Production of Cytomegalovirus Dense Bodies by Scalable Bioprocess Methods Maintains Immunogenicity and Improves Neutralizing Antibody Titers


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ABSTRACT

With the goal of developing a virus-like particle-based vaccine based on dense bodies (DB) produced by human cytomegalovirus (HCMV) infections, we evaluated scalable culture, isolation, and inactivation methods and applied technically advanced assays to determine the relative purity, composition, and immunogenicity of DB particles. Our results increase our understanding of the benefits and disadvantages of methods to recover immunogenic DB and inactivate contaminating viral particles. Our results indicate that (i) HCMV strain Towne replicates in MRC-5 fibroblasts grown on microcarriers, (ii) DB particles recovered from 2-bromo-5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole riboside (BDCRB)-treated cultures and purified by tangential flow filtration (TFF-DB) or glycerol tartrate gradient sedimentation (GT-DB) constitute 92% or 98%, respectively, of all particles in the final product, (iii) epithelial cell-tropic DB particles are recovered from a single round of coinfection by AD169 and Towne strain viruses, consistent with complementation between the UL130 and UL131A expressed by these strains and restoration of the final product, (iv) equivalent neutralizing antibody titers are induced in mice following immunization with epithelial cell-tropic DB or gH pentamer-deficient DB preparations, (v) UV-inactivated residual virus in GT-DB or TFF-DB preparations retained immunogenicity and induced neutralizing antibody, preventing viral entry into epithelial cells, and (vi) GT-DB and TFF-DB induced cellular immune responses to multiple HCMV peptides. Collectively, this work provides a foundation for future development of DB as an HCMV-based particle vaccine.

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uman cytomegalovirus (HCMV) is an important pathogen that remains a priority for vaccine development to prevent disease affecting immunocompromised individuals as well as populations at risk of transmitting congenital cytomegalovirus disease (1, 2). We and others have demonstrated that noninfectious dense body (DB) preparations are favorable candidates for vaccination (3–7). These preparations benefit from an advajant effect of the particle and a protein composition similar to that of virions and present a reduced risk because they lack viral DNA (vDNA) (3–7). The neutralizing antibodies induced by vaccination are neutralizing in preventing viral entry into susceptible cell types. The neutralizing antibodies in serum from naturally infected individuals target a number of HCMV envelope glycoproteins, including glycoprotein B (gB), gH/gL/gO (gH trimer), gM/gN, and gH/gL/UL128-UL131A (gH pentamer) (8–12). Clinical studies support the utility of an HCMV gB subunit vaccine with MF59 adjuvant, which reduced HCMV acquisition in adolescent girls, in women, and in solid organ transplant patients (13–15). The multiple glycoproteins presented on DB (5, 6) may improve on past vaccine approaches with the gB subunit alone. A class III viral fusogen, gB acts in concert with gH/gL or the gH trimer during entry into cultured fibroblasts, whereas the gH pentamer is necessary for efficient entry into epithelial and endothelial cells as well as some dendritic cells (16–21). In a previous report, we showed that vaccination with a DB preparation induced neutralizing antibody in mice that was capable of preventing infection of both cultured fibroblasts and epithelial cells (7). In addition to their glycoprotein composition, DB carry tegument proteins that induce relevant cellular immune responses. Evaluation of the

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memory T cell compartment of naturally infected, healthy individuals has identified CD4+ and CD8+ T cell responses specific to 151 of the 213 HCMV open reading frames (ORF) and revealed that the responses to specific targets is highly variable among individuals (22, 23). In transplant patients, HCMV-specific cytotoxic CD8+ T cells targeting tegument proteins were effective in reducing HCMV disease and viremia (24, 25). The ability to induce both broad cellular immunity and potent neutralizing antibodies may be necessary for an effective HCMV vaccine. Previously, we established that DB induce cellular responses to multiple proteins (7).

Purification of DB requires separation of the DB from the DNA-containing virions and DNA-free noninfectious particles (NIEPs) that are produced during HCMV infection. Purification by ultracentrifugation employs sequential negative-viscosity, positive density gradients made with glycerol and potassium tartrate (3, 26). Our previous comparison of glycerol tartrate gradient sedimentation-purified DB (GT-DB) and purified, soluble gB with adjuvant MF59 highlighted the advantages of DB (7). Here we focus on alternatives to glycerol tartrate gradient sedimentation purification. We developed a combined process whereby a viral terminase inhibitor is employed during infection to reduce the production of virions and demonstrate that tangential flow filtration (TFF)-purified DB (TFF-DB) are as immunogenic as GT-DB. In addition, we evaluated microcarriers for scalable culture and a coinfection strategy to include gH pentamer glycoproteins in the DB preparations.

MATERIALS AND METHODS

Viruses, cells, and evaluations of infectivity. MRC-5 and ARPE-19 cells and strain Towne, green fluorescent protein (GFP)-expressing Toledo (Toledo-GFP), and VR1814 viruses were cultured as previously reported (7) unless otherwise described. The isolation of Towne and Toledo-GFP from cosmid clones was previously described (27, 28). VR1814 was a gift from Lenore Pereira, University of California, San Francisco, CA. Virus strains AD169 (ATCC) and VR1814 were expanded at a low multiplicity of infection (MOI) on MRC-5 and ARPE-19 cells, respectively. To prepare experimental stocks, infected cell supernatants were clarified by low-speed centrifugation, followed by ultracentrifugation; resuspension of pellets in growth medium supplemented with 0.2 M sucrose, 0.01 M phosphate, 0.005 M glutamate (SPG buffer; HyClone; Thermo Fisher Scientific); and stored at −80°C. Viral titers were determined by infection of MRC-5 or ARPE-19 cells and evaluated directly (Toledo-GFP) or by immunodetection using antibodies CH-160 (Virusys, Taneytown, MD) and goat anti-mouse IgG F(ab’)_2, conjugated to Alexa Fluor 488 (Invitrogen Life Technologies, Grand Island, NY) (AD169, Towne, and VR1814 and virus from coinfections). Infected cell numbers were determined from the fluorescence using an IsoCyte reader (Dynamic Devices, Wilmington, DE).

Recovery and characterization of GT-DB and TFF-DB. Purification of GT-DB and virion fractions from glycerol tartrate gradients was completed as previously reported (3, 7, 26). To recover TFF-DB, MRC-5 cells were infected in tissue culture flasks and treated with 15 μM 2-bromo-5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole riboside (BDCRB; a gift from John Drach). After harvest, 1 volume of 10X SPG buffer was added to 9 volumes of infected cell supernatant, and the stabilized harvest was filtered through a 1.2-μm-pore-size Polygard CN filter (Millipore). The clarified harvest was then loaded onto a TFF cartridge with a 0.2-μm pore-size hollow fiber membrane, concentrated 3-fold by ultrafiltration (3×UF), diafiltered with 6 volumes 1X SPG buffer (6×DF), and then concentrated 1.7-fold by ultrafiltration (1.7×UF). The retentate was centrifuged at 110,000 × g in an SW 32 Ti swinging-bucket rotor for 3 h in a Beckman Optima L-90K ultracentrifuge to concentrate it. Following recovery in phosphate-buffered saline (PBS), the absorbance at 280 nm was used to adjust the concentrations to 400 to 800 μg/ml, prior to flash freezing in a dry ice-methanol bath and storage at −80°C. Impurity levels were assessed by measuring host cell protein (HCP) content, vDNA, host cell DNA (HCD), and residual infectivity by plaque assay. Protein concentrations were confirmed using a bichinchoninic acid protein assay (Pierce) prior to experimental use. UV inactivation was performed with a Spectrolinker cross-linker (model XL1000UV; Spectronics Corporation, Westbury, NY) with ≥275 mJ/cm2 UV energy. Inactivation was determined from infectivity assays completed with MRC-5 cells.

Characterization of HCMV particles. The methods used to evaluate the sizes and compositions of the particles in the GT-DB fractions were described previously (7), and the results were directly compared with those for TFF-DB. The sizes of the GT-DB and TFF-DB, the presence of capsid or DNA, and the integrity of the GT-DB and TFF-DB were compared following transmission electron microscopy (TEM) of samples prepared by vitrification (cryo-TEM; Nanolimaging Services, Inc., San Diego, CA). HCMV particle classifications (i.e., DB, virion, noninfectious particle) were determined from 80 to 300 total particles per sample, and the results were compared to those obtained by TEM following negative staining (JFE Enterprises, Belmont, MA). The particle sizes determined from TEM measurements were compared with those determined by nanoparticle tracking analysis (NTA) (29) to provide data from particles in solution, as previously reported (7). With NTA, the size of individual particles, tracked over time, was determined from diffusion rates. Here, data from five repetitions of 35-s tracking measurements obtained by injection of particles into the sample chamber of a NanoSight LM10 apparatus equipped with a 640-nm red laser and a charge-coupled-device camera were evaluated. As the particles were polydisperse, measurements were taken in the extended dynamic range mode using manual shutter and gain adjustments. Diffusion rates were calculated with the aid of NTA (version 2.1).

The protein compositions of GT-DB and TFF-GB were evaluated by several methods. Identification of gB and pp65 following separation by polyacrylamide gel electrophoresis and immunoblotting utilized monoclonal antibodies to gB (monoclonal antibody CA005-1) and pp65 (monoclonal antibody CA003-1), both from Virusys (Taneytown, MD). The viral proteins gB and pp65 were quantified by gB and pp65 enzyme-linked immunosorbent assays (ELISAs) using methods previously reported (7). Briefly, GT-DB and TFF-DB samples were disrupted with 8 M guanidine hydrochloride and sonication. ELISA plates were coated with serial dilutions of DB samples (starting at 15 μg/well) or protein standards (gB or pp65, starting at 0.4 μg/well and 10 μg/well, respectively). The plates were blocked with Superblock blocking reagent (Thermo Fisher Scientific, Waltham, MA) before addition of primary antibodies (antibody CH28 for gB and antibody 3A12 for pp65; Virusys, Taneytown, MD), subsequent detection with goat anti-mouse IgG (HL) horseradish peroxidase (HRP)-coupled antibodies, and reaction with 3,3′,5,5′−tetramethylbenzidine (TMB) substrate. Data on the absorbance at 450 nm were analyzed using the gB or pp65 protein standard dilution series to produce a standard curve, and Softmax Pro software (Molecular Devices, Sunnyvale, CA) was used to calculate the 50% effective concentration (in micromolars per milliliter). The quantity of HCP in GT-DB and GL-DB was determined from an immunoenzymatic assay (ELISA) specifically developed for detection of MRC-5 cell proteins by the manufacturer (product number F300; Cygnus Technologies).

Detection of residual viral DNA in GT-DB and TFF-DB followed methods previously reported (7). Briefly, DB samples were first reacted with Benzonase (EMD Millipore, Billerica, MA) prior to quantitative PCR (qPCR) in reaction mixtures with Brilliant III Ultra-Fast qPCR master mix (Agilent Technologies, Santa Clara, CA) and HCMV UL54 gene-specific primers and probe. The forward and reverse primers and probe sequences were CCCAGGTGGTTGACTACA (forward primer), GGAAGGTTAGGGTGGC (reverse primer), and 5′−FAM−CCCCGT GGCCGTGTTCGACT−HQ1−3′ (where FAM is 6-carboxyfluorescein
and BHQ-1 is black hole quencher dye 1). Results from DB samples were compared to standard curves generated from HCMV strain AD169 genomic DNA (Advanced Biotechnologies, Columbia, MD) to determine the viral DNA copy number.

Animals and study design. Animal studies were approved and performed in accordance with MedImmune Institutional Animal Care and Use Committee policies. Female BALB/c mice (age, 7 to 8 weeks) from Charles River Laboratories were housed under pathogen-free conditions in the MedImmune animal facility. Immunizations were completed on experimental days 0, 13, or 14, and 27 or 28 with anesthesia by isoflurane followed by intramuscular injection of GT-DB or TTF-DB in a 200-μl volume (100 μl per quadriceps) without the use of adjuvant. In one study, the adjuvant glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE), obtained from Immune Design Corporation (Seattle, WA), was used for evaluation of the particles produced by VR1814 or Towne. For all studies, blood samples were collected retro-orbitally prior to each vaccination and at 14 days after the last immunization, and the sera were stored at −20°C.

Microneutralization assays, antibody characterizations, and immune cell evaluations. Microneutralization assays were completed as previously described (7) and included 1% guinea pig complement (Lonzan, Walkersville, MD) where indicated. Infection of MRC-5 or ARPE-19 cells by Toledo-GFP or VR1814 was evaluated directly (Toledo-GFP) or by immunodetection with CH-160 (Virusys, Taneytown, MD) and goat anti-mouse IgG F(ab’2) conjugated to Alexa Fluor 488 (Invitrogen Life Immunodetection with CH-160 (Virusys, Taneytown, MD) and goat anti-mouse IgG F(ab’2) conjugated to Alexa Fluor 488 (Invitrogen Life Technologies, Grand Island, NY) antibodies (VR1814). Infected cell numbers were determined from the fluorescence using an IsoCyte reader (Dynamic Devices, Wilmington, DE). Neutralizing titers are reported as numbers determined from the fluorescence using an IsoCyte reader (Dynamic Devices, Wilmington, DE). Neutralizing titers are reported as numbers.

FIG 1 Infectious HCMV produced by cultures adaptable to a scalable bioprocess can be controlled by addition of the terminase inhibitor BDCRB as late as 48 h postinfection. (A) Image of MRC-5 cells grown on Cytodex-1 microcarriers under conditions optimized to minimize seed cell number and promote growth to confluence by 4 days. (B) Production of HCMV strain Towne evaluated over multiple days from MRC-5 cells grown on Cytodex-1 microcarriers and infected at a low multiplicity (MOI, 0.1). The time of addition of 30 μM BDCRB (in hours postinfection) is indicated. (C) Viral yield and impact of BDCRB following infection (MOI, 1) of MRC-5 cells grown in conventional tissue culture flasks with addition of 15 μM BDCRB. NA, not applicable.

RESULTS AND DISCUSSION

Evaluation of scalable cell culture methods for DB preparation. To develop a scalable cell culture process, we evaluated HCMV infections of MRC-5 fibroblasts grown on Cytodex microcarriers. Cytodex-1 microcarriers composed of DEAE-dextran were a suitable support substrate for these cells, which became confluent by 4 days postseeding (Fig. 1A). MRC-5 cells failed to grow to confluence when Cytodex-3 microcarriers were employed under the same conditions (data not shown), consistent with the expectation that collagen would not enhance the growth of fibroblasts. Contacts between cells on different Cytodex-1 microcarriers were minimal during the first 3 days of culture (data not shown), and these cultures were infected with Towne virus at a low MOI of 0.1 to evaluate viral yields (Fig. 1B). The titers increased to >10^7 PFU/ml by day 6 postinfection, and the viral concentration was maintained until the end of the evaluation at day 10. HCMV titers from cells in tissue culture flasks also peaked at >10^7 PFU/ml (Fig. 1C). Previously, we used BDCRB, an antiviral agent that prevents genome packaging during HCMV virion maturation (30), to enrich for dense bodies in the infected cell supernatant (7). Here, we evaluated the impact of BDCRB on the infectious virus titer over a concentration range of 15 to 50 μM and by addition of BDCRB at various times postinfection (Fig. 1B and C and data not shown). Addition of 30 μM BDCRB at intervals of 1 to 48 h following infection reduced the virus titer by 3 logs. At 72 h postinfection, BDCRB addition reduced peak viral titers by less than 1 log, while cultures treated at 96 h produced virus titers similar to those for the untreated controls, suggesting that viral spread within the Cytodex-1 cultures was largely completed by 72 h. These results highlight the potential for development of a scalable culture process for HCMV DB production with addition of BDCRB to reduce the infectious virus titer by 3 to 6 logs (Fig. 1B and C and data not shown). For further purification optimization, materials from tissue culture flasks (Fig. 1C) were used for subsequent bioprocess development, as these cultures produced supernatant with the lowest concentrations of virus.

Evaluation of scalable purification methods for HCMV particles. The conventional method for HCMV dense body purification (GT-DB) requires multiple, sequential negative-viscosity, positive density gradients (3, 26) that pose challenges for scale-up
As BDCRB decreased the levels of infectious virions in the culture supernatants (Fig. 1), we next evaluated the composition of particles and contaminating materials using an alternate process to concentrate particles and exchange the medium (Fig. 2 and 3, Table 1, and data not shown). The key differences between the conventional gradient purification process (process A) and a potentially more scalable method for supernatant purification (process B) are summarized in Fig. 2. Multiple lots of infected cell supernatant recovered from cells infected at a low MOI were processed by each method. Then, the morphology and composition of TFF-DB particles recovered by process B were determined by transmission electron microscopy (TEM) and nanoparticle tracking assessment (NTA) (Fig. 3 and Table 1). Three types of HCMV-derived particles are produced naturally during infection: DB that do not contain capsids and two categories of capsid-containing particles, one with viral DNA (virions) and one without viral DNA (noninfectious enveloped particles [NIEPs]) (3). Addition of BDCRB enhanced the production of DB over that of both virions and NIEPs (31). We categorized particles as capsid positive (virions plus NIEPs) or capsid negative (DB) and report the frequencies of each of these categories in the virion (Fig. 3A) and GT-DB (Fig. 3B) fractions or from the TFF-DB process (Fig. 3C) (Table 1). TEM evaluation (Fig. 3A to C) indicated that 92% of the particles in the TFF-DB fraction lacked capsids and ranged in size from 170 to 300 nm (Table 1). Particles in the GT-DB fraction were 99% capsid negative and exhibited a slightly larger particle size range of 250 to 344 nm. As a control, the virion fraction was also analyzed to reveal that 80% of these particles included capsids and the particles in this fraction had an expected smaller average size that ranged from 180 to 215 nm. NTA confirmed the broader size range observed by TEM for TFF-DB particles than for GT-DB particles and the smallest size range for virion fractions (Fig. 3D). The particle size determinations assessed by both methodologies (TEM and NTA) gave similar results, confirming the usefulness of NTA as a rapid and accurate means of assessment of the particle size distribution and its appropriateness for lot-to-lot comparisons.

The protein compositions of multiple lots of GT-DB and TFF-DB were analyzed by several methods (Fig. 3E to I, Tables 1 and 2, and data not shown). Protein lysates were evaluated by their reactivity against a pp65-specific (Fig. 3E) or gB-specific (Fig. 3F) monoclonal antibody by conventional immunoblotting to confirm the presence and molecular weights of the gB and pp65 proteins in the materials. Coomassie staining of the protein lysates from GT-DB and TFF-DB (Fig. 3G) indicated additional protein bands for TFF-GB potentially corresponding to HCMV glycoproteins, tegument proteins, and possibly, host cell proteins. Due to
the complexity of the protein profile and the lack of resolution of one-dimensional (1D) gel electrophoresis and 1D Western blotting, protein lysates from TFF-DB and GT-DB were separated by two-dimensional gel electrophoresis (Fig. 3H and I). Overall, the protein spot diversity for TFF-DB was greater than that for GT-DB (1,000 versus 700 protein spots). Previously, the viral and HCP profiles of GT-DB were evaluated by mass spectrometry (7). Here, these previous identifications supported analyses of the gB and pp65 spot intensities, and the results showed a pp65/gB ratio of 3:1 for TFF-DB versus one of 6:1 for GT-DB. We also observed additional protein spots corresponding to viral proteins and HCP in TFF-DB lysates compared to the number of such protein spots in GT-DB lysates. These increases in HCP content and modified ratios of pp65/gB in TFF-DB were confirmed for multiple lots of TFF-DB and GT-DB particles using an ELISA kit that specifically recognizes proteins from MRC-5 cells (HCP) (Table 2). In addition, there was a trend for an increased vDNA content in TFF-DB preparations, and for two of three lots, the TFF-DB DNA content was higher than the GT-DB DNA content (Table 2). In addition, 8% of particles in TFF-DB contained capsid particles, whereas 1% of particles in GT-DB contained capsid particles (Table 1), even though the residual titers of virus recovered from the different lots of GT-DB and TFF-DB were similar (Table 2). Together, these data suggest that the TFF purification potentially inactivated residual viral particles and/or that the increased amount of capsid particles was due to increased amounts of NIEPs in TFF-DB compared with the amount in GT-DB. Overall, these results indicate the potential of tangential flow filtration to provide a method to enrich and purify DB from large-volume cultures.

**TABLE 1** Summary of particle size and composition of virions, GT-DB, and TFF-DB

<table>
<thead>
<tr>
<th>Particle</th>
<th>Size (range) or size range (nm) determined by a</th>
<th>% b</th>
<th>Capsid d</th>
<th>DB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virion*</td>
<td>180 (100–250) 180–215</td>
<td>80</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>GT-DB</td>
<td>300 (250–450) 250–344</td>
<td>1</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>TFF-DB</td>
<td>260 (150–450) 170–300</td>
<td>8</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>

a NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy.  
b With or without DNA.  
c Virions were purified by process A from infections without BDCRB.  
d GT-DB were purified from 15 μM BDCRB-treated cultures by process A.  
e TFF-DB were purified from 15 μM BDCRB-treated cultures by process B.
and their safety profiles have been established in clinical trials (32–39). Neither strain expresses the gH pentamer due to 1 or 2 nucleotide insertions in UL131A (AD169) or UL130 (Towne), respectively (19, 40). Strategies to rescue these mutations include multiple passages of infected epithelial cells until replicating virus emerges (41–43). Following cloning, an AD169-rescued virus derived by this strategy induced neutralizing antibodies in rabbits and rhesus macaques that blocked HCMV infection of epithelial cells in vitro (41). As an initial step toward production of DB with gH pentamer, we evaluated changes in viral tropism resulting from coinfection of fibroblasts with AD169 and Towne and, subsequently, recovery of virus in the supernatant at 6 days postinfection by centrifugation. MRC-5 cells were infected with AD169, Towne, or mixtures of each of these viruses at starting ratios of 1:1

![FIG 4](https://jvi.asm.org/) Towne, AD169, and VR1814 particles induce similar neutralizing antibody titers. (A) ARPE-19 cell/MRC-5 cell (ARPE/MRC-5) infectivity ratio determined for AD169, Towne, virus produced by coinfection with AD169 and Towne, or low-passage, epithelial cell-tropic strain VR1814. (B and C) Neutralizing antibody (NAb) titers in mouse serum following immunization with 100 μg total particles produced by AD169, Towne, 1:1 mixtures of AD169 and Towne, or coinfections. Total viral particles (virions, noninfectious particles, and dense bodies) were evaluated directly or following inactivation by UV (*). Neutralizing antibody titers were determined from VR1814 infection of MRC-5 fibroblasts (B) or ARPE-19 epithelial cells (C) and are compared to those for Cytogam, which is purified, concentrated human IgG (50 μg/ml IgG). (D and E) Neutralizing antibody titers in mouse serum following immunization with 10 μg total particles produced by VR1814 or Towne and formulated with GLA-SE adjuvant. Neutralizing antibody titers were determined from VR1814 infection of MRC-5 (D) or ARPE-19 (E) cells and are compared to those for Cytogam, as supplied (50 μg/ml IgG). Bars in panels B to D, geometric mean neutralizing titers.

### TABLE 2 Summary of GT-DB and TFF-DB preparations evaluated for immunogenicity

<table>
<thead>
<tr>
<th>Material</th>
<th>No. of particles/μg protein</th>
<th>Amt of residual virus (FFU/μg protein)</th>
<th>No. of vDNA-positive particles/μg protein</th>
<th>pp65/gB ratio</th>
<th>Amt of host cell protein (ng HCP/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT-DB replicate 1</td>
<td>2.5 × 10⁶</td>
<td>3.0 × 10⁵</td>
<td>2.4 × 10⁵</td>
<td>41</td>
<td>0.22</td>
</tr>
<tr>
<td>GT-DB replicate 2</td>
<td>2.8 × 10⁸</td>
<td>1.28 × 10⁷</td>
<td>4.45 × 10⁸</td>
<td>45</td>
<td>0.35</td>
</tr>
<tr>
<td>TFF-DB replicate 1</td>
<td>1.0 × 10⁸</td>
<td>1.5 × 10⁷</td>
<td>5.6 × 10⁷</td>
<td>4</td>
<td>6.63</td>
</tr>
<tr>
<td>TFF-DB replicate 2</td>
<td>ND</td>
<td>2.42 × 10⁷</td>
<td>1.43 × 10⁷</td>
<td>ND</td>
<td>8.12</td>
</tr>
<tr>
<td>TFF-DB replicate 3</td>
<td>1.95 × 10⁸</td>
<td>9.28 × 10⁷</td>
<td>1.46 × 10⁷</td>
<td>15</td>
<td>5.78</td>
</tr>
</tbody>
</table>

* Determined by nanoparticle tracking analysis.

* Assessed using an IF immunofluorescence assay. FFU, focus-forming units.

* The number of particles containing viral DNA per microgram of protein. The DNA copy number was assessed by reverse transcription-qPCR.

* pp65, gB, and HCP concentrations were determined by ELISA.

* ND, not determined.
to 1:4 and multiplicities of 2 (1:1 ratio) to 5 (1:4 ratio). Subsequently, the epithelial cell tropism of released virus was determined by comparison of infectivity on MRC-5 or ARPE-19 cells (Fig. 4A and data not shown). The ratio of 1:1 and the input MOI of 2 produced virus with the greatest increase in epithelial cell tropism (data not shown), and the epithelial cell infectivity was increased by 30- to 60-fold compared with that of AD169 or Towne (Fig. 4A). In comparison with the infectivity of control VR1814 (an epithelial cell-tropic virus) (44), coinfection materials were 7.9-fold less infectious for epithelial cells. These results suggest partial restoration of the gH pentamer on virions by coinfection and a potential method to alter the DB composition.

In mice, induced neutralizing antibody titers were similar whether virus and DB from coinfection or AD169, Towne, or control VR1814 infection were compared (Fig. 4B and C), and for epithelial cells, all neutralizing antibody titers were lower than those in undiluted Cytogam, a purified and concentrated pool of IgG from adults with high titers of HCMV antibodies. The failure to improve neutralizing antibody titers by epithelial cell-tropic virus was unexpected on the basis of published observations from Fu et al. (41). On the other hand, highly variable responses to UL130 and a failure to improve the titers induced by gB alone following coadministration of DNA vaccines have been reported in mice (45). In our experiments, low levels of cell-free epithelial cell-tropic virus may have contributed. In the case of VR1814, the quantities produced necessitated the use of a low dose and adjuvant (GLA-SE) (46, 47), which may also have contributed (Fig. 4D and E). To confirm that gene expression from the HCMV genome did not influence subsequent antibody titers in mice, a portion of the pelleted virus with DB was UV inactivated prior to injection in mice (Fig. 4B and C). In this case, HCMV enters mouse cells and can express IE1; however, no other viral genes are expressed and the virus fails to replicate (48). The neutralizing antibody titers produced were the same before and after UV inactivation, indicating that viral gene expression did not contribute (Fig. 4B and C). Overall, these studies highlight a need to further delineate the properties of antigens that provide elevated neutralizing antibody titers by administration of gH pentamer components with gB and other viral glycoproteins. As described below, we focused the subsequent studies on DB produced by HCMV strain Towne.

Neutralizing activity induced by natural infection. Previously, our work highlighted one feature of the DB immunogen, the induction of a humoral response, including neutralizing antibodies preventing infection of multiple cell types (7). Here, we determined the neutralizing antibody titers of cytomegalovirus (HCMV)-seropositive, human female donors in microneutralization assays targeting VR1814, as it is known that maternal immunity correlates with reduced congenital disease severity (49). Serum samples from a total of 37 female donors representing a racially diverse subset of the American population were evaluated (Table 3). The serum samples came from healthy 20- to 40-year-old female volunteers collected under informed consent at local blood banks and stored at −20°C. Serum samples with gB-binding titers below the assay limit of detection (LOD), equivalent to 4 log2, were designated seronegative. Among these 37 female donors, 12 individuals were HCMV seronegative and 25 were HCMV seropositive. A 67.6% seropositivity rate is typical for women of this age range (20 to 40 years) in the United States (50).

<table>
<thead>
<tr>
<th>Demographic parameter</th>
<th>Result by HCMV serostatus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n = 12)</td>
</tr>
<tr>
<td>Median ± SD age (yr)</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>Age range (yr)</td>
<td>21–35</td>
</tr>
<tr>
<td>No. (%) of patients by race:</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>African-American</td>
<td>2 (16.6)</td>
</tr>
<tr>
<td>Asian</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>Declined to state</td>
<td>2 (16.6)</td>
</tr>
</tbody>
</table>

**TABLE 3** Demographics of female cohort

![FIG 5](http://jvi.asm.org/) Human serum includes predominantly complement-independent neutralization activity preventing HCMV infection of MRC-5 or ARPE-19 cells. Sera (n = 12 seronegative serum samples and n = 25 seropositive serum samples) were heat inactivated and evaluated for VR1814 neutralizing titers with 1% guinea pig complement (1% GC) and without guinea pig complement (no GC). The results for the concentrated IgG product Cytogam are included for comparison, and titers are reported relative to those for the concentrated IgG product (50 μg/ml IgG). For human serum, the results and the mean titer (bars) for individual donors are shown. For Cytogam, results from 5 replicate dilutions are reported. (A) MRC-5 fibroblasts; (B) ARPE-19 epithelial cells. Sero(−), seronegative; Sero(+), seropositive. ∗, P < 0.05.
To determine neutralizing antibody titers, MRC-5 fibroblasts (Fig. 5A) and ARPE-19 epithelial cells (Fig. 5B) were infected with VR1814 following microneutralization in the presence or absence of 1% guinea pig complement. The fibroblast entry assay revealed complement-independent neutralizing titers (mean titer, 6.8 log₂) and increased activity following addition of complement (mean titer, 8.0 log₂), suggesting complement-dependent and -independent neutralizing activity (Fig. 5A) in the serum of naturally infected women. The epithelial cell entry assay indicated that these women had predominantly complement-independent antibodies (mean titers, 10.6 log₂ in the absence of guinea pig complement and 10.3 log₂ in the presence of guinea pig complement) (Fig. 5B). These titers provided a relevant benchmark for further characterization of GT-DB and TFF-DB.

TFF-DB-induced neutralization antibody preventing infection in fibroblasts and epithelial cells. The humoral immune responses for two lots each of Towne-derived GT-DB and TFF-DB (Fig. 2, process B, and Table 2) were compared following immunization of mice (Fig. 6A and B). When the responses were evaluated by VR1814 infection of MRC-5 fibroblasts, TFF-DB-induced complement-dependent neutralizing activity, while addition of complement increased the neutralizing titer by 9.2-fold (Fig. 6A). GT-DB also induced complement-independent neutralizing activity, while addition of complement increased the neutralizing titer by only 1.7-fold. The GT-DB- and TFF-DB-induced neutralizing activity preventing VR1814 infection of ARPE-19 epithelial cells was complement independent (Fig. 6B). Moreover, the increased titers of TFF-DB made the levels more similar to those in naturally seropositive women (Fig. 5B). More specifically, the epithelial cell entry assay indicated that the titers induced by GT-DB were 14.9-fold lower than the titers from seropositive women. However, the titers induced by TFF-DB narrowed this difference to 5.3-fold. In contrast, immunization of mice with gB with MF59 adjuvant failed to induce any detectable neutralizing antibody titer in the epithelial cell infection assay (7). Thus, these results suggest that TFF-DB might improve humoral immunity relative

FIG 6 Neutralizing antibody titers in mouse serum following immunization with GT-DB, TFF-DB, or UV-inactivated TFF-DB. BALB/c mice were immunized with 100 μg of GT-DB and TFF-DB derived from HCMV strain Towne. As a negative control, BALB/c mice were immunized with PBS (closed circles). At 2 weeks after dose 3, neutralization antibody titers in heat-inactivated mouse serum were determined in vitro by neutralization assays. (A and B) In vitro neutralization assays with VR1814 and infection of MRC-5 fibroblasts (A) or ARPE-19 cells (B) in the presence of 1% (final concentration) guinea pig complement (1% GC) or the absence of guinea pig complement (no GC) in mouse serum. Closed squares, GT-DB replicate 1; closed triangles, GT-DB replicate 2; open squares, TFF-DB replicate 1; open triangles, TFF-DB replicate 2. Group means and SDs are shown. *, P < 0.05; **, P < 0.005; ****, P < 0.0001 (Student t test, pairwise comparison). (C) In vitro neutralization assay with Toledo-GFP and infection of MRC-5 cells. The group means and SDs are shown.
to that achieved with gB with MF59 adjuvant. Furthermore, in the neutralization assays with VR1814 infection of MRC-5 fibroblasts, TFF-DB immunizations increased the titers within both complement-independent and -dependent subsets, ranging, on average, 111- to 588-fold higher (Fig. 6A) than those achieved with GT-DB and 119- to 478-fold higher than those in human serum (Fig. 5A). Next, TFF-DB were inactivated by UV prior to inoculation (Fig. 6C). Immunization with 100 μg TFF-DB or UV-inactivated TFF-DB (TFF-DB + UV) induced comparable neutralizing activity, indicating that gene expression from residual infectious viruses was not required to induce the observed neutralizing antibody response. Overall, TFF-DB retained or improved the neutralizing antibody responses induced by immunization with GT-DB.

The gB-specific antibody response is comparable between GT-DB and TFF-DB. TFF-DB were composed of particles with higher gB/pp65 antigen ratios than GT-DB (Table 2). Given this difference and the importance of gB for infection of all cell types, we determined whether the superior neutralization potency correlated with gB-specific antibody titers (Fig. 7). Quantitation of gB-specific antibody titers by ELISA revealed that sera from mice immunized with GT-DB or TFF-DB had similar titers of gB-specific total IgG antibodies (Fig. 7A), corresponding to similar IgG1 and IgG2a antibody titers (Fig. 7B). The titer of the IgG1 isotype was lower than the titer of the IgG2a isotype after GT-DB or TFF-DB (P = 0.04) immunization, suggesting a Th1 bias for both immunogens. These data indicate that the overall magnitude of the gB antibody response induced by TFF-DB does not directly correlate with the increased potency revealed by neutralization of VR1814 infection of fibroblasts. Identification of the antigen-specific factors underlying the differences in antibody functional repertoire requires further investigation.

**TFF-DB induced a robust T cell response.** Cellular immunity is expected to be an important component of a successful HCMV vaccine (51–53). GT-DB induce a broad and robust T cell response (7). To compare the cellular immune profiles obtained following immunization with GT-DB and TFF-DB, we initially determined the specific responses to pp65, an immunodominant T cell antigen (22) (Fig. 8A). Splenic lymphocytes were evaluated in IFN-γ ELISPOT assays with pools of overlapping peptides that span the pp65 protein or with a putative immunodominant major histocompatibility complex (MHC) class I H2-Dd-specific epitope (LGPSGHVL) (54). These assays revealed pp65-specific T cell responses to TFF-DB, with the number of spot-forming cells (SFC) per 10⁶ splenocytes being 203 ± 70 (50-μg dose), 317 ± 109 (100-μg dose), and 296 ± 193 (200-μg dose) after *in vitro* stimulation with the pp65 overlapping peptide pool and 157 ± 57 (50-μg dose), 219 ± 121 (100-μg dose), and 204 ± 107 (200-μg dose) after stimulation with the H2-Dd-specific epitope (Fig. 8A). Combined with previous results for GT-DB (7), this suggests that the TFF-DB and GT-DB immunogens each induced pp65-specific CD8⁺ T cells primarily directed against the LGPSGHVL epitope. Next, the T cell response breadth was determined from pooled splenic lymphocytes stimulated *in vitro* with 17 different HCMV ORF-specific peptide pools using an IFN-γ ELISPOT assay (22). As expected for TFF-DB (7), IFN-γ-secreting cells were identified by stimulation with the IE1, IE2, pp65, UL32, UL48, UL55, UL82, UL94, US24, and US32 peptides (Fig. 8B). In comparison, peptides from ORFs IE1, IE2, pp65, UL32, UL48, UL55, and UL94 also stimulated the IFN-γ-secreting cells induced by TFF-DB, highlighting a broad T cell response for either immunogen. Previously, we found that UV inactivation of the GT-DB immunogen was sufficient to abrogate IE1-specific T cell responses (7), consistent with the need for viral gene expression to produce an immune response to this nonstructural protein. To evaluate the role of gene expression from contaminating virus in TFF-DB, cell-mediated immune responses specific to the IE1 gene were evaluated, using the pp65 gene as a control, to determine differences in responses with and without UV inactivation (Fig. 8C). UV inactivation abrogated induction of IE1-specific T cell responses but did not impact the pp65-specific T cell response. These results are consistent with mouse cell restrictions on HCMV gene expression, except for the expression of immediate/early genes, that limit HCMV replication and prevent gene expression in mice (48). Overall, a slightly smaller breadth of detectable T cell responses was induced by TFF-DB immunizations, which may result from differences between the GT-DB and TFF-DB protein composition and/or protein expression levels, while gene expression-specific responses (IE1 and IE2) indicate that residual infectious virus can easily be inactivated. Recent analysis by Sylvester et al. (23) revealed that T cell target specificity varies greatly between individuals, which suggests that a successful HCMV vaccination strategy should include as many protein targets as possible, making DB an attractive immunogen,

![Fig 7](http://jvi.asm.org/Downloaded_from/doi:10.1128/JVI.03352-16)

**FIG 7** GT-DB and TFF-DB induce comparable gB-specific antibody responses. The titers of HCMV gB-specific total IgG (A) or IgG1 and IgG2a (B) isotypes in BALB/c mouse serum following three inoculations with 100 μg of GT-DB or TFF-DB derived from HCMV strain Towne were determined by ELISA. As a negative control, BALB/c mice were immunized with PBS. The group mean is shown in panel A, and the group mean and SD are shown in panel B. AU, arbitrary units.
of TFF-DB and GT-DB. Several limitations important for the development of HCMV DB particles as vaccine candidates have been addressed with the process and analytic methods, and in combination, the culture and filtration approach eliminates the need for conventional glycerol tartrate gradients. We did not determine the BDCRB levels remaining in TFF-DB but expect that the TFF process would remove BDCRB. We have used BDCRB for initial work, but other viral terminase inhibitors would potentially provide additional benefits, such as increased potency with further reductions in contaminating virus or easier removal, and could be evaluated using the methods established here (55, 56). Importantly, TFF-DB induced cell-mediated immunogenicity comparable to that induced by GT-DB and induced humoral immune responses potentially superior to those induced by GT-DB, demonstrating that introduction of a scalable process did not impair the desirable immunogenic properties of GT-DB and may have improved them. Though they were not explored here, potential explanations for the improved neutralization titers following TFF-DB injections include, among others, the potential of glycerol tartrate to alter DB. In contrast, we found no improvement in neutralizing antibody titer when strain Toledo was used as the target virus (data not shown). These strain-specific results were not explored. We also report that the TFF-DB composition increased the ratio of gB/pp65 compared to that achieved with GT-DB, which may also have impacted the fibroblast assay results. Importantly, the TFF-DB process left intact the antigens required for promoting the neutralizing antibody responses that became apparent using VR1814 and epithelial cell infection. Recently, two other evaluations, the first using a Vaxfectin adjuvant-formulated gB DNA vaccine and the second using a transfection-based enveloped viral particle, have demonstrated the sufficiency of gB in promoting neutralizing antibodies effective at preventing infection of both fibroblasts and epithelial cells (45, 57). We did not evaluate the levels of gH/gL/gO or gM/gN on TFF-DB, leaving unanswered many questions regarding DB immunogenicity determinants. UV inactivation provided evidence that TFF-DB induce neutralizing antibody and T cell responses without the need for viral gene expression or replication. Thus, the immunogenicity of TFF-DB may differ from that of the attenuated virus, allow the delivery of higher antigen doses than the live attenuated approach, and overcome the low immunogenicity observed in Towne virus-immunized vaccinees. Overall, these studies establish that in mice, Towne-derived TFF-DB were able to induce neutralizing antibodies preventing infection of fibroblasts and epithelial cells and also a broad HCMV ORF-specific cell-mediated immune response without the need for an adjuvant or a requirement for the coformulation of multiple viral antigens purified separately. HCMV does not replicate in mice, precluding an evaluation of replicating virus relative to that from the TFF-DB approach. Nonetheless, the safety profile of Towne, established previously in clinical trials, combined with the process and analytical tools presented here and in our previous work (7), establish the feasibility of development of a noninfectious HCMV particle as a vaccine candidate.

FIG 8 Cell-mediated immune responses to HCMV peptides of mouse splenocytes following TFF-DB or UV-inactivated TFF-DB immunization. (A) Responses to a pp65-specific overlapping peptide pool and to a putative immunodominant MHC class I H2-Dd pp65 T cell epitope. Responses were determined 2 weeks following three immunizations with 50, 100, or 200 μg of TFF-DB (TFF-DB replicate 1) and are graphed as the number of SFC per 10^6 splenocytes. As a negative control, BALB/c mice were immunized with PBS. (B) Comparison of the breadth of the immune response between GT-DB (GT-DB replicate 1) and TFF-DB (TFF-DB replicate 1) immunization. Responses were determined in triplicate by an IFN-γ ELISPOT assay with peptides spanning 17 HCMV ORFs. Group means and responses from individual animals are shown in panel A. For panel B, splenocytes from 3 to 5 animals were pooled prior to analysis. (C) BALB/c mice were immunized with 100 μg of TFF-DB or UV-inactivated TFF-DB (TFF-DB + UV) derived from HCMV Towne. At 2 weeks after dose 3, IE1- and pp65-specific responses were assessed in IFN-γ ELISPOT analyses following restimulation of splenocytes with IE1 and pp65 overlapping peptide pools. Responses are graphed as the number of SFC per 1 x 10^6 splenocytes.

especially if they are considered relative to subunit vaccine approaches, which encompass only a few viral protein targets.

In combination, these data provide scalable production methods for HCMV DB particle-based vaccine manufacture and provide relevant evaluations of the composition and immunogenicity of TFF-DB and GT-DB. Several limitations important for the development of HCMV DB particles as vaccine candidates have been addressed with the process and analytic methods, and in combination, the culture and filtration approach eliminates the need for conventional glycerol tartrate gradients. We did not determine the BDCRB levels remaining in TFF-DB but expect that the TFF process would remove BDCRB. We have used BDCRB for initial work, but other viral terminase inhibitors would potentially provide additional benefits, such as increased potency with further reductions in contaminating virus or easier removal, and could be evaluated using the methods established here (55, 56). Importantly, TFF-DB induced cell-mediated immunogenicity comparable to that induced by GT-DB and induced humoral immune responses potentially superior to those induced by GT-DB, demonstrating that introduction of a scalable process did not impair the desirable immunogenic properties of GT-DB and may have improved them. Though they were not explored here, potential explanations for the improved neutralization titers following TFF-DB injections include, among others, the potential of glycerol tartrate to alter DB. In contrast, we found no improvement in neutralizing antibody titer when strain Toledo was used as the target virus (data not shown). These strain-specific results were not explored. We also report that the TFF-DB composition increased the ratio of gB/pp65 compared to that achieved with GT-DB, which may also have impacted the fibroblast assay results. Importantly, the TFF-DB process left intact the antigens required for promoting the neutralizing antibody responses that became apparent using VR1814 and epithelial cell infection. Recently, two other evaluations, the first using a Vaxfectin adjuvant-formulated gB DNA vaccine and the second using a transfection-based enveloped viral particle, have demonstrated the sufficiency of gB in promoting neutralizing antibodies effective at preventing infection of both fibroblasts and epithelial cells (45, 57). We did not evaluate the levels of gH/gL/gO or gM/gN on TFF-DB, leaving unanswered many questions regarding DB immunogenicity determinants. UV inactivation provided evidence that TFF-DB induce neutralizing antibody and T cell responses without the need for viral gene expression or replication. Thus, the immunogenicity of TFF-DB may differ from that of the attenuated virus, allow the delivery of higher antigen doses than the live attenuated approach, and overcome the low immunogenicity observed in Towne virus-immunized vaccinees. Overall, these studies establish that in mice, Towne-derived TFF-DB were able to induce neutralizing antibodies preventing infection of fibroblasts and epithelial cells and also a broad HCMV ORF-specific cell-mediated immune response without the need for an adjuvant or a requirement for the coformulation of multiple viral antigens purified separately. HCMV does not replicate in mice, precluding an evaluation of replicating virus relative to that from the TFF-DB approach. Nonetheless, the safety profile of Towne, established previously in clinical trials, combined with the process and analytical tools presented here and in our previous work (7), establish the feasibility of development of a noninfectious HCMV particle as a vaccine candidate.

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