antibodies in the fetal circulation may have influenced the timing of CNS infection, as well as the type and number of cells infected.

Because there are no seronegative breeding-age females at the primate center, it is not possible to model congenital HCMV infection that follows primary infection in gravid humans. However, we are proposing this model specifically for the investigation of the pathogenesis of intrauterine CMV infection. The rhesus monkey model is one in which the timing of fetal infection, the role of maternal immunity in neuropathogenesis and novel vaccine strategies as a means for preventing fetal disease can be explored. Given the close genetic and evolutionary similarities between the human and nonhuman primate and the strong conservation between RhCMV and HCMV, antiviral strategies against RhCMV will also be particularly relevant to investigations that focus on the understanding, treatment, and prevention of CMV disease in humans. Much of the uncertainty surrounding the pathogenesis of congenital HCMV infection centers on our lack of understanding of intrauterine CMV infection and the role of passively acquired antibodies in limiting...
the extent of disease. The model we propose can directly address these questions and should prove invaluable for this purpose.

References


