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Vectored co-delivery of human cytomegalovirus gH and gL proteins elicits potent complement-independent neutralizing antibodies

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ABSTRACT

Human cytomegalovirus (hCMV) is prevalent worldwide with infection generally being asymptomatic. Nevertheless, hCMV infection can lead to significant morbidity and mortality. Primary infection of seronegative women or reactivation/re-infection of seropositive women during pregnancy can result in transmission to the fetus, leading to severe neurological defects. In addition, hCMV is the most common viral infection in immunosuppressed organ transplant recipients and can produce serious complications. Hence, a safe and effective vaccine to prevent hCMV infection is an unmet medical need.

Neutralizing antibodies to several hCMV glycoproteins, and complexes thereof, have been identified in individuals following hCMV infection. Interestingly, a portion of the CMV-specific neutralizing antibody responses are directed to epitopes found on glycoprotein complexes but not the individual proteins. Using an alphavirus replicon particle (VRP) vaccine platform, we showed that bicistronic VRPs encoding hCMV gH and gL glycoproteins produce gH/gL complexes *in vitro*. Furthermore, mice vaccinated with these gH/gL-expressing VRPs produced broadly cross-reactive complement-independent neutralizing antibodies to hCMV. These neutralizing antibody responses were of higher titer than those elicited in mice vaccinated with monocistronic VRPs encoding gH or gL antigens, and they were substantially more potent than those raised by VRPs encoding gB. These findings underscore the utility of co-delivery of glycoprotein components such as gH and gL for eliciting potent, broadly neutralizing immune responses against hCMV, and indicate that the gH/gL complex represents a potential target for future hCMV vaccine development.

1. Introduction

Human cytomegalovirus (hCMV), a member of the *Herpesviridae* family, establishes life-long, persistent infections with cycles of latency and reactivation within their hosts. Infections are generally asymptomatic except among immunocompromised populations and *in utero* [1]. Passive transfer of antibodies and T cells can reduce the effects of hCMV in immunosuppressed transplant recipients, demonstrating that adaptive immune responses protect against disease [2–5]. The risk of transmission to the fetus during pregnancy, which can lead to severe birth defects [6], is much greater among women who have a primary hCMV infection than those becoming reinfected during pregnancy [6–8], demonstrating that natural immunity can prevent disease. In addition, administration of hyperimmune globulin to pregnant women with a primary hCMV infection can significantly reduce the risk of congenital disease [9]. The congenital disease burden, approximately 4000–8000 cases per year in the United States [10,11], could be lessened with an effective vaccine [12,13].

Envelope glycoproteins represent attractive vaccine candidates since they are expressed on the viral surface and can elicit virus-neutralizing immune responses. These proteins serve critical functions in viral attachment, assembly, and fusion. hCMV requires multiple glycoproteins for these activities [14,15]. Fibroblast entry requires the core herpesvirus glycoproteins gB, gH, gL, gM, and gN [16]. Entry into epithelial and endothelial cells requires an additional five-member complex of gH and gL with UL128, UL130 and UL131 [14,17–19]. hCMV gH is intimately associated with gL [20–23] and is often found in larger complexes, gH/gL/gO and

Abbreviations: hCMV, human cytomegalovirus; VRP, alphavirus replicon particle; VEE, Venezuelan equine encephalitis virus; SIN, Sindbis virus; ATCC, American type culture collection; IU, infectious units.

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gH/gL/UL128-131. The gH/gL/UL128-131 complex mediates entry into epithelial and endothelial cells [18,24] whereas gH/gL+/−gO enables entry into fibroblasts [25,26]. Since gH/gL and larger complexes containing gH/gL mediate cellular tropism and gH/gL is likely a regulator of gB-mediated fusion [20,27–33], the gH/gL complex is a promising candidate for vaccine development.

Studies in fibroblast cultures have demonstrated that sera from hCMV-seropositive individuals have neutralizing activity *in vitro* and that epitopes within gB comprise about 50% of this neutralizing activity [34–37]. Similarly, gH appears to be an important target of antibodies that neutralize infection of cultured fibroblasts [38–40]. A recent study suggested that gH/gL-specific antibodies were more important than gB-specific antibodies for the ability of hCMV-hyperimmune immunoglobulin to block hCMV infection of cultured fibroblasts [41]. Human sera is greater than 100-fold more potent in neutralizing hCMV infections of endothelial cells than of fibroblasts [42] and separate laboratories have subsequently shown that potency correlates with antibodies specific for the gH/gL/UL128-131 complex [38,41,43].

We have developed bicistronic alphavirus replicon particles (VRPs) that express the hCMV gH/gL glycoprotein complex in infected cells. VRPs expressing gH/gL elicited stronger and qualitatively different neutralizing antibodies than those elicited by VRPs encoding gB. The hCMV-neutralizing antibody responses elicited by VRPs expressing gH/gL are complement-independent and broadly cross-neutralizing with primary hCMV isolates. Based on these data, we believe gH/gL warrants consideration for inclusion into hCMV vaccines.

2. Materials and methods

2.1. Cells and viruses

All cells were cultivated in media supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). ARPE-19 cells (ATCC, Manassas, VA, USA) were cultured in DMEM/F-12/10% FBS, MRC-5 cells (ATCC) were cultured in DMEM/10% FBS, and BHKV cells (S. Schlesinger, Washington University, St. Louis, MO, USA) were cultured in DMEM/5% FBS. hCMV virus stocks of TB40GFP [44] (C. Sinzger, University of Tübingen, Tübingen, Germany), VR1814 [45] (G. Gerna, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy), Towne (ATCC), AD169 (ATCC), 8819 (A. Feire, Novartis Institute for Biomedical Research (NIBR), Cambridge, MA, USA), 8822 (A. Feire) were propagated in MRC-5 (TB40GFP, AD169, Towne) or ARPE-19 cells (VR1814, 8819, 8822). Stocks consisted of clarified culture medium harvested 2–3 days after 75–90% of cells showed cytopathic effect.

2.2. Antibodies

Rabbit anti-gH (directed against amino acids 523–538) and anti-gL (directed against amino acids 27–39) were obtained from D. Johnson (OHSU, Portland, OR, USA). Mouse anti-gH antibody was obtained from GenWay Biotech, Inc. (San Diego, CA, USA), mouse anti-Venezuelan equine encephalitis virus (VEE) immune ascites fluid was obtained from R.B. Tesh (UTMB, Galveston, TX, USA), goat anti-mouse IgG-HRP was obtained from Amersham (GE Healthcare Biosciences Corp., Piscataway, NJ, USA), and mouse anti-hCMV IE-1 conjugated with Alexa Fluor 488 was obtained from Millipore (Billerica, MA, USA).

2.3. DNA constructs

Codon-altered genes (GeneArt, Regensburg, Germany) encoding target antigens from the Merlin strain of hCMV were cloned into an alphavirus replicon vector plasmid encoding a chimeric VEE/Sindbis virus replicon (VEE/SIN replicon) [46]. The gB sol gene encoded the first 750 amino acids of gB and the gH sol gene encoded the first 715 amino acids of gH. Bicistronic replicons contain a second subgenomic promoter/gene of interest.

2.4. VRP production

VRPs were prepared using previously described methods [46]. Briefly, linear plasmid DNA templates encoding the SIN capsid, SIN E1/E2 glycoproteins, and a VEE/SIN replicon were transcribed and LiCl-precipitated using the Ambion mMessage mMachine SP6 kit (Life Technologies Corp., Carlsbad, CA, USA). Following quantitation and analyses to confirm RNA integrity, RNAs were co-electroporated into BHKV cells with a GenePulser (Bio-Rad Laboratories, Inc, Hercules, CA, USA) using 2 pulses of 220 V, 1000 μF, with exponential-decay. The cells were diluted with DMEM/10% FBS and cultivated for 24 h and the culture supernatant was collected, clarified and either frozen at −80 °C for subsequent titration and *in vitro* analyses or subjected to purification as described below.

2.5. VRP titrations

Infectious titers of VRPs were determined by infecting BHKV monolayers with serial dilutions of VRPs, followed by overnight incubation, fixation with 50:50 methanol:acetone, and immunostaining the infected cells by incubation with anti-VEE immune ascites fluid, followed by incubation with goat anti-mouse IgG HRP and staining with True Blue Peroxidase Substrate (KPL Inc., Gaithersburg, MD, USA). The positive cells were counted by visual inspection and this value was used to calculate the infectious units (IU) per mL.

2.6. VRP purification for animal studies

VRPs were concentrated by centrifugation into a shallow 20/50% sucrose cushion for 2 h at 153,720 *g*. The sucrose fractions were collected and buffer-exchanged using an Amicon Ultra-15 concentrator (100,000 MW cut-off; Millipore), followed by aliquoting and storage at −80 °C.

2.7. Immunizations

Mice were inoculated two or three times with VRPs administered three weeks apart (10^6 IU/mouse; 5 Balb/C mice/group) in a volume of 50 μL/each quadricep muscle. In cases of co-administration studies, 10^6 IU of each VRP were mixed together and administered as above. Sera were collected three weeks after the final immunization. All studies were approved by the Novartis Institute for Biomedical Research animal care and use committee.

2.8. Neutralization assay

Serum samples were heat-inactivated at 56 °C for 30 min, serially diluted in 2-fold steps (2 replicates per dilution), mixed with an equal volume of hCMV virus diluted to a target concentration of 200–250 infected cells/counting field in media ± 10% guinea pig complement (Cedarlane Labs, Burlington, NC, USA), and incubated for 2 h at 37 °C/5%CO_2. These serum/virus samples were added to ARPE-19 cells or MRC-5 cells prepared in 96-well half-area cell culture plates (Corning Inc., Corning, NY, USA). After a 2-hour infection, the infected monolayers were incubated under 0.75% methylcellulose for 48 hours (± 8 h) at 37 °C/5%CO_2, fixed with 10% buffered formalin (EMD Chemicals Inc., Gibbstown, NJ, USA) for 1 hour, washed three times with PBS/0.05% Tween-20, blocked with PBS/2.5% FBS, 0.5% saponin, 0.1% sodium azide, and incubated with anti-hCMV IE1 conjugated with AlexaFluor 488 (diluted in