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A live guinea pig cytomegalovirus vaccine deleted of three putative immune evasion genes is highly attenuated but remains immunogenic in a vaccine/challenge model of congenital cytomegalovirus infection

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ABSTRACT

Live attenuated vaccines for prevention of congenital cytomegalovirus infections encode numerous immune evasion genes. Their removal could potentially improve vaccine safety and efficacy. To test this hypothesis, three genes encoding MHC class I homologs (presumed NK evasins) were deleted from the guinea pig cytomegalovirus genome and the resulting virus, 3DX, was evaluated as a live attenuated vaccine in the guinea pig congenital infection model. 3DX was 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. Protection, assessed by maternal viral loads and pup mortality following pathogenic viral challenge during pregnancy, was comparable between 3DX and WT and significant compared to naïve animals. These results suggest that the safety and perhaps efficacy of live attenuated human cytomegalovirus vaccines could be enhanced by deletion of viral immunomodulatory genes.

1. Introduction

Human cytomegalovirus (HCMV) is ubiquitous in the human population and is estimated to infect ~60% of women of childbearing age in the developed world. Primary infection during pregnancy results in infection of approximately 30–40% of fetuses [1]. Consequently, in the US approximately 40,000 babies are born infected with HCMV each year and of these 5–18% (up to 18,000/year) display sequelae of infection, such as hearing loss or mental retardation [2–5]. Prior immunity to HCMV provides some protection against congenital infection, reducing the incidence of infection to less than 2% [6,7], and in those infants that are infected, prior immunity reduces disease severity [8].

At present there is no licensed vaccine for prevention of HCMV infections. Protective immunity may require both B and T cell-mediated responses [9–12], which makes live attenuated vaccine strategies attractive insofar as they express antigens that stimulate both the humoral and cellular arms of host immunity. A live vaccine based on the attenuated Towne strain has been extensively tested in humans and has demonstrated efficacy in preventing HCMV disease post-renal transplantation [13–17]. However, the Towne vaccine did not protect mothers from acquiring primary infection from their children who were actively shedding virus [18]. Hence, in this setting Towne appears deficient at eliciting immune responses sufficient to confer protection. Moreover, there remains a theoretical concern that a live attenuated vaccine administered inadvertently to a pregnant woman might itself lead to congenital HCMV infection.

Current live virus vaccine candidates such as Towne encode numerous immune evasion genes that may enhance pathogenesis or impair the vaccine's ability to induce robust host immune responses [19]. That removal of immune evasion genes can promote safety without impairing protection was recently shown by a vaccine/challenge study in which murine cytomegalovirus (MCMV) was rendered avirulent by deletion of 32 immune evasion genes yet induced levels of immunity and protection similar to the parental wild type virus [20].

The ability to evade or impair natural killer (NK) functions may be particularly relevant to congenital infection, as evasion of NK control may be critical for viral dissemination to or transmission across the placenta [21]. Cytomegaloviruses aggressively down-regulate host MHC I from the infected-cell surface [22], and although this would normally result in potent activation of NK

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cells to secrete IFN-γ and to kill the MHC I^{low} infected cells, viral factors termed "NK evasins" attenuate this activation and allow infected cells to escape NK-mediated killing. Moreover, NK evasins presumably diminish the NK-mediated IFN-γ response that occurs at the earliest times of viral infection [23,24]. Hence, removal of NK evasin genes from a live attenuated vaccine might improve safety by restoring effective NK restriction of viral dissemination. At the same time, unopposed activation of NK cells by MHC I^{low} virus-infected cells would increase early IFN-γ levels, which, by enhancing downstream adaptive responses, might ultimately improve vaccine efficacy.

Testing the hypothesis that removal of NK evasin genes will enhance both the efficacy and safety of live attenuated vaccines will initially require *in vivo* studies using animal models. Since HCMV does not replicate in species other than humans, related cytomegaloviruses such as MCMV, rat cytomegalovirus (RCMV), and guinea pig cytomegalovirus (GPCMV) have been used as *in vivo* models. HCMV and MCMV encode proteins that have been shown to impair NK activation *in vitro* [25–30] and/or to play important roles in viral pathogenesis *in vivo* [31,32]. Among these are viral-encoded MHC I homologs that are postulated to block NK activation by providing inhibitory signals to NK cells in a manner similar to that of host MHC I. Unfortunately, MCMV and RCMV cannot be used to evaluate vaccines intended to prevent fetal infection or disease as they do not cross the placenta [33–35]. In this respect, GPCMV, which can cross the placenta [36–38], provides an important model system for testing vaccines or other intervention strategies aimed at preventing congenital cytomegalovirus infection or for elucidating the roles of specific viral factors in congenital transmission and pathogenesis [39–45].

In order to identify potential NK evasins encoded by GPCMV, the sequence of the GPCMV genome [46] was examined for open reading frames (ORFs) that could encode proteins having either sequence or predicted structural homologies to MHC I. Three ORFs designated *gp147*, *gp148*, and *gp149* were identified as encoding potential MHC I homologs, designated gp147, gp148, and gp149, respectively [46]. These ORFs lie adjacent to one another near the right terminus of the GPCMV genome (Fig. 1a).

In this study the importance of gp147, gp148, and gp149 was examined *in vivo*. A recombinant virus, 3DX, deleted of *gp147–gp149*, was found to replicate with wild type kinetics *in vitro* but was essentially avirulent *in vivo*. Even so, immunization with the 3DX vaccine induced potent humoral immunity that protected pups from congenital infection, with efficacy similar to that observed following immunization with parental wild type (WT) virus. These results demonstrate that preconception immunization with a live, highly attenuated CMV vaccine, engineered to delete putative immune modulation genes, is capable of conferring protection against congenital infection and disease in the guinea pig model.

2. Methods

2.1. Animal studies

Hartley guinea pigs were purchased from Harlan Laboratories (Indianapolis, IN) or Elm Hill Laboratories (Chelmsford, MA). All animals were determined to be GPCMV seronegative prior to vaccination by ELISA screening [42]. Animals were housed under conditions approved by the American Association of Accreditation of Laboratory Animal Care, in accordance with Institutional Animal Use Committee approved protocols at the University of Minnesota, Minneapolis.

2.2. Virus and cells

GPCMV strain 22122 (ATCC VR682) was propagated in guinea pig lung fibroblast (GPL) cells (ATCC CCL 158) and maintained in F-12 media supplemented with 10% fetal calf serum (FCS; HyClone Laboratories), 10,000 IU/L penicillin, 10 mg/L streptomycin and 7.5% $NaHCO_3$. BAC-derived viruses were also propagated in GPL cells, maintained in minimum essential media (Gibco-BRL) supplemented as described above.

2.3. Construction of mutant virus 3DX lacking the gp147–gp149 region

Linear recombination was used to delete the *gp147–gp149* region from BAC pN13R10, which contains the GPCMV strain 22122 genome [47]. Two regions of the GPCMV genome, approximately 500 bp upstream and downstream of the *gp147–gp149* gene cluster, were PCR-amplified using primers 3D1F (5′ GTCGGGCGATAACATGTAAGG) (forward, left side) and 3D2R (5′ AGATGCAGTACTGCGGCCGCAACGACAGAGACTATGAGGGA) (reverse, left side) or 3D3F (5′ CGCCGGCGAGTACTGCATCTCATCGAGGACAACTTTTGGGT) (forward, right side) and CIM-R (5′ GCTAGCAAGAATCCTTGAAGAAGAAT) (reverse, right side). The two products were then annealed through homologous non-viral sequences that were added to create a Not I restriction site (underlined in 3D2R and 3D3F) and PCR-amplified using primers 3D1F and CIM-R to generate a 1-kb product comprised of a Not I restriction site flanked by the two regions of viral sequence homology. This product was T/A cloned into pCR®8/GW/TOPO® (Invitrogen) to produce plasmid pGP238. A marker gene cassette encoding kanamycin-resistance and *lacZα* (kan^r/lacZ) was excised from pYD-Tn1721 [48] (kindly provided by Dong Yu) by Not I digestion and ligated into Not I-digested pGP238 to create pGP239. SW102 *E. coli* cells (the kind gift of Søren Warming) [49] containing pN13R10 were induced to express phage λ recombinases by growth at 42 °C for 5 min, then transformed with the PCR product generated by amplification of pGP239 using primers 3D1F and CIM-R. Candidate clones were selected as blue kanamycin-resistant colonies and screened for the predicted HindIII restriction pattern (data not shown). The loss of the *gp147–gp149* sequences (nucleotides 223464–230728 as numbered in: Accession number FJ355434) [46] was confirmed by Southern blot hybridization (Fig. 1b) and PCR (data not shown). One BAC clone was selected and designated pN13R10-3DX.

2.4. Viral reconstitution and growth curve analysis

Infectious viruses were reconstituted by transfection of GPL cells with BAC DNAs as described previously [47]. In both BACs the BAC origin of replication can be excised by co-transfection with plasmid pCre (constructed by Wolfram Brune and kindly provided by Gabriele Hahn). As a green fluorescent protein (GFP) marker gene lies within the excised sequences, properly excised viruses can be isolated by limiting-dilution and screening for lack of GFP expression [47]. Virus 3DX was obtained by co-transfection of pN13R10-3DX with pCre and isolation of a GFP-negative virus, while a GFP-negative wild type (WT) virus was similarly derived from the parental BAC pN13R10. For neutralization studies a GFP-positive 3DX virus (3DX-GFP) was derived by transfection of pN13R10-3DX without pCre. Virion DNAs were prepared as previously described [47] and digested with HindIII and NheI to confirm the expected restriction patterns for WT and 3DX (Fig. 1c) and for 3DX-GFP (not shown). Multi-step growth curves were conducted using an MOI of 0.01 as described previously [47].