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Restoration of viral epithelial tropism improves immunogenicity in rabbits and rhesus macaques for a whole virion vaccine of human cytomegalovirus

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ABSTRACT

Maternal immunity to human cytomegalovirus (HCMV) prior to conception is ~70% protective against congenital transmission and in utero infection of HCMV. Both functional antibodies capable of neutralizing virus and effective T-cells are believed to be important for the protection. Previous HCMV vaccines have rarely been shown able to induce neutralizing antibody titers comparable to those seen in naturally infected HCMV seropositive subjects. Recent studies link a glycoprotein H (gH) complex to receptor-mediated viral entry of endothelial/epithelial cells and leukocytes. This pentameric gH complex, composed of five proteins (gH, gL, UL128, UL130 and UL131 proteins), is notably missing in all HCMV vaccine previously evaluated in clinic. Here we showed that a HCMV virus, with restored expression of the pentameric gH complex, can induce 10-fold higher neutralizing antibody titers than an attenuated AD169 virus or a recombinant glycoprotein B vaccine in multiple animal species in which viral replication is not expected. Encouragingly, the peak neutralizing titers post vaccination in rabbits and monkeys were within 2–4-fold of the levels determined in HCMV seropositive subjects. Functional antibodies by vaccination could further be improved when formulated with a novel adjuvant, and the titers of the antiviral antibodies were sustained in rabbits for over a year after vaccination. These results indicate that the pentameric gH complex is associated with greatly improved functional antibodies following vaccination, and support a vaccine concept based on a nonreplicating whole HCMV with the pentameric gH-associated epithelial tropism restored.

1. Introduction

Congenital infection of human cytomegalovirus (HCMV) is a frequently cited cause of birth defects. Developing a prophylactic vaccine for prevention of congenital infection and disease has been assigned a top priority by Institute of Medicine [1–3]. Early vaccine efforts focused on developing attenuated viruses, AD169 and Towne [4–6]. These viruses, also called laboratory strains, had been adapted for growth to high titers in human fibroblasts, such as MRC-5 cells. However, loss of HCMV tropism to endothelial/epithelial cells is a consequence of sequential passage and adaption in fibroblasts [7]. A glycoprotein H (gH) complex has been linked to viral endothelial/epithelial tropism missing in all laboratory strains, due to various mutations in viral UL131-128 locus [8–10]. The epithelial tropism in AD169 can be restored by genetically repairing its frame-shift mutation in UL131 ORF [10]. Biochemical studies show that this gH complex comprises five proteins, and its configuration and incorporation into mature virions require proper assembly of all three UL128, UL130, and UL131 proteins onto the gH/gL scaffold [9,11]. This complex is termed pentameric gH complex to distinguish it from the trimeric gH/gL/gO complex [12].

The loss of endothelial/epithelial tropism has been speculated a deficiency in attenuated AD169 and Towne vaccines [13,14]. Neutralizing antibodies in sera from seropositive humans have 10–15-fold higher activity against viral epithelial entry than against fibroblast entry [15]. Humans with primary HCMV infection develop neutralizing antibodies against viral endothelial/epithelial entry rapidly but against viral fibroblast entry only gradually [16]. Furthermore, neutralizing activity against viral epithelial/endothelial entry is low in the immune sera from human subjects received Towne vaccine [15]. Moreover, a panel of human mAbs from four donors with HCMV infection has been described, and the potent neutralizing clones recognized antigens of the

Abbreviations: HCMV, human cytomegalovirus; NT50, reciprocal of serum dilution to neutralize 50% of input virions; GMT, geometric mean titer; EC50, effective concentration of IgG to block 50% viral epithelial entry; ORF, open reading frame; gH, glycoprotein H; gB, glycoprotein B; AAHS, amorphous aluminum hydroxylphosphate sulfate; OiW, oil-in-water emulsion adjuvant; MOI, multiplicity of infection.

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pentameric gH complex [17]. A recent publication also demonstrated that the majority of neutralizing activity in sera from HCMV seropositive donors targeted the pentameric gH complex [18]. These observations have established the importance of the pentameric gH complex as a target of potent neutralizing antibodies in seropositive humans, and have suggested its role in HCMV vaccine designs.

Horizontal HCMV transmission is through infection of epithelial cells at the contact mucosal surface. A vaccine aimed at preventing primary HCMV infection should ideally be able to block virus entry into these cells. To validate the role of the pentameric gH complex as a vaccine target, we restored the expression of this pentameric gH complex in AD169 virus. The antigenic composition of the virus with the epithelial tropism, named revertant virus, differs from that of the parental AD169 only by the pentameric gH complex. Here we show that the revertant virus can elicit potent neutralizing antibodies against viral epithelial entry as a nonreplicating whole virion vaccine in rabbits and rhesus macaques. Since HCMV is not expected to replicate in these species, the improvement of neutralizing antibody titers is unlikely dependent on viral replication. The relevance of our observations to the development of an effective vaccine for preventing congenital HCMV infection is discussed.

2. Materials and methods

2.1. Reagents, cells and viruses

ARPE-19 and MRC-5 cells were cultured as previously described [23]. Clone B8.6 ($IgG_{2a}\kappa$) is a mAb specific to gB. Clones 3E3 ($IgG_1\kappa$) and 3C5 ($IgG_1\kappa$) are mAbs specific to UL130 protein [10], provided by Thomas Shenk of Princeton University. AD169 was obtained from ATCC, and propagated in MRC-5 cells [20]. The revertant virus was generated by serial passage adaptation of AD169 in culture as described [13]. ARPE-19 cells seeded in 25 cm^2 flask were infected with AD169 at a multiplicity of infection (MOI) of 10 pfu/cell. The cells were passed every 3–4 days at 1:3 split and were closely monitored for CPE. At passage 4, CPE became prominent. The viruses from the supernatant were subcloned twice by limiting dilutions in ARPE-19 cells seeded in 96-well plates. The revertant virus was propagated in ARPE-19 cells. VR1814 and VR3908 viruses were provided by M. Grazia Revello of Servizio di Virologia, Italy, and were propagated and titered in ARPE-19 cells [21,22].

2.2. Animals

Mice and rabbits were maintained in the animal facility of Merck, West Point, PA, whereas rhesus macaques (*Macaca mulatta*) were maintained at New Iberia Research Center (NIRC), New Iberia, LA. All animal studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by Merck, and NIRC when applied, Institutional Animal Care and Use committee (IACUC). Specific pathogen-free female mice of 4–8 weeks old were purchased from Taconic Farm (Balb/c). Mice were immunized by i.m. injection of both quadriceps in 50 μL volume per site without anesthesia. Female New Zealand White (NZW) rabbits of 3–4 months of ages were purchased from a specific pathogen-free colony at Covance (Denver, PA). The animals were housed individually and were immunized by i.m. injection of quadriceps in 0.5 mL volume without anesthesia. Rhesus monkeys were anesthetized and the vaccines were delivered i.m. in 0.5 mL volume into deltoid muscles.

2.3. Viral neutralization assay

An immunostaining-based HCMV neutralization assay has been described elsewhere [23]. No complement was added in the neutralization assays. Immune sera were heat-inactivated at 56 °C for 30 min prior to the neutralization assay. Immune IgG was purified from rabbit immune sera using immobilized recombinant protein A beads (Repligen, Inc., Waltham, MA) and quantified by optical spectrometry.

2.4. Vaccines and adjuvants

DNA vaccines were prepared as described previously [24]. The sequence corresponding to native gB ectodomain (residue 1–700) was based on AD169 strain (protein ID: CAA35414.1). Full length ORFs for UL128, UL130 and UL131 were based on Merlin strain (protein ID: AAR31451, YP081565 and YP081566) [25]. Recombinant gB protein, expressed in a mammalian expression system (US Patent 5,834,307), was purchased from Sinobiological, Inc. (Beijing, China). Vaccines of AD169 and its epithelial revertant virus were cultured in MRC-5 or ARPE-19, respectively, in multilayer flasks (HYPER*Flask*™, Corning). Virions were purified from the supernatant by ultra centrifugation at 64,000 × *g* at 20 °C through a 20% Sorbitol cushion in PBS. Purified virion pellets were resuspended in Hank's balanced salt solution and stored at −70 °C. All vaccines were formulated in saline, with adjuvant if indicated, on the day of immunization. Amorphous aluminum hydroxylphosphate sulfate (AAHS) in saline was supplied by Merck Manufacturing Division. An oil-in-water (OiW) emulsion similar to MF59 adjuvant in composition was constructed as described in US patent 6299884. ISCOMATRIX™ adjuvant was provided by CSL Ltd. (Victoria, Australia).

2.5. Statistical analysis

All analyses were calculated using Prism® 5 program from GraphPad® (San Diego, CA). The comparison was conducted using unpaired, two-tailed *t*-test. Geometric means and 95% confidence intervals were calculated using algorithms in Prism® 5 program.

3. Results

3.1. HCMV UL130 protein contains important neutralizing epitopes against viral epithelial entry

Balb/c mice were immunized with DNA vaccines encoding the gB ectodomain, or full length of UL128, UL130 or UL131 proteins. NT50 were determined in ARPE-19 epithelial cells (Fig. 1A) or in MRC-5 fibroblast cells (Fig. 1B) [23]. Only the group vaccinated with UL130 DNA vaccine demonstrated elevated titers against viral epithelial entry above those seen in the naïve control. The titers within this group were widely spread ranging 32–2480, and although some animals showed robust antibody responses, as a group, they were not statistically different from the controls because of the wide range of NT50. NT50 of this group against viral fibroblast entry were not notably different from those of the other vaccines. This result suggests that UL130 protein contains important epitopes for antibodies to block viral epithelial entry, but the epitopes may not be consistently presented when delivered as a DNA vaccine or not in the context of the pentameric gH complex.

3.2. Generation of epithelial tropic revertant virus

UL130 protein is a part of the pentameric gH complex, linked to viral tropisms to epithelial/endothelial cells [9,10]. We explored the option of using HCMV with epithelial tropism restored as a