

# Nonhuman Primate Models of Intrauterine Cytomegalovirus Infection

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## Abstract

Congenital human cytomegalovirus (HCMV) infection has long been recognized as a threat to the developing fetus, even though studies have shown that only a subset of congenital infections results in clinical signs of disease. Among the estimated 8000 children who develop sequelae from congenital CMV infection each year in the United States alone, most suffer permanent developmental defects within the central nervous system. Because there is currently no approved vaccine for HCMV, and anti-HCMV drugs are not administered to gravid women with congenital infection because of potential toxicity to the fetus, there is a clear clinical need for effective strategies that minimize infection in the mother, transplacental transmission of the virus, and/or fetal disease. Animal models provide a method to understand the mechanisms of HCMV persistence and pathogenesis, and allow for testing of novel strategies that limit prenatal infection and disease. The rhesus macaque model is especially well suited for these tasks because monkeys and humans share strong developmental, immunological, anatomical, and biochemical similarities due to their close phylogenetic relationship. This nonhuman primate model provides an invaluable system to accelerate the clinical development of promising new therapies for the treatment of human disease. This review addresses salient findings with the macaque model as they relate to HCMV infection and potential avenues of discovery, including studies of intrauterine CMV infection. The complexity of the natural his-

tory of HCMV is discussed, along with the ethical and logistical issues associated with studies during pregnancy, the recent contributions of animal research in this field of study, and future prospects for increasing our understanding of immunity against HCMV disease.

**Key Words:** congenital infection; cytomegalovirus; immune ontogeny; intrauterine pathogenesis; nonhuman primate model of HCMV pathogenesis; rhesus macaque; sensorineural deficits; transplacental transfer of maternal immunoglobulin G

## Natural History of Human Cytomegalovirus

Human cytomegalovirus (HCMV<sup>1</sup>) belongs to the Herpesviridae family of viruses, all of whose members are distinctly characterized by a virion structure and a large double-stranded DNA genome (130-230,000 base pairs) (Mocarski 1993). HCMV is ubiquitous in humans, with seroprevalence rates in adults ranging from ~50 to 90% (Alford and Britt 1993). Seroprevalence is generally related inversely to socioeconomic status, with higher rates observed in developing countries and in lower socioeconomic groups of developed nations. Virus can be transmitted at any time during prenatal and postnatal life. Horizontal transmission can occur by exchange of almost any bodily fluid across a mucosal surface, although perinatal transmission and maternal-infant transfer via breast milk appear to be the principal modes (Arvin et al. 2004). HCMV is a virus with low pathogenic potential because a large majority of primary infections do not result in clinical signs of disease in individuals with functional immune systems (Alford et al. 1990). Mononucleosis is the predominant clinical outcome associated with primary infection in young adults, but it occurs < 7% of the time (Taylor 2003). Otherwise, primary

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<sup>1</sup>Abbreviations used in this article: ABR, auditory brainstem response; CD, cluster of differentiation; CMV, cytomegalovirus; CNS, central nervous system; EGFP, enhanced green fluorescent protein; gB, glycoprotein B; HAART, highly active antiretroviral therapy; HCMV, human CMV; IE1, immediate-early 1; IgG, immunoglobulin G; IUGR, intrauterine growth restriction; NT<sub>90</sub>, 90% neutralizing antibody titer; PCR, polymerase chain reaction; PFU, plaque-forming unit; pp65, phosphoprotein 65; RhCMV, rhesus CMV; SPF, specific pathogen-free.

infections are asymptomatic or can result in occasional mild fever and/or flu-like symptoms (Adler 1988; Nigro et al. 2003; Zanghellini et al. 1999). Because HCMV-related disease is the exception following primary infection in immunocompetent hosts, antiviral immune responses to HCMV infection are considered protective.

Although host immune responses usually protect from HCMV sequelae, they are not sufficient to eliminate reservoirs of viral genomes. Like all herpesviruses, HCMV establishes a lifelong persistence within an infected host, characterized by latent viral genomes that periodically reactivate to produce infectious virus that is shed at mucosal surfaces. HCMV genomes have been found in multiple cell types in which there is an absence of active viral gene expression and virus production. This characteristic is particularly well described for cluster of differentiation (CD<sup>1</sup>)34<sup>+</sup> myeloid progenitor cells of long-term seropositive individuals (Bolovan-Fritts et al. 1999; Hahn et al. 1998; Larsson et al. 1998; Maciejewski et al. 1992; Soderberg-Naucler and Nelson 1999; Soderberg-Naucler et al. 2001; Taylor-Wiedeman et al. 1991, 1993; Zhuravskaya et al. 1997). These HCMV-infected cells are noted for either a complete lack of or an extremely limited pattern of viral transcription (Kondo et al. 1996). The genomes are capable of replicating because they can be reactivated and can enter a productive cycle of gene expression following allogeneic stimulation *in vitro* (Soderberg-Naucler et al. 1997, 2001). Despite HCMV latency at the cellular level, HCMV is a persistent virus at the level of the infected host. HCMV can be detected in bodily fluids derived from multiple tissues of long-term seropositive hosts, including saliva, breast milk, urine, and semen (Aynaud et al. 2002; Dworsky et al. 1983; Gautheret-Dejean et al. 1997; Hamprecht et al. 1998; Howard et al. 1997; Kashiwagi et al. 2001; Mansat et al. 1997; Stagno et al. 1975). As with most primary infections, persistent HCMV expression is essentially asymptomatic. A virus-host relationship has evolved whereby sufficient immune responses are stimulated to prevent clinical symptoms during primary or recurrent infection, yet are insufficient to eliminate antigen-positive cells.

The critical role that the host immune response plays in preventing HCMV disease is highlighted by the course of HCMV infection in individuals who lack a fully functional immune system. Those at risk for HCMV morbidity and mortality include chemically immunosuppressed transplant recipients for whom HCMV represents the single most important infectious post-transplantation pathogen (Pereyra and Rubin 2004). A large majority of solid organ and bone marrow transplant recipients will develop activated HCMV infection within 1 yr of transplantation. Additionally, HCMV was once a particularly common opportunistic infection in immunodeficient AIDS patients before the advent of highly active antiretroviral therapy (HAART<sup>1</sup>) (Griffiths 2004). Since the introduction of anti-HIV HAART drug cocktails in Western Europe and the United States, the frequency of HCMV-related disease has declined in AIDS patients in these particular geographical areas. Finally,

congenitally infected fetuses represent an especially vulnerable group to HCMV sequelae (Weller 1971a,b).

HCMV is the most common congenital infection in the United States, infecting 0.5 to 2% of all live-born infants (40,000 in the United States) (Alford et al. 1990). It is estimated that 10% of these children present with HCMV disease at birth. Moreover, a significant proportion (15%) of those congenitally infected newborns who are healthy at birth develop subsequent neurological sequelae several years later, which primarily manifest as progressive hearing loss (Arvin et al. 2004; Dobbins et al. 1992; Ista et al. 1995). Factors that influence the potential for both transplacental transmission of HCMV and sequelae in congenitally infected infants are not well known. Multiple maternal, fetal, and viral factors are likely to be involved. The frequencies of both maternal-fetal transmission and clinically apparent congenital infections are closely associated with the serostatus of the mother at the time of conception. Between 30 and 40% of fetuses become congenitally infected when the mothers undergo a primary infection during pregnancy (Stagno et al. 1982, 1986). Approximately 10% of these fetuses display clinical signs of infection at birth. Clinically apparent infections are characterized by permanent sensory and neurological deficits and/or transient multiple organ pathology such as petechiae and hepatosplenomegaly (Alford et al. 1990).

The developing central nervous system (CNS<sup>1</sup>) is highly vulnerable to the effects of intrauterine HCMV, which result in permanent neurological damage and include mental insufficiencies and sensorineural hearing loss. In addition to the physical and emotional tolls of HCMV infection on children and their families, there is a large financial burden (~\$2B/year) on the US health care system (Arvin et al. 2004). Less than 0.5% of women with preconceptional immunity to HCMV transmit virus transplacentally to the fetus (Fowler et al. 1992). As a result of reactivated HCMV infection, the vast majority of congenitally infected infants do not present with clinically apparent HCMV disease. However, actual frequencies of disease remain to be determined. A report by Boppana and colleagues (1999) indicates that symptomatic outcomes in infants of mothers with seroimmunity to HCMV before conception may be more common than previously suspected. More recent studies from this ongoing natural history study of congenital HCMV infections have suggested that the overall number of infants with clinically symptomatic disease following nonprimary maternal infection is similar to that observed following primary maternal infection (S. Boppana and W. J. Britt, submitted for publication).

Taken together, these data demonstrate that maternal anti-HCMV immune responses reduce the potential for dissemination of HCMV to the fetus. However, the fact that prenatal disease is observed in a minority of congenitally infected fetuses, following either primary or nonprimary maternal infection, strongly suggests that undefined factors limit HCMV pathogenic potential after transmission. Once the maternal-fetal interface is breached by HCMV, the fetal

milieu is the sole determinant of HCMV's pathogenic potential. Accordingly, the ability of HCMV to replicate and cause disease is likely a complex interaction of developmental processes that may include transplacental transfer of maternal immunoglobulin G (IgG<sup>1</sup>) and fetal de novo immune responses. Transplacental transfer of IgG is thought to initiate during the second trimester with kinetics of transfer increasing through parturition (Eitzman 1970; Fujimoto et al. 1983a; Mussi-Pinhata et al. 2003; Simister 2003; Stiehm 1975). The protective effects of maternal IgG in the fetus are dependent on the quality of maternal antiviral antibodies (Boppana and Britt 1995; Mussi-Pinhata et al. 2003) and the dynamics of transplacental transfer (Eitzman 1970; Fujimoto et al. 1983a; Mussi-Pinhata et al. 2003; Simister 2003; Stiehm 1975).

Additionally, the developmental status of the fetus at the time of infection may also influence the severity of HCMV disease. Several studies suggest that HCMV infection early in gestation, particularly during the period of organogenesis, results in more severe developmental outcomes than infections occurring later in gestation (Barkovich and Lindan 1994; Hayward et al. 1991; Twickler et al. 1993). Similarly, development of a functional immune system by the fetus may augment the protective immune responses conferred by maternal IgG. Accordingly, the damaging effects of intrauterine HCMV infection on the fetus may be a function of the stage of development during which infection occurs. Investigations into these aspects of intrauterine HCMV infection are not feasible in the human clinical setting. Further complicating the issue is a lack of knowledge regarding key aspects of infection, such as the time during gestation when the damaging effects of fetal infection are most likely to occur. An understanding of immunological and developmental factors that influence outcome of intrauterine HCMV infection is necessary for the design of efficacious vaccines and antiviral therapies.

It should be noted that HCMV is highly species specific in that it can infect cells of human origin or primary cells only from our closest evolutionary relation, the chimpanzee (Perot et al. 1992). Direct infection of nonhuman species with HCMV is not possible, and the use of relevant animal CMV surrogates is essential to address many of the issues focused on HCMV persistence, pathogenesis, and therapeutic modalities. For additional information on important research related to other, nonprimate models of CMV, readers are referred to the article of Dr. Mark Schleiss in this issue of *ILAR Journal* (Schleiss 2005).

## **Nonhuman Primate Model of HCMV Persistence and Pathogenesis**

CMV has been isolated from numerous species of nonhuman primates, and it is likely that every simian species harbors its own indigenous CMV that has co-evolved with its host. Of the many monkey CMV isolates described to date, rhesus CMV (RhCMV<sup>1</sup>), isolated from rhesus ma-

caques (*Macaca mulatta*), is the best characterized. RhCMV infection of rhesus macaques closely recapitulates HCMV infection in immunocompetent and immunocompromised individuals, as well as viral genetics, immunology, and protein functions (Alcendor et al. 1993; Andrade et al. 2003; Barry et al. 1996; Chang et al. 2002; Hansen et al. 2003; Huff et al. 2003; Kaur et al. 2000, 2002, 2003; Kravitz et al. 1997; Lockridge et al. 1999, 2000; London et al. 1986; McCormick et al. 2003; North et al. 2004; Penfold et al. 2003; Sequar et al. 2002; Swanson et al. 1998; Tarantal et al. 1998; Vogel et al. 1994; Yue et al. 2003). RhCMV can also induce severe disease in fetal macaques that directly parallels the pathogenesis of HCMV (Chang et al. 2002; London et al. 1983; Tarantal et al. 1998), as described below.

Like HCMV infection in humans, RhCMV is ubiquitous in rhesus macaque populations. In breeding colonies of rhesus macaques, 50% of infants are seropositive for RhCMV by 6 mo of age, and almost 100% are seropositive by 1 yr of age (Vogel et al. 1994). Similarly high rates of infection have been observed in rhesus macaques captured in the wild (L. Jones-Engle and P. Barry, unpublished data). Although the routes of transmission have not been determined, RhCMV is probably transmitted horizontally from infected to uninfected animals via excretion of virus in breast milk, saliva, and urine (Alford and Britt 1993), all known routes of transmission in humans. Seropositive animals persistently secrete infectious virus during their lifetime (Asher et al. 1974; Huff et al. 2003).

Importantly, vertical transmission of RhCMV has never been reported in monkeys, although low rates (< 1 – 2%) of congenital infection cannot be excluded (Vogel et al. 1994). There have been no descriptions of spontaneously aborted monkey fetuses or neonates exhibiting histopathological or clinical sequelae consistent with congenital CMV infection. Spontaneously aborted fetuses in which RhCMV disease might be observed are usually not available for analysis. The absence of reports of naturally occurring congenital RhCMV disease should not be interpreted to mean that fetal infection never occurs in nonhuman primates. If it does occur, however, the apparently low frequency is most likely a function of the seroprevalence rates in sexually mature monkeys. Rhesus macaques become sexually mature around 4 yr of age, a time when all breeding-age females are seropositive for RhCMV (Vogel et al. 1994). Because congenital HCMV infection rates in seropositive humans are < 0.5% (Fowler et al. 2003), transplacental transmission of RhCMV is likely to be comparably infrequent ( $\leq 1\%$ ). The ability to study RhCMV-related fetal disease (Chang et al. 2002; London et al. 1983; Tarantal et al. 1998) has required direct fetal inoculation with virus to ensure sufficient animal numbers in defined study groups. Accordingly, the macaque system is a model of intrauterine HCMV pathogenesis, not a model of transmission. Ongoing programs at nonhuman primate breeding facilities may enable maternal-fetal transmission studies in the future (discussed below).

## Intrauterine RhCMV Inoculation

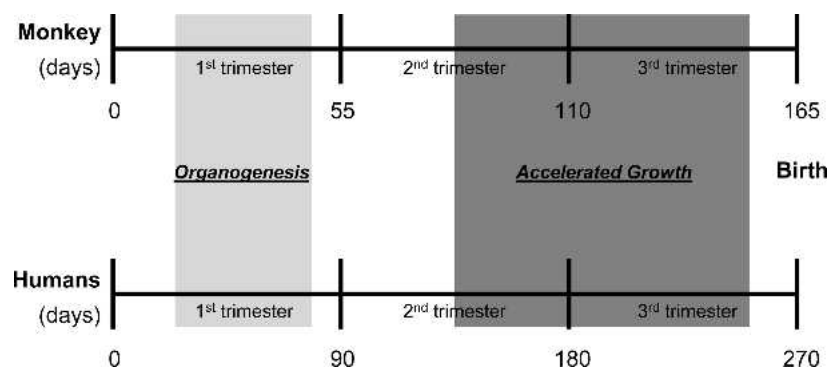
Studies involving RhCMV pathogenesis in fetal macaques take advantage of breeding programs in national primate research centers and other nonhuman primate facilities. Pregnancy in the rhesus monkey is divided into trimesters by 55-day increments, with 0 to 55 days gestation representing the first trimester, 56 to 110 days gestation representing the second trimester, and 111 to 165 days gestation representing the third trimester (term  $165 \pm 10$  days) (Tarantal and Gargosky 1995) (Figure 1). Animals are time mated using established protocols, and pregnancy is typically confirmed by ultrasound in the early first trimester (Tarantal 2005; Tarantal and Hendrickx 1988a,b). Fetal development in humans and macaques has many similarities, including spatial and temporal patterns of organ development, placental structure, length of gestation, growth characteristics, hematopoietic and immune system ontogeny, and organ system maturation (Hoar and Monie 1981; Jones et al. 1993; Tanimura and Tanioka 1975; Tarantal and Gargosky 1995). Fetuses are sonographically evaluated to confirm normal growth and development before RhCMV inoculation. Using established ultrasound-guided procedures (Tarantal 1990), at the end of the first trimester, the fetus is directly injected with a 0.3-mL volume of RhCMV using either an intraperitoneal or intracranial approach (see below) (Chang et al. 2002; Tarantal et al. 1998).

## Intrauterine RhCMV Pathogenesis

RhCMV can cause a range of developmental abnormalities in rhesus fetuses that are similar to those observed in humans congenitally infected with HCMV. Three studies of direct fetal inoculation with RhCMV are described in the literature (Chang et al. 2002; London et al. 1986; Tarantal et al. 1998). Despite study differences in the viral strain, titers of virus, and routes of inoculation, a common theme for all three studies was the susceptibility of the CNS to intrauterine RhCMV exposure (Table 1). In the first study (London et al. 1986), fetuses were inoculated with 0.2 mL of

RhCMV ( $10^5$  50% tissue culture infectious doses/mL) by either the intramniotic route (50 days) or the intracranial route (80 days). The fetuses were delivered at term by caesarian section and were analyzed for virological and histopathological parameters of infection. Of the 20 fetuses inoculated by either route, 16 developed CNS abnormalities ranging from mild to severe. Examples included focal to extended denudation of the ependymal lining with gliosis in the subependymal white matter (16/20, 80%), severe ventricular dilatation (6/20, 30%), granular calcification with focal areas of necrosis and karyorrhexis (15/20, 75%), and choroid plexitis (6/20, 30%). The intracranial route of inoculation generally resulted in more severe disease. Interestingly, no evidence of RhCMV histopathology was observed in other fetal tissues, although virus was recovered from the pancreas and lung of two of five inoculated fetuses that were analyzed 7 to 14 days after inoculation. Placental abnormalities were also noted in 13 of 16 (81%) placentas examined, including deciduitis, infarction, calcification, hyalinization, and lymphocytic infiltrates.

In a previous publication from our group (Tarantal et al. 1998), four fetuses were inoculated with the 68-1 strain of RhCMV by the intraperitoneal route at 60 days of gestation (early second trimester), and fetal development was evaluated sonographically until the third trimester (130 days gestation). All four fetuses exhibited sonographic evidence of RhCMV disease within 2 to 4 wk of inoculation, ranging from mild (flocculent amniotic fluid, renal and hepatic echogenic foci, 2/4) to severe (intrauterine growth restriction [IUGR<sup>1</sup>], microcephaly, and ventriculomegaly, 2/4). At gross examination, the two fetuses with ventriculomegaly had extensive degenerative changes of the cerebral parenchyma, including lissencephaly and multiple areas of calcification. Histological analysis of the brains revealed polymicrogyria, gliosis, leptomeningitis, periventricular calcifications, and inclusion-bearing cells. Some of the neuropathological findings, particularly lissencephaly and microcephaly, are consistent with early insults to CNS development in humans (Barkovich and Lindan 1994; Hayward et al. 1991; Twickler et al. 1993). The other



**Figure 1** Comparison of gestation in rhesus macaques and humans. Each trimester corresponds to 55 and 90 days, respectively, although developmental landmarks such as organogenesis and accelerated growth occur at the same relative times.

**Table 1 Experimental approaches and outcomes following fetal inoculation with rhesus cytomegalovirus (RhCMV)**

| Reference (see text) | RhCMV strain | No. of animals | Route <sup>a</sup> | Time of inoculation <sup>b</sup> | Harvest                | Outcomes   |
|----------------------|--------------|----------------|--------------------|----------------------------------|------------------------|--|
| London et al. 1986   | Primary      | 2              | IA                 | 50                               | 57-64                  | Virus isolated in lung at 14 days (n = 1)  |
|                      |              | 3              | IC                 | 80                               | 57-64                  | Virus isolated in brain and lung at 14 days (n = 1) and brain and pancreas at 14 days (n = 1)  |
|                      |              | 11             | IC                 | 80                               | 160                    | Ventriculomegaly (n = 4), leptomeningitis (n = 3), brain parenchyma abnormalities (n = 7), placental abnormalities (n = 6 of 7 examined)   |
|                      |              | 9              | IA                 | 50                               | 160                    | Ventriculomegaly (n = 2), leptomeningitis (2 = 3), brain parenchyma abnormalities (3 = 7), placental abnormalities (n = 7)   |
| Tarantal et al. 1998 | 68-1         | 4              | IP                 | 60                               | 130                    | Ventriculomegaly/lissencephaly (n = 2), IUGR (n = 1), microcephaly (n = 1), polymicrogyria (n = 1), increased AF flocculence (n = 4), echogenic foci (n = 3), splenomegaly (n = 1) |
| Chang et al. 2002    | 68-1/EGFP    | 4              | IC                 | 50                               | 60-75                  | Microcephaly (n = 4), ventriculomegaly (n = 3), IUGR (n = 2), lower limb deformity (n = 3), ileocolic stricture (n = 3), severe hydrops (n = 3), multiorgan disease (n = 3)        |
| These authors        | 68-1         | 4              | IC                 | 50                               | 71-80                  | RhCMV histopathology and RhCMV antigen-positive cells within cochlea (discussed herein)  |
| These authors        | 68-1         | 13             | IP                 | 50                               | ≤ 30 days <sup>c</sup> | See Tables 2 and 3 and text for details  |
|                      |              | 7              | IP                 | 65                               | ≤ 30 days              | See Tables 2 and 3 and text for details  |

<sup>a</sup>IC, intracranial; IA, intramniotic; IP, intraperitoneal; IUGR, intrauterine growth restriction; AF, amniotic fluid.

<sup>b</sup>Days gestation.

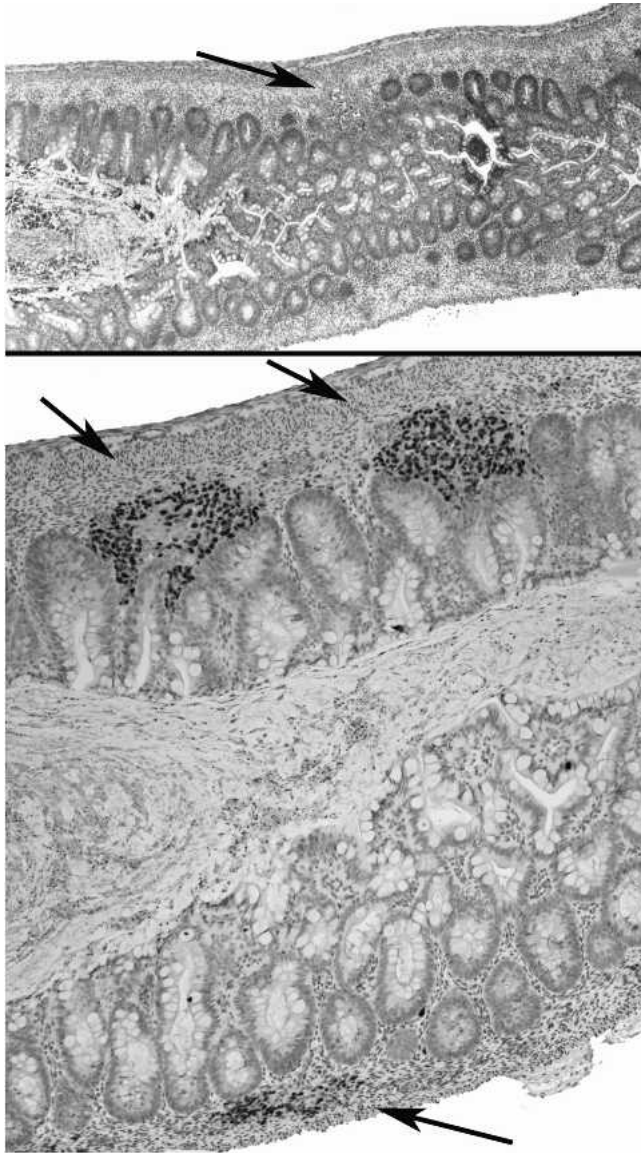
<sup>c</sup>Postnatal age.

two fetuses exhibited normal cranial growth and development, although viral inclusions and focal areas of active degenerative processes were detected in the brain of one of the two fetuses. DNA from multiple fetal tissues was analyzed by polymerase chain reaction (PCR<sup>1</sup>) for the presence of RhCMV genomes. The majority of tissues from these four fetuses was positive for RhCMV DNA, and the relative copy numbers were exceedingly high in selected tissues such as brain, kidney, and lung (these authors, unpublished data). In addition, infectious virus was recovered from amniotic fluid samples collected in utero and at the time of tissue harvest. Systemic and non-CNS RhCMV sequelae (i.e., IUGR and renal and hepatic foci), together with the wide tissue distribution of RhCMV, supported the interpretation that RhCMV efficiently disseminates to and

induces multiorgan fetal abnormalities following in utero inoculation.

The finding described above was confirmed in a subsequent study in which a recombinant variant of RhCMV (10<sup>5</sup> plaque-forming units [PFUs<sup>1</sup>]) was administered by intracranial injection under ultrasound guidance into the lateral ventricles of four fetuses at day 50 of gestation (late first trimester) (Chang et al. 2002). For this study, a mammalian expression cassette for the enhanced green fluorescent protein (EGFP<sup>1</sup>) was introduced into a noncoding region of RhCMV. One goal of this study was to demonstrate that insertion of a heterologous gene cassette did not alter the pathogenic potential of RhCMV. Fetuses inoculated with the EGFP variant of RhCMV rapidly developed severe focal and systemic manifestations of RhCMV. Manifestations in-

cluded microcephaly, ventriculomegaly, IUGR, hydrops, diaphragmatic defects, and lower limb deformities similar to findings associated with congenital contractures. Three fetuses also developed ileocolic strictures with associated colonic distension (Figure 2), a finding that has also been observed following intraperitoneal inoculation (unpublished data, these authors). Foci of cells positive for the RhCMV immediate-early 1 (IE1<sup>1</sup>) antigen were present at the stric-



**Figure 2** Ileocolic stricture in rhesus macaque fetuses inoculated intracranially with RhCMV/EGFP (Table 2). Top (10×): 4-micrometer section of the site of the stricture stained with hematoxylin and eosin. Arrow indicates focal lesion consisting of cells with intranuclear and cytoplasmic inclusions consistent with RhCMV cytopathology at the site of the stricture. The tissue section is oriented caudal (left) to cephalic (right). Bottom (20×): serial section stained for the immediate-early 1 (IE1) antigen of RhCMV. Arrows indicated three focal areas of IE1-positive cells at the site of the stricture.

ture, suggesting a causal relationship between RhCMV and this particular pathological outcome. Development of the stricture was especially rapid as evidenced in one of the fetuses that was terminated 10 days after inoculation due to imminent fetal demise. The other two fetuses were terminated 21 and 23 days after inoculation based on ultrasound findings of ileocolic stricture and severe hydrops. HCMV-associated colonic strictures have been observed in congenitally infected infants, although the reported frequency is rare (Reyes et al. 1997). HCMV has also been associated with strictures in other tissues such as kidney and liver following allograft transplantation (Egawa et al. 2001; Lowell et al. 1994). Focal areas of RhCMV histopathology were observed in multiple tissues of the inoculated fetuses, confirming the findings of earlier studies that indicated RhCMV disease was not limited to the developing CNS.

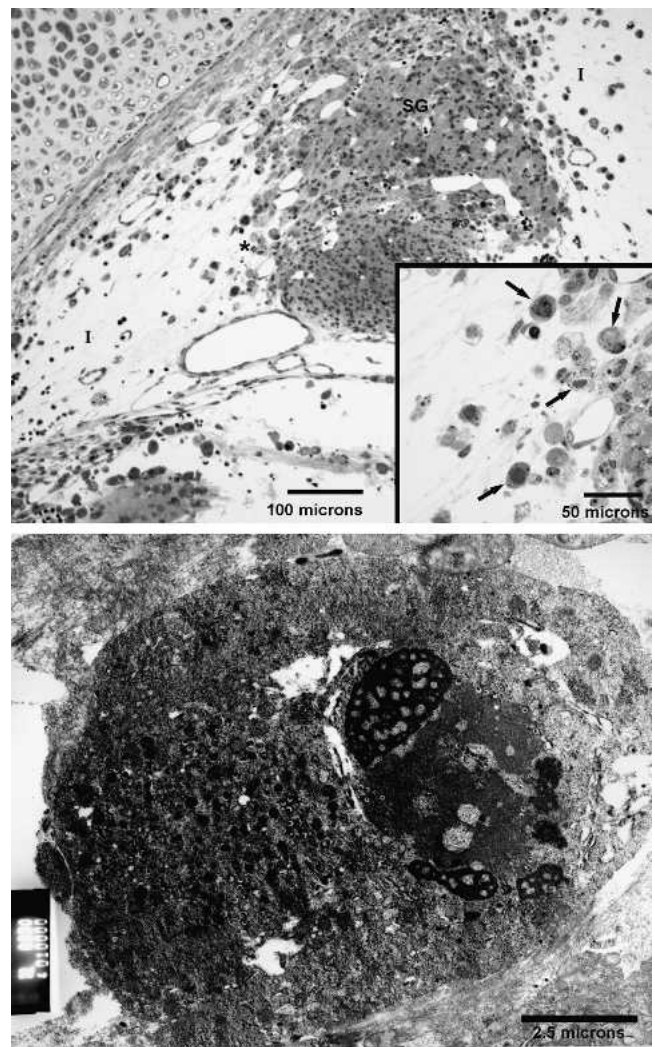
The neuropathological findings following cranial inoculation were similar to those observed in congenitally infected humans (Chang et al. 2002). Extensive inflammatory changes and neuronal migration defects were observed in the fetuses examined, particularly the two that were terminated 21 and 23 days after inoculation. Immunohistochemical analysis of either EGFP or viral IE1 expression localized the majority of RhCMV-positive cells to the granular layer of the neuroepithelium immediately surrounding the lateral ventricles, although antigen-positive cells were observed scattered throughout the cerebral hemispheres. The periventricular zone is the site of neuronal stem/progenitor cells, from which immature neuronal and glial cells migrate outward during neurodevelopment (Rakic 1988). Studies of congenitally infected human fetuses and experimental studies with murine CMV have suggested that cells within this region are highly susceptible to infection (Li and Tsutsui 2000; Perlman and Argyle 1992). One implication of these findings is that infection of these cells at a time during development that occurs before neuronal migration, differentiation, and organization could result in more severe outcomes than when infection occurs after developmental processes are completed. Abundant RhCMV protein expression was detected within the choroid plexus of one of the fetuses terminated 10 days after inoculation, suggesting that epithelial cells within this region may act as an early portal for CMV entry into the fetal CNS and across the blood-brain barrier. The findings of developmental abnormalities outside the brain indicate the presence of viral particles in the fetal circulation.

### Sensorineural Abnormalities in Inoculated Fetuses

Congenital HCMV infection has been identified as a significant cause of nonfamilial childhood hearing loss in congenitally infected children, including both those exhibiting clinical signs of HCMV infection at birth and those with asymptomatic infections (Hicks et al. 1993). Progressive bilateral sensorineural hearing loss can result from damage to the sensory portion, the neural portion, or both portions of

the auditory network. Published studies on fetal inoculation with RhCMV in experimental animal models have not yet addressed this issue; however, our group has recently investigated whether RhCMV can replicate within cells of the auditory system, thereby inducing direct and/or indirect damage within areas critical for hearing (S.P.T., S.S.Z., P.A.B., manuscript in preparation). Four fetuses were inoculated intracranially at 50 days gestation with  $1 \times 10^6$  PFU of RhCMV strain 68-1, and samples were fixed at 21 to 30 days after inoculation by transcardial perfusion with mixed aldehydes. The inner ears of all of the fetuses were examined and found to be positive as assessed by immunohistochemistry for RhCMV antigens, the presence of cells with pathognomonic CMV inclusion morphology, and viral particles observed by electron microscopy. RhCMV histopathology was largely confined to the developing spiral ganglion and eighth cranial nerve, although viral positive inflammatory infiltrates were also present in vestibular structures (Figure 3). RhCMV IE1-positive cells were localized in the developing spiral ganglion and the inflammatory infiltrate of the modiolus, scalae, and the connective tissue of the exterior cochlear surface (not shown). Positive cells, however, were absent from the developing organ of Corti and stria vascularis, although some positive cells were present in the inflammatory infiltrate of the connective tissue matrix filling the nascent scala tympani and vestibuli. Although no RhCMV-infected cells were noted within the organ of Corti in general, and hair cells in particular, secondary damage to these structures was observed. In one notable example, a profound protein exudate in the scalae vestibuli displaced Reissner's membrane and compressed the scalae media and the organ of Corti into unrecognizable remnants (not shown). These studies indicate that RhCMV can replicate within certain regions of the developing inner ear, resulting in damage to both the sensory and neural portions of the cochlea that could, theoretically, contribute to hearing loss.

In an effort to explore further the potential hearing loss associated with prenatal RhCMV infection, auditory brainstem responses (ABRs<sup>1</sup>) have been evaluated in a subset of animals inoculated using the intraperitoneal route on either 50 or 65 days gestation (N = 5 and 6, respectively) and followed prenatally and then for 1 mo postnatal age (S.M.G., A.F.T., P.A.B., unpublished data). The ABR protocols used were similar to published studies with young rhesus macaques (Wennberg et al. 1993). Although no definitive ABR deficits were observed in the infants that were inoculated in utero compared with age-matched untreated controls (N = 5), differences in defined parameters for the inoculated animals (e.g., I-IV interpeak latency, a measure of brainstem conduction) approached significance. Histological analysis of the inner ears of these animals demonstrated that although cochlear structure was essentially intact, there appeared to be a qualitative loss of cellularity within the spiral ganglion, spiral ligament, stria vascularis, and organ of Corti (not shown). These samples were fixed by immersion in 10% buffered formalin (vs. perfusion as



**Figure 3** RhCMV cytopathology in the cochlea of a fetus inoculated intracranially with rhesus cytomegalovirus (RhCMV) at 50 days gestation and harvested at 71 days gestation. Top: 71 days gestation. Low-magnification image demonstrating inflammatory infiltrate (I) surrounding the developing spiral ganglion (SG) in the cochlea. \* = area of inset demonstrating RhCMV-infected cells exhibiting pathognomonic cytopathology (arrows). Bottom: Electron microscopic detail of a RhCMV infected cell from the developing cochlear labyrinth demonstrating various stages of viral particle formation (arrows).

noted above), and changes associated with immersion fixation could not be ruled out entirely. However, mixed lymphocytic infiltrates were observed, consistent with a host immune response to RhCMV within the cochlea. These results indicate that additional studies in the nonhuman primate model of HCMV infection are warranted to identify an association between loss of sensorineural function and changes in structure.

In summary, the studies described above indicate that inoculation of fetuses with RhCMV recapitulates the broad tissue tropism and disease observed during congenital

HCMV infection. Subsequent studies have highlighted an intriguing aspect of intrauterine pathogenesis. Specifically, direct inoculation of macaque fetuses with high titers of virus ( $10^5$ – $10^6$  PFUs) does not result in 100% adverse outcomes. Similar to congenital infection with HCMV, there are likely to be various factors that limit RhCMV disease potential.

### Fetal Outcome in Relation to Fetal Age at the Time of RhCMV Inoculation

We addressed whether pathogenesis was related to the timing of intrauterine infection. To that end, fetuses of seropositive dams were inoculated with RhCMV by the intraperitoneal route in the late first trimester (day 50 of gestation;  $N = 13$ ), or early second trimester (day 65 of gestation;  $N = 7$ ), delivered at term by caesarean section and nursery reared through 1 mo postnatal age. Of the 20 fetuses inoculated, 54% (7/13) inoculated at 50 days gestation and 14% (1/7) inoculated at 65 days gestation either resulted in intrauterine demise or were harvested because of imminent demise (Table 2). Differences in the frequency of fetal loss following inoculation at the different time points approached but did not achieve statistical significance ( $p = 0.16$ , one-tailed Fisher's exact test). Comparable frequencies of RhCMV sequelae (macroscopic and microscopic) were observed in the two inoculation groups. Of the 20 fetuses inoculated, 77% (10/13) inoculated at 50 days gestation and 57% (4/7) inoculated at 65 days gestation exhibited pathological outcomes following RhCMV inoculation ( $p = 0.61$ ).

Tissues were available for analysis from six of the seven terminated fetuses (inoculated on day 50 of gestation). Four of these fetuses exhibited pronounced multiorgan RhCMV disease, which was characterized by the presence of cells bearing intranuclear and cytoplasmic inclusions. Two other fetuses displayed diaphragmatic defects that resulted in herniation of the liver into the thoracic cavity and pulmonary hypoplasia. One infant in each inoculation group developed severe hydrocephalus. One infant inoculated at 50 days gestation and two inoculated at 65 days gestation exhibited gliosis, lymphocytosis, calcification, and cystic degeneration; one of these four fetuses also exhibited IUGR. Non-CNS tissues were histologically normal in all of the infants. The latter observation underscores the following critical aspect of congenital HCMV infection: although non-CNS sequelae observed at birth in congenitally infected humans may be transient (e.g., petechial rash, hepatosplenomegaly), CNS-sequelae (e.g., sensorineural hearing loss, neurological deficits) are lifelong.

### Tissue Distribution of RhCMV

Molecular analysis of DNA from available tissues of every animal inoculated intraperitoneally for the presence of

**Table 2 Outcomes following fetal inoculation with rhesus cytomegalovirus (RhCMV)**

| Inoculation Timepoint | No. | Outcome (n)   | Percentage      |
|-----------------------|-----|---|-----------------|
| Control               | 8   | Normal: No clinical or histological findings          | 100             |
| Day 50 <sup>a</sup>   | 13  | Nonviable fetus <sup>b</sup> (7/13)                   | 54 <sup>e</sup> |
|                       |     | Live births (6/13)                                    | 46              |
|                       |     | Fetus/infant with RhCMV sequelae (10/13) <sup>c</sup> | 77              |
| Day 65                | 7   | Fetus/infant without RhCMV sequelae (3/13)            | 23              |
|                       |     | Nonviable fetus (1/7)                                 | 14 <sup>e</sup> |
|                       |     | Live births (6/7)                                     | 86              |
|                       |     | Fetus/infant with RhCMV sequelae (4/7) <sup>d</sup>   | 57              |
|                       |     | Fetus/infant without RhCMV sequelae (3/7)             | 43              |

<sup>a</sup>Day of gestation.

<sup>b</sup>6 of 7 nonviable fetuses (69–120 days gestation; 2nd to early third trimester) and 1 of 7 stillborns with diaphragmatic defect and pulmonary hypoplasia.

<sup>c</sup>RhCMV pathological outcomes included non-central nervous system (CNS) (multiorgan) and CNS (microcephaly, ventriculomegaly, calcification, gliosis, cystic degeneration, and lymphocytosis) disease. Two fetuses with diaphragmatic defects were nonviable. Total ( $n = 10$ ) includes 7 fetuses and 3 infants.

<sup>d</sup>RhCMV neuropathological outcomes in the infants included microcephaly, ventriculomegaly, cystic degeneration, lymphocytosis, and intrauterine growth restriction.

<sup>e</sup> $p = 0.11$  (one-tailed Fisher's exact test).

RhCMV DNA sequences demonstrated that almost every tissue obtained from terminated fetuses supported RhCMV replication (Table 3). The only exception was bone marrow, in which RhCMV DNA was undetectable in all of the samples. The reason for this latter observation is not yet known. Other studies have detected RhCMV DNA in bone marrow (these authors, unpublished data). Decidua and membranes were also negative for RhCMV DNA, although the placenta (a combination of fetal and maternal cells) was positive in the samples assayed (2/2, 100%, data not shown). It should be noted that the wide tissue tropism and high copy numbers of RhCMV were observed in the fetuses with histologically apparent RhCMV disease. Very low copy numbers of RhCMV DNA were detected in only seven tissues of the one fetus that was stillborn due to pulmonary hypoplasia (described above) (15–45 copies per  $10^6$  cells).

RhCMV DNA was detected in the majority of tissues (14 of 20, 70%) from four infants that had been inoculated at 50 days gestation (Table 3). The number of RhCMV

**Table 3 Virus isolation from specimens obtained from fetuses and infants inoculated intraperitoneally with rhesus cytomegalovirus (RhCMV) at 50 or 65 days gestation**

| Specimen                 | Fetus (inoculation 50 or 65 days) |   | Infant (inoculated 50 days) |                            | Infant (inoculated 65 days) |                          |
|--------------------------|-----------------------------------|---|-----------------------------|----------------------------|-----------------------------|--------------------------|
|                          | No. (+) tissues/no. fetuses       | Range <sup>a</sup>                        | No. (+) tissues/no. fetuses | Range <sup>a</sup>         | No. (+) tissues/no. fetuses | Range <sup>a</sup>       |
| Brain                    | 3/4                               | 600 - 4 × 10 <sup>4</sup>                 | 4/6                         | 30 - 1.2 × 10 <sup>3</sup> | 6/6                         | 50 - 5 × 10 <sup>3</sup> |
| CSF <sup>b</sup>         | ND <sup>b</sup>                   | —   | 0/6                         | 0                          | 1/2                         | 150                      |
| Thymus                   | 2/4                               | 2 × 10 <sup>3</sup> - 2 × 10 <sup>6</sup> | 1/6                         | 10                         | 0/6                         | 0                        |
| Lung                     | 4/4                               | 10 - 2 × 10 <sup>6</sup>                  | 1/6                         | 1 × 10 <sup>3</sup>        | 0/6                         | 0                        |
| Spleen                   | 2/3                               | 6 × 10 <sup>3</sup> - 5 × 10 <sup>6</sup> | 1/6                         | 100                        | 0/6                         | 0                        |
| Liver                    | 3/3                               | 10 - 4 × 10 <sup>6</sup>                  | 1/6                         | 10                         | 0/6                         | 0                        |
| Pancreas                 | 4/4                               | 40 - 2 × 10 <sup>6</sup>                  | 1/6                         | 30                         | 0/6                         | 0                        |
| Kidney                   | 2/3                               | 6 × 10 <sup>3</sup> - 2 × 10 <sup>6</sup> | 2/6                         | 10                         | 1/6                         | 1.5 × 10 <sup>3</sup>    |
| Adrenal                  | 3/4                               | 1 × 10 <sup>4</sup> - 2 × 10 <sup>6</sup> | 0/6                         | 0                          | 1/6                         | 30                       |
| Stomach                  | 3/4                               | 2 × 10 <sup>3</sup> - 1 × 10 <sup>7</sup> | 1/6                         | 10                         | 0/6                         | 0                        |
| Duodenum                 | 3/3                               | 3 × 10 <sup>3</sup> - 4 × 10 <sup>7</sup> | 1/6                         | 1 × 10 <sup>3</sup>        | 1/6                         | 30                       |
| Jejunum                  | 2/3                               | 200 - 4 × 10 <sup>7</sup>                 | 1/6                         | 30                         | 0/6                         | 0                        |
| Ileum                    | 3/4                               | 400 - 4 × 10 <sup>4</sup>                 | 2/6                         | 10 - 60                    | 0/6                         | 0                        |
| Colon                    | 3/4                               | 100 - 9 × 10 <sup>5</sup>                 | 2/6                         | 10 - 30                    | 0/6                         | 0                        |
| Axillary LN <sup>b</sup> | 3/3                               | 30 - 5 × 10 <sup>4</sup>                  | 0/6                         | 0                          | 0/6                         | 0                        |
| Inguinal LN              | 1/3                               | 60  | 0/6                         | 0                          | 0/6                         | 0                        |
| Mesenteric LN            | 1/2                               | 5 × 10 <sup>4</sup>                       | 1/6                         | 200                        | 0/6                         | 0                        |
| Muscle                   | 2/2                               | 9 × 10 <sup>3</sup> - 7 × 10 <sup>7</sup> | 1/6                         | 10                         | 0/6                         | 0                        |
| Skin                     | 3/3                               | 40 - 1 × 10 <sup>4</sup>                  | 0/6                         | 0                          | 0/6                         | 0                        |
| Bone marrow              | 0/4                               | 0   | 0/6                         | 0                          | 0/6                         | 0                        |

<sup>a</sup>RhCMV genomes/10<sup>6</sup> cells.

<sup>b</sup>CSF, cerebrospinal fluid; ND, not determined; LN, lymph nodes.

DNA-positive tissues in any individual animal ranged from 1 to 13, and the RhCMV copy numbers were relatively low (10-1,200 RhCMV genomes per 10<sup>6</sup> cells). Of the six analyzed infants inoculated at 50 days gestation, two that were histologically normal had no tissues with detectable RhCMV DNA (limit of detection: 5 copies per 10<sup>6</sup> cells). Of the six infants that had been inoculated at 65 days gestation, only five different tissues among the six infants had detectable RhCMV DNA (30-150 RhCMV copies per 10<sup>6</sup> cells), although positive detection was usually confined to a single tissue within any one animal. The one tissue in this inoculation group that was consistently positive for RhCMV DNA was the brain. In fact, the frequency of RhCMV detection in the brain was quite striking compared with all other tissues. RhCMV DNA was present in 13 of 16 fetal and infant samples analyzed, far exceeding any other tissue in frequency. All of the infants with neuropathological evidence of RhCMV infection were positive for RhCMV DNA.

Because only a small quantity of tissue (~100 mg) of the cerebral cortex and associated meninges was processed for DNA, the PCR results for the brain suggest a relatively high viral burden within this one tissue, even in clinically inapparent infections. The results indicate that the developing

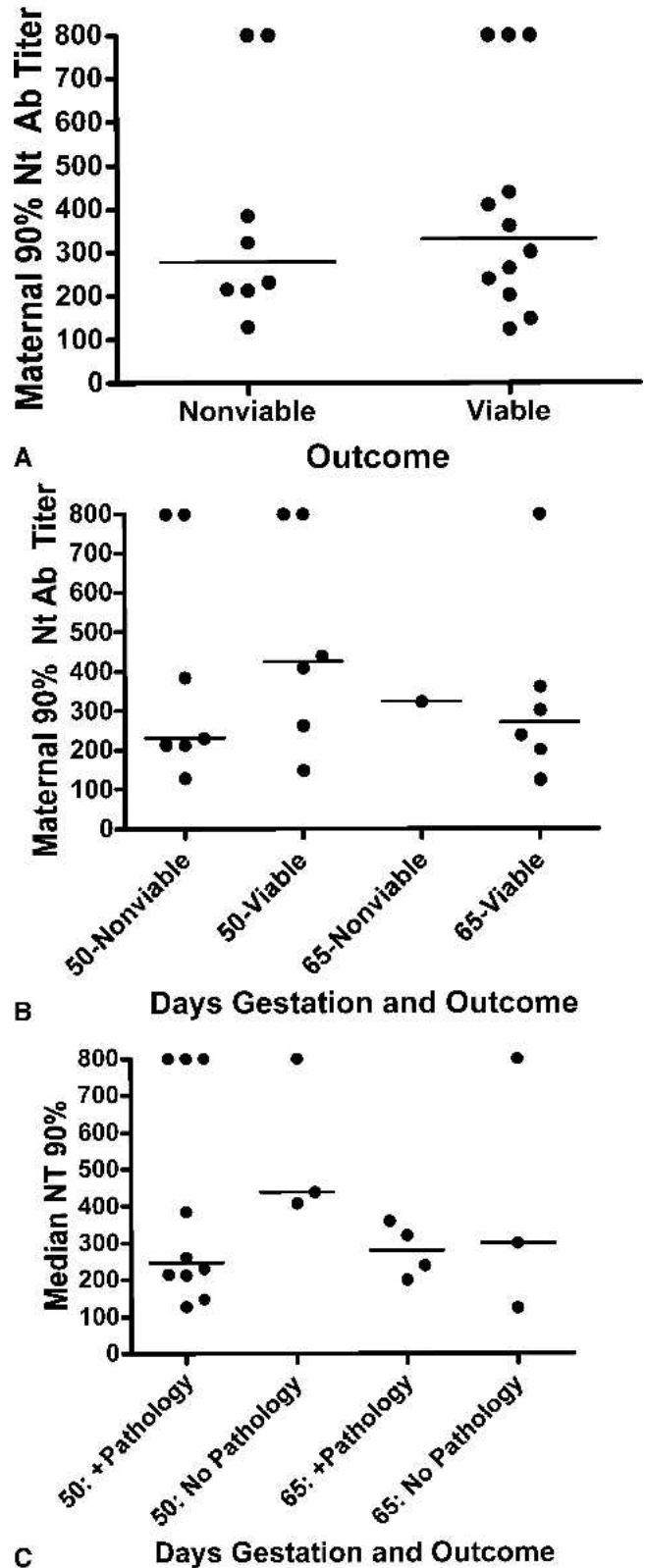
CNS is highly susceptible to RhCMV infection. The comparable frequencies of RhCMV DNA detection in the brains of fetuses inoculated either at 50 or 65 days of gestation further indicated that the window of susceptibility did not diminish from the late first trimester to the early second trimester. The results of London and colleagues (1983), described above, also demonstrated that the fetal brain remains vulnerable to intrauterine RhCMV until at least mid-gestation (day 80).

Studies on congenital HCMV infection have found that severe CNS outcomes are consistent with fetal infections that occur early in gestation (Hayward et al. 1991; Barkovich and Lindan 1994; Twickler et al. 1993). As discussed above, disruption of neuro-ontogeny at an early developmental time point would likely result in profound disruption of subsequent developmental landmarks, compared with infection at later stages of gestation. Fetal susceptibility may be a function of the relative timing of intrauterine infection and stage of organogenesis. There are also other developmentally regulated processes related to antiviral immune responses that may determine the outcome of intrauterine infection. Two variables that could differentially affect the course of viral infection during gestation are transplacental transfer of maternal

IgG and the ontogeny of the fetal immune system. The RhCMV copy numbers in the fetal tissues were generally orders of magnitude greater than those in the infants (Table 3). These results suggest two nonmutually exclusive scenarios. A threshold of antiviral immunity was present in some fetuses at the time of inoculation, or soon thereafter, which prevented the establishment of high viral loads in fetal tissues. Alternatively, or in combination, antiviral immune responses in the fetus and/or infant effectively cleared tissues of high viral loads by 1 mo postnatal age.

### Maternal Anti-RhCMV Antibody Titers and Fetal Outcome

Maternal neutralizing antibody titers were determined by end-point dilution (Lockridge et al. 1999) to assess whether fetal outcomes were related to maternal neutralizing antibodies at the time of fetal inoculation. There was essentially no difference between the median 90% neutralizing titers ( $NT_{90}^1$ ) of the dams (irrespective of inoculation time) in which fetuses were nonviable in utero and those whose fetuses were viable at term ( $NT_{90} = 227$  and  $331$ , respectively) (Figure 4A). When the dams of fetuses inoculated at 50 days gestation were analyzed separately, those with fetuses that expired in utero had lower median  $NT_{90}$  titers than those of fetuses that survived to term ( $231$  vs.  $424$ , respectively) (Figure 4B). Although the difference was not significant ( $p = 0.22$ ), the difference approached significance when dams of fetuses and infants with RhCMV sequelae were compared with those with normal infants ( $NT_{90} = 247$  and  $438$ , respectively;  $p = 0.11$ ) (Figure 4C). No difference was observed for dams of fetuses inoculated at 65 days gestation (Figs. 4B and C). In addition, fetal outcomes were unrelated to maternal RhCMV-binding antibody titers quantified by enzyme-linked immunosorbent assay at the time of fetal inoculation (not shown). Taken together, the results present a conflicting portrait of the potential protective effect of maternal antibodies in the fetal circulation at the time of fetal inoculation. Although the results suggest that higher maternal neutralizing titers ameliorated pathological outcomes following inoculation at 50 days gestation, there was no obvious protection conferred when inoculations were performed at 65 days gestation. However, some factor(s) intrinsic to the fetus protected the vast majority of fetuses inoculated at 65 days gestation (86%, Table 2). Because the fetuses were directly inoculated with RhCMV, the ability of maternal antiviral IgG to offer any protection to the fetus would have been directly related to the level of maternal IgG in the fetal circulation. The protective effect of maternal antibodies in the fetal circulation may be especially pronounced in the presence of lower titers of virus encountered following transplacental transmission compared with the high titers used for direct fetal inoculation.

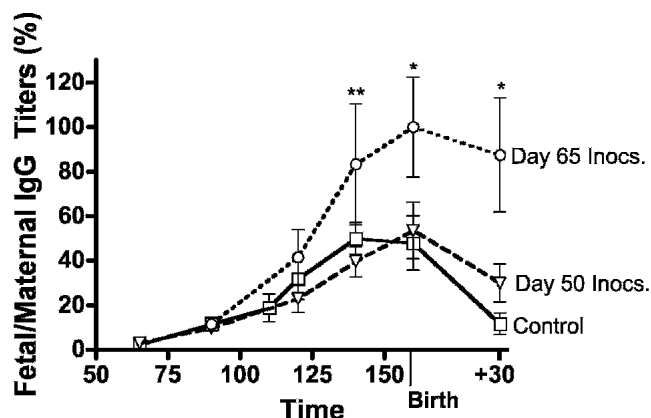


**Figure 4** Comparison of maternal 90% neutralizing antibody (Nt Ab) titers to (A) fetal survival outcomes (irrespective of time of inoculation), (B) survival outcomes following inoculation at either 50 or 65 days gestation, and (C) disease outcomes following inoculation at either 50 or 65 days gestation.

## Kinetics of Transplacental Transfer of RhCMV IgG and Fetal Antibody Responses to Viral Antigens

To address the hypothesis that transplacental transfer of maternal IgG may alter the course of intrauterine infection, the kinetics of transfer were evaluated at different times during gestation. Maternal and fetal antibody titers to viral antigens were prospectively analyzed following fetal inoculation during obtaining paired maternal/fetal blood samples at 90, 120, and 140 days gestation, birth, and 30 days postnatal age. Fetal blood samples were obtained by cardiocentesis (Tarantal 1990). Maternal RhCMV IgG titers were first assayed longitudinally to determine whether there was evidence of a maternal anamnestic immune response following inoculation. The range and mean titers of antiviral IgG in the dams at the time of fetal inoculation were similar to control dams at equivalent time points during gestation (data not shown). Individual titers remained essentially constant throughout gestation, consistent with an absence of anamnestic immune responses to RhCMV antigens in any of the dams following fetal inoculation.

Using paired control maternal/fetal plasma samples, the kinetics of transplacental transfer of maternal antiviral IgG to their fetuses were determined. In Figure 5, the mean kinetic values of transfer for control dams and fetuses are shown, beginning at 65 days gestation (the earliest sample collected) until 1 mo postnatal age. Because there was a large range of maternal RhCMV IgG titers, fetal titers are presented as a percentage of each respective maternal titer. Mean antiviral IgG titers in control fetuses at 65 days gestation were approximately 1 to 2% of maternal titers, increasing to 12.5% of maternal titers by 90 days gestation



**Figure 5** Mean fetal anti-rhesus cytomegalovirus (RhCMV) antibody titers relative to maternal titers at the corresponding times of gestation (expressed as a percentage relative to maternal titers). Vertical bars represent the standard error of the mean. Only the fetal titers at birth and +30 days after birth in the animals that were inoculated at 65 days gestation were significantly different from controls (asterisks).  $**p = 0.0002$ ;  $*p < 0.0001$ ; IgG, immunoglobulin G.

(late second trimester). Mean RhCMV antibody titers for control fetuses increased during gestation and peaked just below 50% maternal titer between 140 days gestation and birth (range: 25-100%). Because maternal titers were constant throughout gestation (not shown), relative increases in control fetal IgG titers reflect increased transplacental transfer of maternal IgG. By 1 mo after birth, neonatal serum concentrations of RhCMV IgG were reduced to approximately 12% of titers assessed at birth. These data suggested that the serum half-life of maternal RhCMV IgG in the neonatal circulation was approximately 15 days. The results of this study are consistent with others demonstrating that the kinetic values of IgG transfer across the placenta are similar among the primates (Coe et al. 1993; Eitzman 1970; Fujimoto et al. 1983a,b, 1988; Jauniaux et al. 1995; Moe and Osburn 1983; Stiehm 1975).

Similar analyses were performed for RhCMV-inoculated fetuses (Figure 5). Fetuses inoculated in the first trimester (50 days gestation) had transfer kinetics similar to control fetuses between 90 and 140 days of gestation. Higher mean RhCMV IgG titers were observed at 30 days postnatal age in these animals, compared with control animals (30 vs. 12% of maternal titers). The differences were not statistically significant. Fetuses inoculated at 65 days gestation had higher RhCMV IgG responses than controls beginning at 120 days gestation. Elevated mean titers were statistically significant for the fetuses inoculated in the second trimester beginning at 140 days of age ( $p = 0.0002$ ) and during the remainder of gestation and through 1 mo postnatal life ( $p < 0.0001$ ). Unlike concurrent controls and fetuses inoculated in the first trimester, neonates inoculated at 65 days gestation had antiviral IgG titers equivalent to maternal titers at birth. In addition, these same neonates did not exhibit the same rate of antiviral IgG decay as concurrent controls, inasmuch as there was greater than 85% of the birth titer remaining at 1 mo, versus 12% for controls. These data are consistent with the interpretation that fetuses inoculated at 50 and, particularly, 65 days gestation developed anti-RhCMV IgG antibodies following inoculation. Thus, not only were the concentrations of maternal antibodies in the fetal circulation greater at the time of inoculation at 65 days gestation, but also the older fetuses appeared to be more capable of developing de novo antibody responses to RhCMV. This interpretation is consistent with studies in rhesus macaques that identified CD20<sup>+</sup>/CD5<sup>+</sup> B cells in multiple fetal tissues by 65 days gestation (Makori et al. 2003). Antigen-specific humoral immune responses following in utero infections with various pathogens, including HCMV, have been documented in human neonates (Aase et al. 1972; Hara et al. 1996; Lynch et al. 1991).

## Fetal Production of RhCMV pp65-specific IgG

To confirm that inoculated fetuses were immunologically competent to develop RhCMV-specific IgG responses in utero, we have also analyzed IgG responses specific to

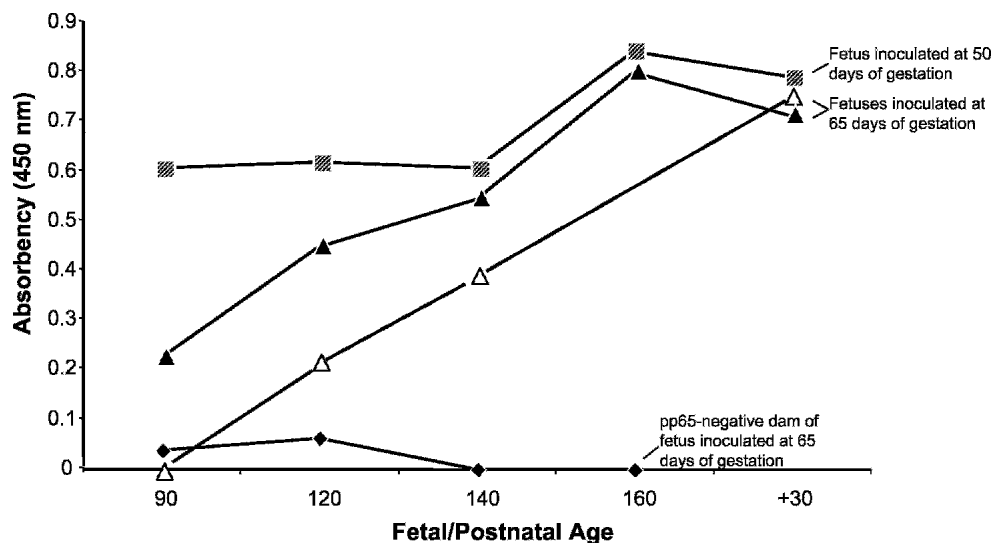
RhCMV phosphoprotein 65 (pp65). Some macaques chronically infected with RhCMV do not develop an IgG antibody response to pp65 (Yue, Zhou, and Barry, submitted for publication). Therefore, we identified experimental dams without pp65 seroreactivity (N = 3) and evaluated their fetuses (N = 1 inoculated at 50 and N = 2 at 65 days of gestation) for pp65 IgG seroreactivity following inoculation. All three inoculated fetuses developed pp65 IgG antibody in the absence of maternal anti-pp65 seroreactivity (Figure 6). The fetus inoculated at 50 days of gestation had higher pp65 IgG absorbency at 90 days of gestation than either of the fetuses inoculated at 65 days of gestation. These data confirmed that inoculated fetuses were immunologically competent to develop RhCMV-specific IgG responses by the mid-second trimester. These results did not, however, resolve whether the fetal antiviral antibodies elicited a protective effect from RhCMV disease. The pp65 protein is a tegument protein localized within the virion (Mocarski 1993), and antibodies against it are unlikely to inhibit viral dissemination. A relevant parameter to assess whether the fetus was able to generate protective antibodies would have been the titration of neutralizing antibodies against the RhCMV protein encoding the majority of neutralizing epitopes, glycoprotein B (gB<sup>1</sup>) (Yue et al. 2003). This possible approach was limited by the fact that all of the dams were seropositive for RhCMV, and all seropositive animals develop and maintain antibodies against gB (Yue et al. 2003). Accordingly, it would have been difficult to distinguish between anti-gB antibodies in the fetal circulation that were of maternal versus fetal origin.

## Summary

Studies to date suggest that the risk of developmental abnormalities following RhCMV inoculation of macaque fe-

tuses ranges between 50 and 100%. Multiple variables are likely to affect the frequency of RhCMV-related disease, including the route of inoculation and the time during gestation when the inoculation is performed. Intracranial inoculation has a high likelihood of marked neuropathological outcomes (Chang et al. 2002; London et al. 1986; these authors, unpublished research), whereas intramniotic inoculation can result in clinically inapparent infections (London et al. 1986). The data also suggest that the frequency of severe outcomes related to infection (e.g., fetal demise) is greater when inoculations are performed at 50 days gestation (Table 2). This finding is consistent with the hypothesis that the constantly changing fetal environment influences RhCMV disease potential.

There are several nonmutually exclusive hypotheses to account for differences in outcomes following fetal infection at different times of gestation. These differences may be related to (1) transplacental transport of maternal IgG, (2) fetal immune competence, and (3) differential fetal susceptibility based on gestational age at the time of infection. The differences share the underlying premise that RhCMV disease potential is related to the time of gestation when fetal infection occurs. The first two hypotheses further imply that a threshold of protective immunity can be established in the fetus, above which there is a reduced likelihood of RhCMV disease. The putative threshold could be established through a combination of maternal transplacental transfer of IgG and de novo fetal immune responses. Although the specificity of a protective immune response is unknown, the effects of protective immunity are probably established relatively early in gestation. RhCMV sequelae (e.g., ventriculomegaly, fetal hydrops, IUGR) are usually detectable by the early third trimester (Table 2; these authors, unpublished research). The absence of disease in the third trimester and beyond for some animals suggests that the developing im-



**Figure 6** Fetal immune responses to rhesus cytomegalovirus (RhCMV) pp65 in one fetus inoculated at 50 days gestation and two fetuses inoculated at 65 days gestation. All the dams were seronegative for RhCMV pp65 at the time of fetal inoculation and remained pp65-seronegative during gestation (only results from one dam are shown). Results are presented as the absorbency at 450 nm.

mune environment during the second trimester (56-110 days gestation) limits the potential for RhCMV disease. The second trimester has been shown to be the period of accelerated transfer of maternal IgG and fetal immune system maturation (Simister 2003).

In humans, transplacental transfer of maternal antibody is critical for protection of the fetus and neonate from pathogens such as HCMV, rubella, and measles virus (Alford and Britt 1993; Caceres et al. 2000; Englund et al. 1998). IgG in the control fetal circulation has been shown to reach appreciable concentrations (12.5% of mean maternal titer) by mid-gestation (90 days of gestation) and to have achieved maximal titers (50% of maternal titer) during the third trimester. It is unknown whether this low level of transplacentally derived antibody at this gestational time period is protective. Based on our findings regarding the kinetic values of antibody transfer, the duration and perhaps the magnitude of RhCMV replication may be very different in fetuses inoculated during the late first trimester compared with the early second trimester. It is expected that there would be a shorter period of time in fetuses at 65 days gestation for RhCMV replication to occur unencumbered by the presence of protective antibodies. Variables such as quality of maternal antibody (i.e., avidity and neutralizing titer [Boppana and Britt 1995]) and fetal immune responses could also determine the window of sustained viral replication and disease potential. It is important to understand these issues in order to design modalities that reduce the frequency and severity of symptomatic congenital infection in humans. Limited natural history studies suggest that more severe outcomes are observed following congenital infections earlier in pregnancy (Barkovich and Lindan 1994; Hayward et al. 1991; Jauniaux et al. 1995). In addition, symptomatic congenital outcomes can occur following non-primary or recurrent maternal infection (Boppana et al. 1999). These data suggest that the immune environment within the human fetus influences HCMV disease outcomes following transplacental transmission.

The contribution of fetal antiviral immune responses to protection from RhCMV disease is not known. It is generally considered that fetuses lack fully competent immune systems, although some functional parameters of immunity are established during prenatal life (Rayfield et al. 1980, 1987; Schelonka and Infante 1998; Schelonka et al. 1998). Antigen-specific humoral immune responses following in utero infections with various pathogens, including HCMV, have been documented in human neonates (Aase et al. 1972; Hara et al. 1996; Lynch et al. 1991). Our results demonstrated that both groups of experimentally inoculated fetuses developed antiviral antibody responses. Salient observations in the inoculated fetuses included (1) higher antiviral antibody titers relative to control titers, (2) pp65 seroreactivity in three inoculated fetuses from dams lacking pp65 seroreactivity, and (3) the presence of antiviral IgM in fetal blood (data not shown).

Extrapolation of the rhesus macaque studies to the human condition points to a window of fetal susceptibility

during which, if HCMV crosses the placenta, there may be no immune-mediated mechanism(s) to prevent unrestricted viral replication and pathogenic outcomes. Before the ontogeny of fetal adaptive immunity and transplacental transport of maternal IgG, innate immunity is most likely the only defense against HCMV disease. Accordingly, the design of vaccines for HCMV must be aimed at restricting access of the virus to the fetus, particularly during the period of organogenesis. Because the CNS is a primary target of intrauterine CMV, and CNS sequelae are permanent, minimizing the window of vulnerability will be critical to success.

## Future Prospects

The preceding discussion clearly supports the importance of the rhesus monkey model of HCMV infection, but also highlights some limitations. For example, the absence of sufficient numbers of seronegative, breeding-age female rhesus monkeys limits the modeling of potential vaccine strategies that restrict congenital infection and disease. Until sufficient numbers of monkeys that are specifically pathogen free (SPF<sup>1</sup>) for RhCMV are available, it will not be possible to perform studies that include vaccination of seronegative individuals followed by viral challenge to assess maternal-fetal transmission.

Efforts are currently under way at the National Institutes of Health-supported national primate research centers to develop breeding populations of SPF monkeys for several viruses endemic in macaques colonies. The primary goal of the SPF effort is to develop sustainable populations of macaques that are free of herpes B virus (*Cercopithecine herpesvirus 1*), an alphaherpesvirus genetically related to herpes simplex virus (Huff and Barry 2003). SPF derivation involves separating the infant from the dam at birth and subsequent hand-rearing in a nonhuman primate nursery. This process results in an infant population that is also seronegative for RhCMV and other endemic infectious agents. The animals remain RhCMV free well past the age of sexual maturity, when they are segregated from RhCMV-infected monkeys (Minamishima et al. 1971; Nigida et al. 1979; these authors, unpublished research). It will be possible to evaluate vaccine strategies that significantly limit transplacental transmission of RhCMV once breeding SPF colonies are established.

Despite this current limitation to full utility of the model, the clear importance of a model of intrauterine pathogenesis is that it will allow studies to address mechanisms of viral pathogenesis, structural and functional analysis of neurological deficits, and ontogeny of the fetal immune system in relation to intrauterine infection. Moreover, other areas where the current model is of great importance include the assessment of anti-HCMV drugs, which are rarely given to mothers of congenitally infected fetuses due to concerns related to adverse effects on the fetus. RhCMV and HCMV are equally susceptible to the

anti-HCMV drugs (e.g., ganciclovir and cidofovir) that have been approved by the US Food and Drug Administration, as well as novel experimental compounds (North et al. 2004; Swanson et al. 1998). Thus, it is now possible to use the fetal pathogenesis model to address transplacental pharmacokinetics, teratogenic potential, and antiviral efficacy of lead compounds similar to studies that have been performed on the fetal pathogenesis model for HIV infection (Tarantal et al. 1994, 1999, 2002).

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