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Developing a Vaccine against Congenital Cytomegalovirus (CMV) Infection: What Have We Learned from Animal Models? Where Should We Go Next?

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Summary

Congenital human cytomegalovirus (HCMV) infection can lead to long-term neurodevelopmental sequelae, including mental retardation and sensorineural hearing loss. Unfortunately, CMVs are highly adapted to their specific species, precluding the evaluation of HCMV vaccines in animal models prior to clinical trials. Several species-specific CMVs have been characterized and developed in models of pathogenesis and vaccine-mediated protection against disease. These include the murine CMV (MCMV), the porcine CMV (PCMV), the rhesus macaque CMV (RhCMV), the rat CMV (RCMV), and the guinea pig CMV (GPCMV). Because of the propensity of the GPCMV to cross the placenta, infecting the fetus *in utero*, it has emerged as a model of particular interest in studying vaccine-mediated protection of the fetus. In this paper, a review of these various models, with particular emphasis on the value of the model in the testing and evaluation of vaccines against congenital CMV, is provided. Recent exciting developments and advances in these various models are summarized, and recommendations offered for high-priority areas for future study.

Keywords

Cytomegalovirus; Animal cytomegalovirus models; Guinea pig CMV; Rat CMV; Murine CMV; Rhesus CMV; Porcine CMV; Cytomegalovirus vaccine; Live; attenuated CMV vaccines; CMV pentameric complex; Guinea pig model; placenta; immune modulation

Maternal infection with human cytomegalovirus (HCMV) during pregnancy can produce severe disease in newborns, and can lead to long-term neurodevelopmental sequelae, particularly sensorineural deafness [1–3]. Development of a vaccine focused on protecting newborns from the sequelae of congenital HCMV infection is therefore a major public health priority, as identified by the National Vaccine Program Office [4] and the Institute of Medicine [5]. Discussions about the proposed deployment of a CMV vaccine have focused primarily on immunization of adolescent girls and women of child-bearing age, toward the goals of optimizing protection amongst women anticipating potential pregnancies in the near future and preventing congenital infection [6]. However, it is not clear what would constitute an optimal protective vaccine in this setting. Moreover, it has become increasingly clear in recent years that both CMV-naïve and CMV-immune women are at risk to acquire CMV infections during pregnancy, with subsequent transmission to the fetus [7]; hence, a targeted vaccination of CMV-seronegative women will not solve the problem of congenital CMV infection. Primary maternal infections carry a greater risk of transmission and severe sequelae for the neonate than do recurrent infections [8], but the burden associated with re-infection is nonetheless quite substantial, and the goal of improving protection of the fetus

by “augmenting” immunity to CMV in a woman who is already CMV-seropositive is a very challenging concept for vaccine development.

The two general approaches to vaccination evaluated in clinical trials have been based on subunit vaccines, consisting of individual immunodominant antigens (administered as purified DNA or proteins, or as “vectored” vaccines) important in the cellular and/or humoral immune response, and live, attenuated vaccines (reviewed in [9]). CMV is a member of the *herpesvirinae* family [10]. Virus particles are enveloped and contain a nucleocapsid (consisting of viral genomic DNA with accessory proteins) and a proteinaceous tegument layer, lying between the nucleocapsid and the surrounding envelope. The double-stranded DNA genome of HCMV is approximately 230 kb in size, the largest among known human viruses, and consists of unique long (U_L) and unique short (U_S) segments, each of which is flanked by inverted repeats sequences [11]. Most of the approximately 200 genes encode protein products. CMV gene nomenclature is complex: most genes are generally named by their position within the genome, although some also have additional descriptive names. For example, UL83 is the 83rd annotated gene in the unique long region of the genome; it encodes a 65 kDa phosphoprotein also known as pp65. Hence, the protein product is both known as ppUL83, and pp65. Several HCMV gene products have been recognized as key immunogens in the host response to infection. These include envelope glycoproteins such as UL55 (also known as glycoprotein B), UL75 (glycoprotein H), and others. Some proteins in the viral tegument are targets of cell-mediated immune responses, in particular the ppUL83 (pp65). As noted, many of these proteins are being employed as subunit vaccines in various clinical trials [9].

Success in HCMV vaccine programs has been limited to date – underscoring the need to continue to use preclinical models to study correlates of protection against CMV infection and disease. Subunit vaccines targeting the major envelope glycoprotein gB (gpUL55) have demonstrated varying degrees of efficacy against CMV infection and/or disease in high-risk populations, including young women, solid organ, and hematopoietic stem cell transplant patients [12–16]. However, it remains uncertain if a vaccine-induced antibody response to a single viral glycoprotein target would be sufficient for a vaccine designed to prevention infection of the fetus. Live, attenuated HCMV vaccines induce both antibody responses as well as broad-based cellular responses, including cytotoxic CD+ T-cell responses [9, 17], and hence may have theoretical benefits compared to subunit approaches. Safety considerations regarding theoretical long-term risks of a CMV live-virus approach, including atherosclerosis, immune senescence, reactivation from latency, and, potentially, even Alzheimer's disease have dampened enthusiasm for the live attenuated vaccine approach [18–20].

Given the striking species-specificity of CMVs, preclinical studies of HCMV vaccine are generally not feasible in animal models. HCMV-specific immunogens, including recombinant proteins, virions, dense bodies, and other vectored vaccines [21–30], have all be evaluated for immunogenicity in a number of animals, including mice, rabbits, hamsters, guinea pigs, and rhesus macaques. However, although *immunogenicity* studies are possible (and provide valuable information), *efficacy* evaluations of these vaccines cannot be conducted, since HCMV will not cause replicate or cause disease in animals. Hence, species-specific CMVs must be studied, in order to model infection and test potential vaccine strategies for HCMV. In this review, the current status of animal models for CMV vaccines is reviewed, with a particularly emphasis on studies of vaccines targeting protection against congenital infections. High-priority areas for future animal model research are proposed and outlined in the executive summary section of this article.

Basis for Species-Specificity of CMVs: Can it be Overcome?

The precise molecular/cellular basis for the species-specificity of betaherpesviruses, including CMV, remains unknown. Early experiments in multiple laboratories attempted to grow CMVs from one species in heterologous cell culture systems from different species. Typically these experiments show some evidence of uptake of heterologous virus, but a block exists preventing full productive replication. A study of human diploid WI-38 cells inoculated with murine CMV (MCMV) demonstrated cytopathic effect, including intranuclear inclusions, but infection was abortive [31]. Rabbit kidney cultures were found in one study to support some level of replication of MCMV [32], and rabbit lung cells have been reported to support low-level HCMV replication [33]. MCMV was also reported to replicate in guinea pig brain cells, and maintain infectivity (although not replicate) for 12 days in human cells [34]. A recombinant MCMV expressing the GFP gene demonstrated evidence of abortive infection in multiple mammalian cell types [35]. A comparative study of various human and non-human cell cultures to determine their sensitivity to the Davis strain of HCMV indicated that mink lung cell cultures appeared more sensitive than other cell cultures tested, in a shell-vial centrifugation assay, although it was not demonstrated that these cells supported productive viral infection [36]; moreover, additional studies suggested that the observed cytopathogenicity of CMV in mink cells might have been related to mycoplasma contamination of HCMV stocks [37]. In other studies, inoculation of HCMV into mixed cultures of guinea pig fibroblasts and epithelial cells demonstrated CPE following adsorption of virus by conventional microscopy, and uptake of virus particles both into the cytoplasm as well as in cytoplasmic vacuoles by electron microscopy [Figure 1]. These experiments demonstrated expression of HCMV antigens by immunofluorescence, but the infection was ultimately nonproductive [38]. The results of these various investigations suggested that the barrier to CMV cross-species infection was downstream of events related to binding and penetration of virions into the cell, and that some limited initial gene expression appears to occur prior to an undefined block in productive replication.

Recently there have been some insights into the molecular basis for species-specificity of CMV infection. Replication of MCMV in human cells was examined in detail several years ago by the late Gerd Maul at the Wistar Institute. Extending the work performed years earlier by other investigators, Maul demonstrated that MCMV can undergo all processes of its life cycle in human cells, but that there were several synergistic mechanisms of suppression of MCMV production, including hydrolysis of newly replicated viral DNA and very low capsid protein transcription [39]. These effects could be ameliorated by adding HCMV tegument proteins and IE 1 protein. Additional insights into the molecular basis for the species-specificity have also been gleaned from innovative work performed by the Brune laboratory. Preliminary work demonstrated that a key factor blocking MCMV replication in human cells was the induction of apoptosis, and that this block to replication could be partially overcome by the overexpression of members of the anti-apoptotic Bcl-2 protein family, including adenovirus E1B-19k [40]. Bcl-2 also allowed rat CMV (RCMV) to replicate and form plaques in human cells [40]. These studies were extended when these authors examined a spontaneously occurring MCMV mutant that had gained the ability to replicate rapidly and to high titers in human cells [41]. Compared to the wild-type virus, this mutant exhibited reduced apoptosis in cells, and sequence analysis of revealed mutations in the *M112/M113*-coding region of the genome, a region encoding a family of “early 1” (E1) proteins, involved in DNA replication. Another recent study reported by the Tang lab examined HCMV-infected mouse cells and MCMV-infected human cells, toward the goal of identifying intrinsic cellular defense mechanisms against cross-species infection at the post-entry level [42]. These studies demonstrated that the `knock-down' of ND10 components (PML, Daxx, and SP100) could result in significantly increased viral protein production, suggesting that ND10 and ND10 constituents might be important defensive factors against

CMV cross-species infections. These various molecular mechanisms underlying the species-specificity of CMVs are summarized in Table 1.

Understanding the mechanisms preventing and/or facilitating cross-species CMV infections could one day potentially allow the testing of HCMV vaccines in animal models. These studies also may have practical implications for solid organ transplant recipients of porcine xenografts, given that HCMV was recent shown to produce infection of porcine endothelium [43, 44]. Even though there was no evidence of productive viral replication, HCMV IE antigens were expressed in infected endothelium, and it was postulated that infection could, by upregulation of E-selectin and vascular cell adhesion molecule-1, result in leukocyte recruitment and an increased risk of rejection. Human fibroblasts have also similarly been shown permissive for PCMV infection in cell culture [45], as have human endothelial cells [46].

Recent studies have also examined the tropism and replication of primate CMVs in human cells. These studies have focused primarily on Rhesus macaque cytomegalovirus (RhCMV). A strain of RhCMV strain, 68–1, was noted to spread slowly when grown in cultured rhesus epithelial cells; this strain was not fully wild-type in sequence in the *UL128-131* locus that has been shown to be important in epithelial and endothelial tropism for HCMV infection [47–49]. Specifically, it was noted that this strain did not code for ORFs corresponding to homologs of HCMV UL128 and the second exon of HCMV UL130. Following repair of mutations of the *UL128-UL131* locus of strain 68–1, as well as repair of a mutation in the UL36 ORF, repaired viruses replicated much more efficiently than virus in rhesus epithelial cells, and, remarkably, replicated efficiently in cultured human epithelial cells and endothelial cells [50]. These results suggest that these gene products, at least in primate CMVs, also play roles in dictating the species-specificity of CMV. Additional reports have examined the replication of HCMV in chimpanzee cells. This work demonstrated that primary skin fibroblast cells derived from chimpanzees supported the replication of HCMV, with infection virus recovered from supernatants that was only 10-fold reduced in titer compared to HCMV grown in HF cells [51].

Other studies have attempted to overcome the problem of species-specificity of CMV infection by examining human tissue explants maintained in compartments in animals (typically, mice) that could allow the inoculation and propagation of HCMV in human cells. These animals in turn could be evaluated with antiviral drugs or other therapeutic interventions specific for HCMV in a system one step closer to an *in vivo* model. These systems have included human fetal thymus/liver [52–54] or fetal retinal tissue implants [55, 56] maintained in SCID/hu mice. Another model involved the use of HCMV-infected human foreskin fibroblasts seeded onto a biodegradable gelatin matrix, Gelfoam [57]. Infected fibroblasts are then implanted subcutaneously into SCID mice, and antiviral drugs targeting HCMV can be administered to assess antiviral effect in an in vivo model. Although these models are to varying degrees useful in evaluation of antiviral drugs [58, 59], there is less information about exploiting the SCID/hu implant model for evaluation of HCMV vaccines and immunotherapies.

Another novel approach to overcoming species-specificity issues in the study of CMV vaccines in animal models was described by Khanna and colleagues [60, 61]. In these experiments, replication-deficient adenovirus vectors expressing the extracellular domain of HCMV gB along with other HCMV epitopes (corresponding to immunodominant peptides from the the IE-1, IE-2, gH, pp150, pp50, and pp65 gene products) were used to immunize HLA-2 transgenic mice. The chimeric vaccine consistently generated strong HCMV-specific CD8+ and CD4+ T-cell responses, as well as virus-neutralizing antibody. Intriguingly, immunization with the HCMV/adenoviral chimeric vaccine protected mice against a

challenge (referred to by the authors as a "quasi-virus challenge") with a recombinant vaccinia virus encoding various combinations of HCMV antigens. Protection was associated with the induction of antigen-specific cellular responses and antibody responses. Although not a true "HCMV challenge" in a heterologous animal model, these experiments nevertheless represent an innovative approach to overcoming the problem of species-specificity in CMV vaccine models, and provide some opportunity to validate and compare various subunit vaccine targets using clinically relevant virological endpoints.

Finally, another approach to dealing with the challenge of the species-specificity of CMVs in the context of vaccine studies is to "humanize" the specific animal CMV by engineering HCMV gene targets into the heterologous viral genome by BAC mutagenesis [62]. One such "swap" mutant approach was described by our lab recently, in which the guinea pig CMV (GPCMV) homolog of the HCMV *UL97* gene, *GP97*, was replaced with its HCMV counterpart [63]. This experiment was of interest, since UL97 is the kinase responsible for phosphorylating the antiviral drug, ganciclovir, to its active form, and GPCMV is intrinsically resistant to ganciclovir. The recombinant virus expressed functional UL97 protein, and had improved susceptibility to ganciclovir, compared to wild-type GPCMV. Engineering GPCMV to express HCMV glycoprotein targets, such as gB, could allow the testing of HCMV vaccines in the guinea pig congenital infection model – in essence, another type of "quasi-virus challenge". Animal model testing of species-specific CMV vaccines based on authentic homologous viral challenge are considered in the following section.

Animal Models to Test Vaccines for Prevention of Congenital CMV Infection

Although there has been, as noted, some progress in elucidating the basis of species-specificity of CMV infection, at the present time there are very limited options for the testing of vaccines against HCMV in any animal models of infection. The following section summarizes salient features of and recent developments in animal models of vertical CMV transmission, with a particular emphasis on how this information may be exploited for testing HCMV vaccines. Ideally, an animal model would have the features of convenience and low expense; clear-cut vertical transmission and fetal/neonatal disease endpoints that would allow assessment of vaccines specifically against *congenital* infection; a wide array of immunological reagents that would allow detailed characterization of immune correlates of protective immunity; a well-characterized genome allowing for the expression of relevant subunit vaccine candidates; and experimental endpoints in newborn animals that mimic those observed in infants. The extent to which the various animal CMVs fulfill these criteria is summarized in the following section. A summary table outlining these various models is also provided (Table 2).

Porcine Cytomegalovirus

Cytomegalic inclusion disease due to porcine cytomegalovirus (PCMV) was first recognized and described in 1964 [64], followed shortly thereafter by recognition of the causative agent by EM [65] and culture [66]. Many perinatal PCMV infections in piglets are probably acquired post-natally, from breast-feeding and other forms of close contact, since interventions such as early weaning of piglets and barrier rearing (interventions undertaken to try to increase the supply of PCMV-negative organs for xenotransplantation) does decrease the prevalence of PCMV infection [67, 68]. On the other hand, studies of pregnancy outcomes in pigs following intranasal exposure to PCMV have demonstrated transplacental infection as well, particularly if challenge is undertaken very early in pregnancy. In one study, five out of 22 embryos were infected when PCMV challenge occurred shortly after insemination. In infected embryos, virus was found in leptomeningeal cells, hepatic sinusoidal cells, peritoneal macrophages, periosteal cells and occasional alveolar cells, but less commonly in placenta [69]. A study of six seronegative sows infected

with PCMV by intranasal route between 31 and 85 days of pregnancy followed by caesarean delivery at term demonstrated 18/60 mummified or stillborn fetuses. PCMV was isolated from 16 piglets from 4 of the 6 litters examined, most commonly from lung and liver but also from spleen, kidney, brain and nasal mucosa [70]. In another study, pregnant sows with PCMV antibody were challenged in pregnancy, and transmission occurred in spite of pre-existing immunity [71] – analogous to the issue of re-infection with vertical transmission that is being increasingly recognized as a concern in pregnant women [72–75]. In the reinfection study reported in sows, virus was detected in 8 of 24 fetuses, and in some fetuses virus could be identified in capillary endothelium and macrophages of the lung, as well as nasal mucosa, spleen and brain [71]. Interestingly, the majority of virus-positive fetuses in this study were reportedly normal in appearance, suggesting that these infections were asymptomatic. However, there is some evidence from the veterinary medicine literature that PCMV can be pathogenic in newborn piglets [76].

Although PCMV does transmit to the fetus and produce some interesting pathologies in the piglet, it has not been further exploited for development as a vaccine model. Genome information is incomplete, although PCR assays for detection of PCMV DNA have been developed [77, 78]. Recently, cloning and purification of a 270 nt region of the PCMV gB gene containing the putative immunodominant epitopes has allowed development of a PCMV ELISA assay [79]. If xenotransplantation with porcine organs continues to be developed as an intervention for humans with end-stage solid organ disease [68, 80], further examination of treatment or prevention of PCMV infections by either vaccination or antiviral therapy may become of practical medical importance [81].

Rat Cytomegalovirus (RCMV)

RCMV is an extensively used model that has been employed by many investigators to study CMV pathogenesis [82, 83]. The RCMV model has proven to be most useful in the context of viral challenge following immunosuppression of the rat, typically via total body irradiation [84], and has been used in this context to study a variety of antiviral therapies [85, 86]. RMCV also provides an excellent model for study of endovascular infection and disease [87], particularly in the setting of transplantation, especially cardiac allografts [88–90], but also in the setting of liver [91] and kidney [92] transplantation. RCMV can be used to model the phenomena of transplant vascular sclerosis (TVS; [93, 94]), a rapidly progressive and serious complication of heart transplantation in humans. Interesting, TVS is driven, in part, by the elaboration of RCMV-encoded chemokines (CKs), including the RCMV chemokine r129 [95], the homolog of the HCMV UL128 protein [96, 97].

To date, there are no reports of the use of the RCMV infection model for the study of CMV vaccines. Recent experiments reported by Loh and colleagues, however, have suggested that the RCMV model might be surprisingly useful for the study of congenital CMV infection and, conceivably, vaccines to prevent congenital transmission. In a study conducted at the Department of Veterinary Pathology and Microbiology at the Universiti Putra Malaysia, a novel strain of RCMV, designated ALL-03, was isolated from the placenta/uterus of a house rat (*Rattus rattus diardii*) and was found to: productively infect rat embryo fibroblasts; produce typical herpesvirus-like cytopathic effects; and demonstrate herpesviral inclusion bodies and virus related particles in the cytoplasm and nucleus of infected cells by EM [98]. Hyperimmune serum generated against another strain of RCMV, the Maastricht strain, was reactive against the ALL-03 strain, as assessed by neutralization test, immunoperoxidase assay, and immunofluorescence studies. Despite demonstrating typical morphological characteristics of a CMV, initial sequence analyses of the viral genome demonstrated that it was significantly different from that of the better-known Maastricht [99] and English [100] strains of RMCV. The finding that this virus infected the placenta and uterus of rats

suggested that it might be vertically transmitted to the fetus. This was confirmed in a follow-up study by this group [101] that demonstrated, following experimental infection of immune compromised Sprague-Dawley rats, that virus challenge during pregnancy lead to infection of of uterus, placenta, and fetus, with virus identifiable in the lung, kidney, spleen, liver and salivary gland of congenitally infected pups. Inoculations were performed at day 10 of pregnancy (average gestational period, 22 days). In this study, virus could not be detected in the rat brain, potentially rendering this model less relevant to the problem of congenital HCMV infection. However, continued characterization of this strain of RCMV [102, 103] may be valuable, and could lead to the development of models of vaccine or antiviral therapies against congenital CMV infection in this model.

There are limited reagents available for the study of RCMV. There are PCR primers described for real-time detection of RCMV DNA [104]. A panel of monoclonal antibodies was developed by the Bruggeman lab several years ago [105], but do not appear to be commercially available. A BAC clone in *E. coli* of the Maastricht strain of RCMV has been developed and used to generate recombinant viruses [95].

Mouse Cytomegalovirus (MCMV)

The MCMV model has been the most powerful animal model for the study of CMV pathogenesis and immunity, due to the vast array of mouse immunological reagents; the plethora of transgenic mice; the well-characterized MCMV genome; the availability of recombinant MCMVs based on bacterial artificial chromosome (BAC) and other technologies; and the lower cost of mice compared to many other animals used in viral models. The MCMV model, particularly its usefulness in the study of latency, reactivation, and immune evasion, has been the subject of several excellent reviews [106–110]. With relevance to CMV vaccines, the MCMV model has been well-characterized, and targets of both the CD8+ T cell response [111–114] as well as the antibody response to infection have been evaluated in experimental models of vaccine-mediated protection. These studies have demonstrated protection against viral challenge following immunization with recombinant plasmids expressing key MCMV immunogens [115–121]; peptide-based vaccines [122, 123]; live, attenuated vaccines [124–129]; and vectored vaccines based on MCMV envelope glycoprotein homologs of gB and gH [113, 114]. Of particular interest has been the use of BAC technologies to selectively engineer live-virus vaccines deleted of viral immune evasion genes to improve immunogenicity and enhance safety [130–132]. In another variation of the BAC-based “designer vaccine” approach, a recombinant MCMV vaccine was engineered expressing the high-affinity NKG2D N cell ligand, RAE-1γ. This vaccine, by eliciting tight innate immune control via activation of NK cells, was highly attenuated, but induced strong and long-lasting protective immunity [133].

A chief limitation of the MCMV model for testing vaccines against congenital CMV infection is the probably inability of the virus to infect the fetus by transplacental route. This may relate to differences between the mouse placenta, which contains three trophoblastic layers, and the human placenta. Placental histology may contribute to the lack of transmission of MCMV across the mouse placenta [134, 135]. However, recently the dogma that the mouse fetus cannot be transplacentally infected has been challenged by studies reported by Woolf [136]. In these studies, timed-pregnant SCID mice were intraperitoneally injected with MCMV at embryonic stages E0–E7, and vertical MCMV transmission was noted at rates up to 53% when dams were challenged at day E4. Later time points of challenge were associated with reduced transmission. MCMV antigens were detected in PCR-positive fetuses, and transplacental MCMV transmission was associated with intrauterine growth retardation and microcephaly. The fact that these experiments were performed in SCID mice may have contributed to this result, since prior efforts to

demonstrate vertical transmission of MCMV were uniformly unsuccessful [134, 135]. This model could conceivably be exploited to study vaccines against congenital MCMV infection, although there would be challenges intrinsic to the modeling of any active vaccination strategies in SCID mice.

Other models of neonatal MCMV disease exist. These have not been fully exploited for the testing of vaccines, although such studies may be of value: even though there may not be congenital transmission *per se*, maternal immunization, particularly with vaccines inducing humoral responses, could be studied as a strategy to improve the outcome following neonatal challenge. Studies performed by Tsuigui's group have pioneered the analysis of MCMV fetal neuropathogenesis using techniques that bypass the placenta to directly inoculate the fetus. Injection of MCMV into mid-term developing embryos is used to study brain abnormalities (neuronal migration defects, microcephaly, polymicrogyria), and eye abnormalities (microphthalmia), although unfortunately no vaccine studies have been pursued to date in this model [137–142]. Another intriguing study has demonstrated the effectiveness of antiviral immune globulin in limiting MCMV neuropathology [143]. In this study, treatment of MCMV infection in newborn mice with MCMV-immune serum or with MCMV-specific monoclonal antibodies reduced the titer of infectious virus detection limit, whereas brains of mice receiving control serum had significant viral load noted. In addition, histopathological and immunohistological analyses revealed more viral foci, mononuclear cell infiltrates, and glial nodules in the brains of untreated mice [143]. Another model developed by the Lokensgard laboratory has recently examined neonatal MCMV infection and cochlear pathology [144]. In this model MCMV was found to preferentially infect both cochlear perilymphatic epithelial cells and spiral ganglion neurons, and induce a chronic inflammatory response, including a prolonged increase in reactive oxygen species production by macrophages. An interesting report from a group at the Huazhong University of Science and Technology (Wuhan, China) examined fetal and newborn outcomes following placental inoculation of MCMV in BALB/c mice at 12.5 days gestation (average gestational period, 19–22 days). This group found that approximately 29% of newborn mice had MCMV brain infection, and these mice demonstrated not only microcephaly and reduced brain weight upon necropsy, but also had diminished performance in the Morris water maze compared to controls, with deficits in learning and memory capability [104]. This model could be very relevant to the HCMV-infected infant, since microcephaly and learning disabilities are commonly encountered. In addition, Jaskoll and colleagues have recently described an elegant *in vitro* model of MCMV cochlear pathogenesis [145]. All of these models of neonatal MCMV induced pathology are powerful, since they recapitulate the pathology observed in infants infected with congenital HCMV. Future studies of antiviral antibodies in many of these models could be considered, including those induced by maternal vaccination prior to delivery, and such data could be very instructive to HCMV vaccine development. Recent advances in real-time imaging of neonatal MCMV infection should also provide valuable new tools for addressing immunotherapies in the MCMV model [146].

Guinea Pig Cytomegalovirus (GPCMV)

The chief value of the GPCMV model, compared to other small animal models, lies in the unequivocal ability of the virus to produce congenital infection following maternal inoculation with this virus. A number of aspects of guinea pig reproductive biology further contribute to the usefulness of this model. Guinea pig gestational periods are lengthy, compared to rodent models, ranging from 65–70 days [147]. Like the human plancenta (but in contrast to the mouse and rat placenta), the guinea pig placenta is hemomonochorial, containing a single trophoblast layer separating maternal and fetal circulation [148–150]. These and histologic and biological similarities to the human placenta add to the value of

this model for vaccine testing. The ability of GPCMV to infect the guinea pig placenta was first described in the 1930s [151]. In the late 1970s, three groups independently described studies of fetal infection following infection of pregnant dams with GPCMV [152–154]. Notably, in one of these reports it was observed that “transplacental transmission of virus with invasion of the fetus was observed…in some mothers with preinoculation evidence of GPCMV antibody” [154], a finding that has significance in light of the increasing recognition of maternal re-infections in pregnancy leading to symptomatic congenital infections in infants [72–75]. Since these reports on transplacental transmission of GPCMV, numerous investigations have employed the guinea pig model for vaccine studies (Table 3). Depending upon the strain of guinea pig (inbred vs. outbred), source of virus (tissue culture-adapted or salivary gland-passaged), and timing of inoculation during pregnancy, a variety of experimental endpoints can be studied in this model [155]. These include mortality in dams, pup infection rate, pup mortality rate, maternal and pup weights, fetal resorption rate, total viral load in dams and pups (assessed by viral culture or PCR), and end-organ disease (including placenta), assessed histologically and/or by viral load analysis. Variability in the strain of animal, route of inoculation, and timing of inoculation has contributed to a range of levels of effectiveness of various vaccine strategies studied to date in this model: Table 4 compares the magnitude of the protective effect reported in these various studies. To date there has unfortunately not been any analysis of the impact of vaccines on functional outcomes in pups following maternal challenge. Of particular interest with respect to the problem of congenital CMV in infants, it has been noted that congenitally infected guinea pig pups can exhibit brain and visceral involvement, inner ear pathology, and sensorineural hearing loss, providing additional useful and highly relevant endpoints that should be emphasized for future vaccine and pathogenesis studies [156–160]. Figure 2 demonstrates both end-organ disease in a dam challenged with virulent, salivary gland-adapted GPCMV mid-gestation (pneumonia) and cochlear pathology in a pup challenged post-natally with GPCMV as examples of pathology induced by viral infection in this model.

The first vaccine study against congenital GPCMV reported in the literature was a study comparing a live, attenuated GPCMV vaccine, and a partially purified, soluble envelope vaccine, administered with Freund's adjuvant, in Hartley guinea pigs [161]. Live vaccine was prepared after 11 serial passages in tissue culture, whereas envelope vaccine was prepared by detergent treatment of GPCMV-derived dense bodies and virions, in a noninfectious vaccine enriched for viral envelope antigens. It is of interest that this vaccine preparation was derived from highly tissue culture-passaged GPCMV, which in retrospect may have selected for a the genome variant of the GPCMV 22122 strain deleted of the 1.6 kb region that encodes GP129, 131 and 133, homologs of the HCMV pentameric complex proteins UL128-131 [162–165]. This envelope vaccine preparation may have therefore been suboptimal in its ability to elicit neutralizing antibodies capable of blocking endocytic entry of virus into cells. In any case, after challenge with virulent GPCMV, animals inoculated with live virus vaccine were protected against acute viremia and death, and a reduced incidence of generalized maternal and fetal infection was observed. In contrast, envelope antigen-vaccinated animals showed acute viremia after similar challenge with virulent virus, but infection was less generalized than that in control animals, and GPCMV was not isolated from fetuses. These data provided the first suggestion in the GPCMV model that superior protection could be noted using live, attenuated vaccines, compared to adjuvanted subunit vaccines.

Another interest study reported by Bia and colleagues in 1984 examined “low-passage” GPCMV vaccine, which had been shown in the prior study to be effective in preventing GPCMV transmission, for the ability to undergo reactivation during pregnancy. In Hartley strain guinea pigs challenged by intraperitoneal route with low-passage vaccine, 27 (41%) of 66 throat swabs obtained during subsequent pregnancies were culture-positive for GPCMV,

in contrast to 28 (24%) of 115 throat swabs obtained from nonpregnant controls. In contrast, high-passage GPCMV vaccine did not cause acute viremia, detectable generalized infection, or any evidence of GPCMV reactivation during subsequent pregnancies. In spite of its attenuation and apparent inability to establish latent infection in the salivary gland, these immune pregnant animals and their fetuses were protected against GPCMV when challenged with virulent salivary gland-adapted virus. Strain 2 guinea pigs were also protected against pneumonitis in a vaccine/challenge study in non-pregnant animals [166]. In retrospect, it seems likely that the experimental low-dose, high-passage GPCMV vaccine reported on by Bia's group in this study [167] – which failed to show any apparent vaccine reactivation during pregnancy – was attenuated due to selection of virus with deletions in the ~1.6 kb region of the viral genome identified by Inoue's group [162, 163] that is essential for conferring *in vivo* pathogenesis for GPCMV.

More recently, BAC-based mutagenesis has allowed further refinement of live, attenuated vaccines in the GPCMV model with targeted `knock-outs' of immune modulation genes. These viruses have been analyzed for protective efficacy in the congenital infection model. We reported on a vaccine study with a targeted deletion of three genes encoding MHC class I homologs (presumed NK evasins): *gp147, gp148*, and *gp149*. The resulting virus, 3DX, grew with wild-type kinetics in cell culture, but was attenuated in guinea pigs. In spite of attenuation, vaccination with 3DX produced elevated cytokine levels and higher antibody titers than wild-type virus, while avidity and neutralizing titers were similar. Protection, assessed by maternal viral loads and pup mortality following challenge with salivary gland-adapted virus during pregnancy, was comparable in animals vaccinated with 3DX and wild-type virus, and statistically significant compared to control (non-vaccinated) animals [168]. In the current issue of *Future Virology*, we report on another live, attenuated viral vaccine, vAM409, a vaccine with a targeted deletion in the pp65 (pUL83) homolog, GP83. This vaccine virus, although attenuated, provided protection against pup mortality in litters born to vaccinated dams compared to unvaccinated controls, with attendant reduction in congenital GPCMV infection. Further exploration of attenuated live-virus vaccines with targeted deletions in genes involved in immune modulation and pathogenesis is warranted in this model.

Since the early descriptions of detergent-treated crude envelope protein vaccines in the GPCMV model [161], subunit approaches to vaccination against congenital GPCMV infection utilized lectin-purified or immuno-affinity purified glycoprotein vaccines have continued to be studied. In a study in inbred guinea pigs, an immunoaffinity-purified glycoprotein was found to induce strong antibody and CMI responses when administered with Freund's adjuvant, and newborn pups were protected against congenital infection and disease [169, 170]. In a similar study, immunity conferred by immunization with a mixture of envelope glycoproteins resulted in substantial protection against pup mortality, with a reduction in pup mortality from 56% to 14% in the immunized group compared to controls ($p<0.001$), and reduced *in utero* transmission in surviving animals [171].

More recently, cloned recombinant technologies have been applied to the study of the GPCMV homologs of gB [172] and pUL83 (GP83) [173] in vaccination against congenital infection. When these ORFs were cloned in plasmid expression vectors [174] for DNA immunization studies, a four-dose series of plasmid vaccine administered epidermally by “gene gun” approach resulted in reduced pup mortality and a significant decrease in the incidence of congenital CMV infection for gB vaccine, but not GP83 [175]. However, both vaccines resulted in a reduced viral load in infected animals, compared to controls. The immunogenicity and efficacy of a DNA BAC vaccine, modified by transposon insertion into an essential gene, *GP48* [176], was also studied. BAC plasmid DNA was adjuvanted by inclusion of the lipid adjuvant, DOTMA/DOPE. Following vaccination of Hartley guinea

pigs with BAC DNA, third-trimester GPCMV challenge demonstrated that immunization resulted in reduced pup mortality, and a correlate of protection appeared to be reduced viral load in vaccinated dams.

Adjuvanted subunit studies with a recombinant, secreted form of gB expressed in insect cells, truncated at Pro_{692} [177] and purified by lectin column, have also been conducted in the GPCMV model [178]. One study compared a three-dose series (50 μg doses) of vaccine administered with either Freund's adjuvant or alum. ELISA titers and complement-dependent virus neutralizing titers were significantly higher in guinea pigs immunized with gB and Freund's compared to gB with alum. Among control dams (n=41 pups), pup mortality was 76% (31/41). In contrast, for pups born to gB-immunized dams, the overall pup mortality rate was reduced to 25% (23/91; $p<0.0001$). Differences were noted in mortality and congenital infection when the Freund's and alum groups were compared. The gB/Freund's adjuvant vaccine was also more effective than alum in reducing maternal viral load. A subsequent vaccine/challenge study examined GPCMV-gB vaccines formulated with the clinically-relevant adjuvant systems, $AS01_B$ and $AS02_V$, or with Freund's adjuvant [179]. Efficacies against pup mortality were estimated at 64%, 84% and 44% for $gB/AS01_B$, $gB/AS02_V$ and gB/FA, respectively. Efficacies DNAemia were estimated at 88%, 68% and 25% for the same vaccines, respectively, but were only significant for the $gB/AS01_B$ ($p<0.001$), and the $gB/AS02_V$ ($p=0.002$) adjuvants. These studies suggest that for optimum protection against congenital CMV conferred by a recombinant gB vaccine, adjuvant choice will be critical.

In addition to DNA vaccines and adjuvanted glycoprotein vaccines, subunit vaccine approaches to congenital GPCMV infection have been performed using vectored approaches. We reported on the use of a propagation-defective, single-cycle, RNA replicon vector system, derived from an attenuated strain of the alphavirus Venezuelan equine encephalitis virus, that was used to produce virus-like replicon particles (VRPs) expressing GP83 [180]. Vaccination with VRP-GP83 induced antibodies and CD4+ and CD8+ T cell responses, and dams vaccinated with VRP-GP83 had improved pregnancy outcomes (13% pup mortality), compared with dams vaccinated with a control VRP expressing influenza hemagglutinin (57% mortality; $P<0.001$, Fisher's exact test). Improved pregnancy outcome was also accompanied by reductions in maternal blood viral load. Another vectored approach was recently reported by Inoue's group [181], using a recombinant adenovirus approach expressing the GPCMV gB. This form of gB was also a truncated, secreted variant form of the gB molecule, truncated at Tyr_{674}. Vaccination of dams with a single dose of a recombinant adenovirus-gB construct in early pregnancy resulted in reduced congenital GPCMV infection, from 75% in controls to 13% of fetuses immunized dams. Notably, placentas were infected less frequently in the gB-immunized animals, and there was reduced intrauterine growth retardation in fetuses in vaccinated dams. These authors postulated that antibodies against gB protect against spread of infection mainly at the placental interface, rather than from the placenta to the fetus, and that the development of strategies to block cell-to-cell viral spread in the placenta would represent the most effective strategy against congenital CMV infection.

In summary, vaccine studies in the GPCMV model (magnitude of protection noted in various vaccine studies is summarized for side-by-side comparisons in Table 4) have provided the following insights into vaccine-mediated protection against congenital infection: 1) there is strong evidence for the importance of both gB and GP83 in protective immunity against congenital CMV infection; 2) for subunit protein vaccines, the choice of adjuvant is critical; 3) the magnitude of the antibody response engendered, particularly the neutralizing antibody response, correlates with the extent of protection; 4) the magnitude of reduction of maternal DNAemia is an important correlate of protection of the fetus, and a

threshold level of DNAemia exists that, above which, there are striking increases in pup mortality; 5) glycoprotein B vaccines to date have not provided “sterilizing immunity” of the maternal, placental, or fetal compartments; 6) reduction of cell-to-cell spread at the level of the placenta may be an important goal of vaccine-induced immunity; 7) live, attenuated vaccines confer superior protection to subunit vaccines. Given the regulatory issues implicit in licensure of a live, attenuated HCMV vaccine, greater value may be realized in future studies in the GPCMV model are focusing on subunit, and not live attenuated, vaccines. In particular, variables leading to optimization of antibody response, inclusion of cellular targets (such as GP83) in multicomponent vaccine studies, and examination of the protective immunity conferred by vaccination with proteins corresponding to the gH/gL/GP129–133 pentameric complex homologs of the HCMV gH/gL/UL128-131 complex are high priority areas for future studies.

Rhesus Macaque Cytomegalovirus (RhCMV)

The final animal model for the study of vaccines against congenital CMV infection is the rhesus macaque model. This model employs RhCMV, a closely related primate CMV that has a high degree of genome identity with the HCMV genome [182–186]. The rhesus macaque model is especially well suited for the study of HCMV pathogenesis, based on the strong developmental, immunological, anatomical, and biochemical similarities that humans and monkeys share by virtue of their close phylogenetic relationship. The RhCMV model has been the subject of several excellent reviews [187–189]. The RhCMV model also provides an excellent model for fetal neuropathogenesis due to CMV infection in the developing central nervous system. In a study of fetal rhesus monkeys inoculated intraperitoneally with RhCMV early in the second trimester, fetuses had evidence of severe CMV disease, including intrauterine growth restriction, ventriculomegaly, microcephaly, lissencephaly, and degenerative changes of cerebral parenchyma [190]. Histopathologic examination in this study revealed the presence of polymicrogyria, gliosis, leptomeningitis, periventricular calcifications, and inclusion-bearing cells. The demonstration 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 infants provides a strong impetus for the continued development of this model to test vaccines against congenital CMV infection. The recent development of specific-pathogen-free colonies of macaques [191] is also of great potential value in helping to control for the issue of pre-existing maternal immunity in the context of vaccine studies.

There have not been, to date, any RhCMV vaccine studies reported in which the primary study endpoint was prevention of vertical transmission, or fetal/neonatal disease. A heterologous DNA prime/protein boost strategy was used in seronegative macaques, consisting of four DNA immunizations against pp65-2, gB, and immediate-early 1 (IE1), followed by two boosts with formalin-inactivated RhCMV virions; this approach was associated with strong cellular and humoral immune responses, and decreased viral replication at a site of primary inoculation [192]. A DNA vaccine study comparing combinations of plasmids expressing various combinations of RhCMV gB, pp65, and virally-encoded interleukin-10 homolog demonstrated a role for gB and pp65 vaccine in altering the course of acute and persistent RhCMV infection [193]. A study of viral shedding in saliva in macaques immunized with modified vaccinia virus Ankara (MVA) expressing gB, pp65, and IE1 demonstrated reduction in shedding in vaccinated animals compared to controls [194], and a prime-boost study in which animals were primed with DNA vaccination and boosted with MVA vaccine demonstrated reductions in viremia following viral challenge [195]. More recently, a MVA vaccine based on the RhCMV homologs of the gH/gL/UL128-131 pentameric complex demonstrated the induction of broadly neutralizing antibody responses and reduced plasma viral load in 8 vaccinated macaques [196].

Other recent studies have highlighted the rhesus macaque model's usefulness in studying the immunopathogenesis of CMV infection, particularly the role of viral immune modulation genes in immunity and infection. As noted, since the genome sequences of rhCMV [182–186] and the related cynomolgus macaque cytomegalovirus [197] are known, the high degree of genetic relatedness of these CMVs to HCMV should make future vaccine studies of particularly high relevance to human health. Of particular interest are studies reported by the Picker laboratory examining the results of mutation of the RhCMV homologs of HCMV *UL128-131* on the CD8+ cell repertoire in RhCMV infected macaques. In this report, it was shown that the absence of *Rh157.5*, *Rh157.4*, and *Rh157.6* (homologs of human CMV UL128, UL130, and UL131 genes) allowed CD8+ T cells to be generated against unusual, diverse and highly “promiscuous” epitopes [198]. The ability to engineer a live-virus vaccine capable of achieving distinct and more diverse patterns of CD8+ T cell epitope recognition may prove useful in both the development of new HCMV vaccines, as well as in the use of HCMV itself as a vaccine “vector” for other infectious diseases. Another innovative, recently reported RhCMV vaccine study targeted the viral homolog of the anti-inflammatory cytokine, viral IL-10 [199], (encoded by the RhCMV genome) as a potential vaccine target. RhCMV-seronegative macaques were immunized with engineered, nonfunctional RhCMV IL-10 variants, which were constructed to abolish binding to the cellular IL-10 receptor. Vaccine recipients developed antibodies that neutralized RhCMV IL-10 function with no cross-neutralization of cellular IL-10. Following viral challenge, vaccine recipients demonstrated reduced replication at the inoculation site reduced Rh CMV shedding in body fluids compared to controls [200]. Neutralization of viral immunomodulation genes represents a novel vaccine paradigm for HCMV vaccines. Future studies examining the role of this and other subunit vaccines in protection of the macaque fetus against congenital RhCMV infection with attendant neuropathogenic intrauterine infection are eagerly awaited.

Future Perspective

As summarized in Table 3, there have been now a number of vaccine studies for congenital CMV infection performed in the most tractable animal model, the guinea pig model. Using GPCMV, a number of live-attenuated and protein subunit vaccine approaches have demonstrated varying degrees of effectiveness. For subunit vaccines, the choice of adjuvant is critical. Notably, even with optimal and potent adjuvants, sterilizing immunity is not observed with gB based vaccines. The recent recognition and characterization of the GPCMV homologs of the pentameric complex proteins (HCMV gH/gL/UL128-131) opens up the exciting opportunity to examine the comparative efficacy of vaccines based on this complex head-to-head with gB vaccines. In addition, cell–mediated targets, such as GP83 (the pUL83 homolog), have been validated as useful vaccines in the GPCMV model. It will be important to more fully characterize the depth and breadth of the guinea pig response to other cellular targets to optimize this model. Finally, the rat and mouse models may be more useful for the study of vaccines against fetal CMV infection than has been previously recognized, and these models deserve further development. The most relevant model, the rhesus macaque, also deserves additional study, since the neurological and neurodevelopmental sequelae of infection can be best studied in this relevant primate system.

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** Of considerable interest

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Executive Summary Points

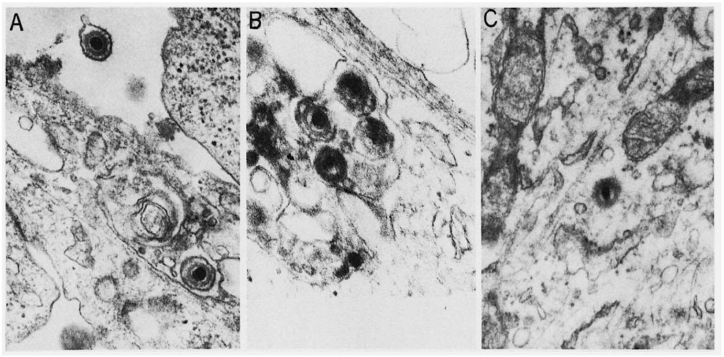
Basis for Species-Specificity of CMVs

- Congenital CMV infection is a major public health problem and CMV vaccines are a major priority, but must be tested in preclinical models of congenital infection.
- CMVs unfortunately only infect animals of their respective species; i.e., there is a mouse CMV, a human CMV, a rat CMV, but these viruses won't infect across species.
- The species-specificity of CMVs is poorly understood, but recent insights from MCMV suggest a role of the M112/113 region of the viral genome as well as constituents of the PML, Daxx, and SP100 proteins in dictating species-specificity. Insights into the molecular basis of species-specificity suggests the very real possibility of directly studying HCMV in animal models in the future.

Animal Models to Test Vaccines for Prevention of Congenital CMV Infection

- Transplacental infection is an important pre-requisite for a model of study of vaccines for congenital infection, but RCMV and MCMV have been underappreciated as potential models of congenital infection.
- Glycoprotein vaccines for congenital CMV require optimized adjuvants.
- Live, attenuated vaccines using molecular mutagenesis techniques should not be dismissed as potential HCMV vaccines, and the safety and efficacy of such approaches should be examined in animal models.
- Future studies should emphasize the pentameric complex, diverse CTL targets, improved adjuvant systems, and more nuanced outcome measures, such as hearing loss and neurological injury, in animal models.
- The recognition of the role of deletion/modification of immune modulation genes in rhCMV (particularly Rh157.5, Rh157.4, and Rh157.6) in directing cell-mediated responses, including CD8+ T-cell responses, to unusual and diverse epitopes, could offer new vaccine design strategies. These concepts should be further developed, given the close similarity in the molecular biology and pathophysiology of RhCMV and HCMV.

A)



B)

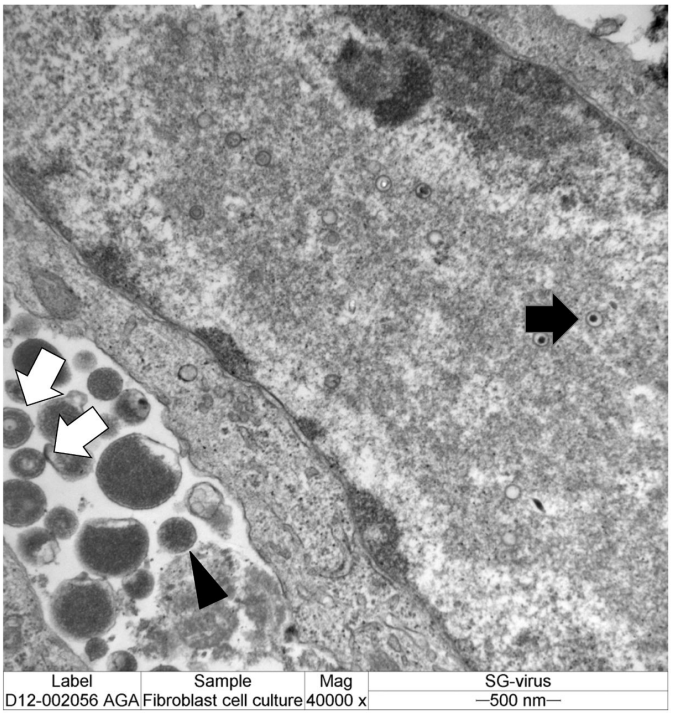


Figure 1.

A) Nonproductive infection of guinea pig cells with human cytomegalovirus. Electron micrographs of thin sections of guinea pig cells (mixed fibroblasts and epithelial cells) infected with human cytomegalovirus, Towne strain. Reproduced from reference [38], with permission. Panel A, virus particles are noted near cell surface and cytoplasmic vacuoles within 15 minutes of infection. Panel B, viral nucleocapsids noted in vacuoles within 1 hour post-infection (x48,000). Panel C, fully unenveloped particle in cytoplasm 1 hour post-infection (x41,000). These and other data (see text) indicate that species-specific block to CMV replication is not due to binding and uptake of virus in cells of heterologous species. B) Productive infection of guinea pig cells with guinea pig cytomegalovirus. Experiment from our laboratory for comparison demonstrating productive infection of guinea pig fibroblast cell culture with salivary gland-adapted GPCMV (x40,000). Images obtained 48 hours post-infection. Intranuclear capsids are noted (black arrow) as well as extracellular virions (white arrows) and dense bodies (black arrowhead).

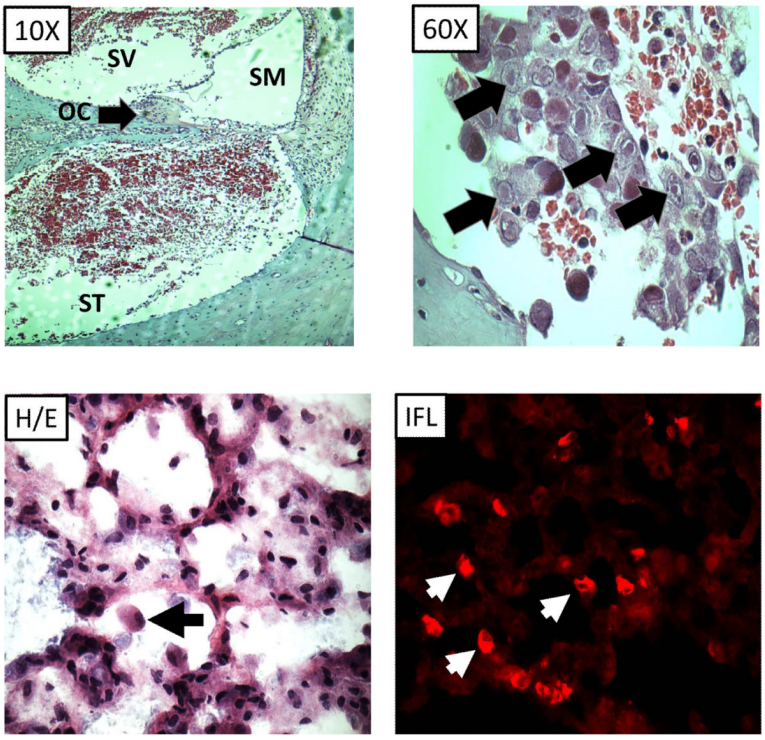


Figure 2.

Pathology of GPCMV infection. A) Cochlear infection of guinea pig. Following GPCMV inoculation inflammatory infiltrates and viral inclusions. Left panel, cochlea (10X magnification) with organ of Corti (oc), scala media (sm), scala tympani (st) and scala vestibule (sv) labeled. Inflammatory infiltrates are most notable in perilymphatic compartment (st/sv). Right panel, 60x magnification of st demonstrating viral inclusions (black arrows). This pup had profound sensorineural hearing loss. B) GPCMV pneumonitis in pregnant dam (40x magnification). Left panel, hematoxylin/eosin stain (H/E) of lung from pregnant dam that died with GPCMV pneumonitis approximately 7 days following early third trimester challenge. Viral inclusion cell is noted (arrow). Right panel, immunofluorescence image (IFL) of lung using an anti-GPCMV gB antibody (IE321) generated in our laboratory demonstrating presence of gB in lung tissue (arrows).

Table 1

Molecular Basis for Species-Specificity of Cytomegaloviruses: Potential Mechanisms Blocking Trans-Species Infections

CMV Used➔Heterologous Cell Types Infected	Mechanism of Suppression of Transpecies Infection	Factors Contributing to Overcoming Species-Specificity Barrier
MCMV ➔Human Foreskin Fibroblasts (HFFs)	• MCMV DNA replication but DNA Degradation	• HCMV IE1: mechanism involving increased amount and stability of MCMV DNA and enhanced activity of HCMV pp71
	• Impaired Encapsidation Due to Lack of Synthesis of Capsid Proteins	• HCMV pp71: possible mechanism of neutralization of human Daxx protein to relieve Daxx-mediated repression of MCMV replication?
	• Lack of Synthesis of “Late” Proteins	• Other HCMV tegument proteins – unknown mechanism?
MCMV, RCMV➔Human Retinal Pigment Epithelial Cells (ARPE)	• Initiation of apoptosis identified as a key event in blocking cross-species infection	• Block to replication overcome by expression of anti-apoptotic Bcl-2 proteins
		• Adenovirus E1b-19K (Bcl-2-like)
		• HCMV pUL37 (vMIA; structurally and functionally distinct from Bcl-2 family)
MCMV➔Retinal Pigment Epithelial Cells; human embryonic lung fibroblasts (MRC-5); HFFs; human umbilical vein endothelial cells (HUVECs)	• Spontaneous isolation of MCMV mutant capable of replication in human cells	• *M112/113* mutations decrease the proapoptotic effect of MCMV infection on human cells
	• Mutations in *M112/M113* appear responsible; this region encodes the E1 proteins, (four differentially spliced isoforms)	• Increase the formation of replication compartments and the efficiency of viral DNA replication
	• Presumed pro-apoptotic function	• The mutations may enhance the ability of MCMV to disrupt promyelocytic leukemia protein-associated nuclear bodies (PMLs) nuclear domains, in the process possibly relieving transcriptional repression
MCMV➔MRC-5 cells; adenovirus-transformed human epithelial kidney cell 293 (HEK293) HCMV➔murine NIH 3T3 cells	• ND10 (nuclear domain 10) proteins P100, Daxx, and PML appeared to be strong repressors of cross-species infection	• Species-specificity barrier could not be overcome in these studies
		• Knock-down of ND10 enhanced cross-species protein synthesis but did not confer replication competence
RhCMV➔MRC5; ARPE; HUVEC	• RhCMV deleted in UL128-131 homolog region unable to replicate in human cells	• Repair of RhCMV UL128-UL131 homologs confers cross-species infection of human cells by RhCMV
		• UL36 homolog influenced epithelial replication (anti-apoptotic function?)

Table 2

Comparison of Animal Models for Potential Study of Congenital CMV Infection

Animal Model	Strengths	Challenges
Porcine CMV (PCMV)	• Congenital/placental Infections observed	• Genome sequence is currently incomplete
	• Reinfection with transmission of virus in immune sows; could be useful model for re-infection	• Few immunological reagents for pig research
	• Fetal disease observed including lung, spleen and brain	• Expense of animals
	• PCR assays for viral genome detection are available	• No prior published reports of vaccine studies being performed in this model
	• Reports of PCMV cross-species infection of human cells; may have implications for xenotransplantation	• No clear endpoints that mimic congenital HCMV but not well studied
Rat CMV (RCMV)	• Excellent model for CMV-associated vascular disease	• Relative paucity of reagents (monoclonal antibodies, immunological reagents for rat research)
	• Several RCMVs sequenced; BAC clones available; role of RCMV genes in pathogenesis elucidated; molecular assays (PCR) available	• Intense immunosuppression required in some studies to observe disease
	• Recent observation of novel RCMV strain (ALL-03) capable of transplacental transmission	• No clear endpoints that mimic pathology in congenially infected infants; absence of brain infection
	• ALL-03 infects placenta, lung, liver, kidney, salivary gland, spleen, not brain	• No reports of vaccine studies in this model
	• Animals relatively inexpensive	
Murine CMV (MCMV)	• Best-established animal model of CMV infection, immunology and pathogenesis	• Congenital/transplacental transmission unclear; conflicting reports in literature
	• Vast array of knock-out mice, immunological reagents available	• In absence of transplacental transmission, vaccine studies specifically for prevention of congenital CMV infection have not been performed
	• Viral genomes sequenced, cloned as BACs for mutagenesis studies	• Neonatal models of pathogenesis are available and may be useful surrogate models for congenital HCMV infection; brain infection, cochlear infection models available following neonatal infection and could be exploited in future vaccine studies
	• Well-developed models for neuropathogenesis; relevant to HCMV	
	• Extensive literature on vaccine studies in this model; protein subunit, DNA vaccines, live attenuated vaccines, vectored vaccines	
	• Humoral and cellular targets elucidated; role of immune evasion genes well-defined; useful model to define roles of innate and adaptive immunity	
Guinea Pig CMV (GPCMV)	• Best-established animal model of placental infection and congenital transmission	• Paucity of immunological reagents for guinea pig model
	• Viral genomes sequenced, cloned as BACs for mutagenesis studies	• Relatively few reagents for GPCMV proteins; proteome not fully characterized
	• Congenital infection recapitulates pathology observed in infants; brain and cochlear infection; intrauterine growth retardation; vertical transmission with viremia and end-organ disease	• Endpoints of pup mortality/severe end-organ disease are extreme; congenital HCMV infection rarely causes mortality

Animal Model	Strengths	Challenges
	• Multiple studies of vaccines specifically targeting congenital transmission	• Long-term neurodevelopmental outcome studies following congenital GPCMV infection have not been performed
Rhesus Macaque CMV (RhCMV)	• Viral genomes sequenced; BAC clones available for mutagenesis studies.	• Fetal pathogenesis requires direct inoculation of virus into developing fetus; bypasses placental route; relevance to congenital HCMV less clear using this route of inoculation
	• Outstanding model for fetal pathogenesis including neuropathogenesis and cochlear infection	• RhCMV-seronegative animals difficult to procure
	• Model for other aspects of HCMV pathogenesis including re-infection, immunosenescence	• Expense of studies is considerable
	• Immunological reagents available	• Relatively few reagents for RhCMV (antibodies, viral mutants) although these are becoming increasingly available
	• Vaccine studies have been reported using a variety of subunit and vectored expression technologies	• No reports of vaccine studies to date specifically targeting congenital infection
	• Novel vaccine strategy of targeting viral immune modulation protein recently described	

Table 3

Vaccines for Prevention of Congenital CMV in the GPCMV Model

Vaccine Approach	Guinea Pig Strain	Findings
Live, attenuated vaccine isolated following 11–34 serial passages of ATCC virus in tissue culture [161, 166, 167]	Hartley	• Reduced incidence of maternal and fetal infection • Vaccine virus did not reactivate or produce fetal infection in subsequent pregnancy • Did vaccine lose pentameric complex genes on TC passage?
	Strain 2	• Protection against viremia, pneumonitis, and mortality in non-pregnant animals • Reduction in end-organ recovery by culture of SG challenge virus in vaccinated animals
Envelope vaccine (dense bodies and virions) with Freund's adjuvant [161]	Hartley	• Neutralizing antibody responses elicited • Vaccinated animals demonstrated reduction in viremia following SG virus challenge • Protection against congenital infection
Immunoaffinity purified envelope glycoprotein with Freund's adjuvant [170]	JY-9	• Neutralizing antibody and cell-mediated responses elicited • Reduced maternal DNAemia • Protection against GPCMV-associated pup mortality and congenital infection
Lectin-purified envelope glycoprotein(s) with Freund's adjuvant [171]	Hartley	• Antibody responses to several glycoproteins including gB • Neutralizing antibody responses • Protection against GPCMV-associated pup mortality and congenital infection in live-born pups
DNA vaccines gB/GP83 and BAC DNA[175, 176]	Hartley	• Vaccination resulted in lower viral load in infected pups • BAC DNA vaccine resulted in decreased pup mortality • BAC DNA immunogenicity augmented by lipid adjuvant
Recombinant gB vaccine with Freund's, alum and MPL adjuvants [178]	Hartley	• Neutralizing antibody response adjuvant-dependent • Decreased pup mortality and reduced viral load • Magnitude of viral load reduction adjuvant-dependent • Best protection against mortality seen with MPL
Adenovirus-vectored gB vaccine [181]	Hartley	• Single vaccination in early pregnancy • Neutralizing and high-avidity antibody • Reduced vertical transmission • Protection at level of reduced placental spread
Single-cycle, RNA replicon-vectored vaccine (VRP) expressing GP83 [180]	Hartley	• Engineered using replication-deficient alphavirus • Antibody, CD4 and CD8 responses elicited

Vaccine Approach	Guinea Pig Strain	Findings
		• Reduced maternal and pup viral load • Reduced pup mortality and improved weights
MHC class I deletion vaccine [168]	Hartley	• 3DX highly attenuated *in vivo* but not *in vitro* • Vaccination with 3DX produced elevated cytokine levels and higher antibody titers than wild type (WT) virus while avidity and neutralizing titers were similar • Decreased maternal viral loads and pup mortality noted in vaccinated animals following SG virus challenge

Table 4

Effectiveness of Vaccines Against Congenital Infection and Pup Mortality in the GPCMV Model

Vaccine Approach	Pup Infection		Pup Mortality	
	Control	Vaccine	Control	Vaccine
Live, attenuated vaccine 11 serial passages in cell culture [161, 166, 167]	7/26 (27%)	1/25 (4%)	13/26 (50%)	1/25 (4%)
Live, attenuated vaccine 33–34 serial passages in cell culture [167]	6/35 (17%)	0/42 (0%)	8/35 (23%)	6/42 (14%)
Envelope vaccine/Freund's adjuvant [161]	7/26 (27%)	0/16 (0%)	13/26 (50%)	5/16 (31%)
Immunoaffinity purified glycoprotein/Freund's adjuvant [170]	12/23 (52%)	4/22 (18%)	10/23 (43%)	1/22 (5%)
Lectin-purified envelope glycoprotein(s)/Freund's adjuvant [171]	16/20 (80%)	24/54 (44%)	27/48 (57%)	9/63 (14%)
GP83DNA vaccine [175]	20/26 (77%)	17/25 (68%)	13/39 (33%)	13/38 (34%)
gB DNA vaccine [175]	20/26 (77%)	11/27 (41%)	13/39 (33%)	14/41 (34%)
Recombinant gB/Freund's adjuvant (study 1) [178]	5/10 (50%)	8/36 (22%)	31/41 (76%)	6/42 (14%)
Recombinant gB/alum adjuvant [178]	5/10 (50%)	15/32 (47%)	31/41 (76%)	17/49 (35%)
GP83VRP vaccine [180]	11/13 (85%)	8/17 (47%)	12/21 (57%)	4/32 (13%)
BAC DNA vaccine [176]	NA	NA	23/35 (66%)	10/34 (29%)
Recombinant gB/Freund's adjuvant (study 2) [179]	36/55 (65%)	24/33 (72%)	37/57 (65%)	12/33 (36%)
Recombinant gB/MPL adjuvant [179]	36/55 (65%)	34/73 (46%)	37/57 (65%)	12/73 (16%)
MHC class I deletion vaccine[168]	NA	NA	20/46 (43%)	6/27 (22%)
Adenovirus-vectored gB vaccine [181]	9/12 (75%)	2/16 (13%)	NA	NA