Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Use of flow cytometry for characterization of human cytomegalovirus vaccine particles

Q1 Josef Vlasak[a], Van M. Hoang[a], Sianny Christanti[a], Richard Peluso[a], Fengsheng Li[b], Timothy D. Culp[a,*]

Q2 [a] Vaccine Bioprocess Research and Development, Merck Research Labs, Merck & Co., Inc., West Point, PA 19486, United States
Q3 [b] Vaccine Discovery, Merck Research Labs, Merck & Co., Inc., West Point, PA 19486, United States

ARTICLE INFO

Article history:
Received 6 October 2015
Received in revised form 9 March 2016
Accepted 19 March 2016
Available online xxx

Keywords:
Cytomegalovirus
HCMV vaccine
Pentameric gH complex
gB glycoprotein
Flow cytometry
Flow virometry

ABSTRACT

Despite a 40-year effort, an effective vaccine against human cytomegalovirus (HCMV) remains an unmet medical need. The discovery of potent neutralizing epitopes on the pentameric gH complex (gH/gL/UL128/130/131) has reenergized HCMV vaccine development. Our whole-virus vaccine candidate, currently in a Phase I clinical trial, is based on the attenuated AD169 strain with restored expression of the pentameric gH complex. Given the complexity of a whole-virus vaccine, improved analytical methods have been developed to better characterize heterogeneous viral particles released from infected cells during vaccine production. Here we show the utility of a commercial flow cytometer for the detection of individual HCMV particles, either *via* light scattering or using fluorescence after labeling of specific antigens. Rapid measurements requiring minimal material provide near real-time information on particle concentration, distributions of different particle types, and product purity. Additionally, utilizing immunoreagents has allowed us to characterize the distribution of key antigens across individual particles and particle types.

1. Introduction

Congenital human cytomegalovirus (HCMV) infection is the leading viral cause of congenital neurological defects and HCMV infections in immunosuppressed individuals can lead to serious complications [1]. Development of an effective vaccine to prevent congenital HCMV is widely recognized as a significant unmet medical need. Early clinical trials with attenuated fibroblast-adapted HCMV strains, Towne and AD169, yielded disappointing results, showing only marginal immune response compared to natural infection (reviewed in [1,2]). More recently, a vaccine based on recombinant gB protein in MF59 adjuvant was evaluated in a Phase II efficacy trial in HCMV seronegative women and was shown to be 50% effective in preventing primary HCMV infection [3]. The same gB-based vaccine also showed an ability to reduce viremia in a Phase II trial with patients receiving solid organ transplants [4].

Over the past decade, the importance of the pentameric gH complex (gH/gL/UL128/130/131) for humoral immunity has become widely recognized. This glycoprotein complex is important for the infection of epithelial and endothelial cells [5] and is the predominant target of the neutralizing activity in human sera with regard to these important cell types [6,7]. In an effort to improve upon immune responses observed following vaccination with either fibroblast-adapted attenuated strains (Towne and AD169) or recombinant gB, we restored the expression of the pentameric gH complex in the attenuated AD169 strain by repairing the frameshift mutation in the UL131-128 locus [8]. This whole-virus vaccine candidate has possible advantages over other vaccine approaches by having the potential to present the full array of native HCMV glycoproteins to drive a humoral response capable of protecting multiple cell types, and by having the potential to elicit a more robust CD8 response than subunit vaccines due to the *de novo* expression of viral antigens. The new vaccine candidate strain is currently being evaluated in Phase I clinical trials.

Despite the potential advantages of an attenuated, whole-virus vaccine for eliciting broad humoral and cellular immunity, developing such a complex vaccine against HCMV presents many analytical challenges. HCMV is a complex virus containing dozens of structural proteins [9,10] and producing multiple particle types [11,12].

Abbreviations: HCMV, human cytomegalovirus; DB, dense bodies; NIEPs, non-infectious enveloped particles; cryo-TEM, cryo transmission electron microscopy; dpi, days post infection; CPE, cytopathic effect; EVs, extracellular vesicles.

Q4 * Corresponding author. Tel.: +1 2156522585.
E-mail address: timothy.culp@merck.com (T.D. Culp).

http://dx.doi.org/10.1016/j.vaccine.2016.03.067
0264-410X/

Infectious virions compose only a small percentage of the total viral particles released from infected cells. These virions are approximately 200 nm in diameter [13] and contain an encapsidated viral genome enclosed by a multi-protein tegument layer surrounded by a viral envelope containing a large number of glycoprotein complexes. In addition to infectious virions, *in vitro* cultures also produce large numbers of dense bodies (DB) – spherical particles of uniform electron density that consist predominantly of the main tegument protein pp65 surrounded by a viral envelope. Dense bodies are non-infectious particles lacking both capsid and viral genome [11] and can be variable in size. Infected cultures also produce a third particle type called non-infectious enveloped particles (NIEPs), which are structurally similar to virions except they lack DNA in the capsid [12]. These three viral particle types (infectious virions, NIEPs and DB) can be separated by density gradient centrifugation [14].

Detection of viruses by flow cytometry was demonstrated over 30 years ago [15]. This technology, however, has not gained widespread use for routine characterization of viral particles due to the lack of sensitivity of most conventional flow cytometers and has been limited to custom-made instruments [15–17]. One instrument that has been used in virology labs is the Virus Counter made by Virocyt (Boulder, CO) that uses proprietary dyes that bind to either proteins or nucleic acid. Potentially infectious viruses are differentiated from other particles because they have both protein and DNA signal. Specific antigens, however, cannot be labeled with the Virus Counter thereby limiting its usefulness for characterizing specific antigen distribution across different particle types. Flow cytometers have had more widespread use in characterizing extracellular vesicles and several reviews have been published [18–20]. The power of flow cytometry for characterization of viral particles was demonstrated in a recent work by Gaudin and Barteneva [17]. Using a customized flow cytometer the authors were able to show a link between virus infectivity and the concentration of viral glycoproteins on the surface of the virus.

Here we demonstrated the utility of an inexpensive, commercially available flow cytometer from Apogee Flow Systems (A50 Micro) to support vaccine development by providing information on multiple attributes of a virus-containing sample. The unique optical design of the Apogee flow cytometer allows detection of particles down to approximately 100–150 nm in diameter by light scattering (depending on the refractive index) and provides exceptional resolution of populations of small particles [20]. Using only light scattering, the A50 Micro was capable of providing near real-time characterization data that accurately predicted a variety of quality attributes including infectious titer, total antigen concentration, and purity. Such data were invaluable for monitoring infected cultures in lieu of labor or time-intensive methods that are not practical for real-time process monitoring. Furthermore, combining immunochemistry with the A50 Micro flow cytometer allowed for characterization of the distribution of two key antigens (pentameric gH complex and gB) across the various particle types independent of labor-intensive separation techniques.

2. Materials and methods

2.1. HCMV virus

The vaccine virus was produced in ARPE-19 cells, a spontaneously immortalized cell line of human retinal pigment epithelium. ARPE-19 cells were grown on plastic culture wares containing proprietary growth media at 37 °C and 5% CO_2. The expanded cells were subsequently planted into final production vessels for infection and production. Samples of cell culture supernatants were taken from the production vessels during the virus production phase to determine virus titer and particle composition. Data shown represent different culture conditions used during production.

The expression level of the pentameric gH complex on the vaccine strain was compared to the expression level on low-passage HCMV grown from virus isolated from infected individuals. These "clinical isolates" are expected to closely represent wild type virus present in the population. Isolates VHL/E, VR3908, VR7863, VR5235, and UXCA were first adapted for culture in ARPE-19 cells for one to three months in DMEM/F12, pen/strep, 10% FBS complete medium. The HCMV strains were then expanded in 500 mL HyperFlasks for about 15–21 days until 70–95% of the cells showed signs of a cytopathic effect (CPE). Culture supernatants were harvested and the virus was purified by centrifugation.

2.2. Glycerol-tartrate centrifugation and fraction collection

The gradient and centrifugation conditions followed the original work of Talbot and Almeida [14]. Briefly, linear gradient ranging from 30% (w/w) glycerol + 15% (w/w) tartrate (top) to 35% (w/w) tartrate (bottom) was prepared in a gradient maker. The sample was layered on top of the gradient and spun for 15 min at 40,000 rpm in a Beckman SW40 rotor (Beckman Coulter, Indianapolis, IN). A different, shallower gradient was used for fractionation because it improved the separation of virions and dense bodies – 30% (w/w) glycerol to 20% (w/w) tartrate, spin for 30 min at 20,000 rpm in Beckman SW40 rotor. After separation individual bands were removed from the gradient, mixed with TN buffer (50 mM Tris, 100 mM NaCl, pH 7.4), and pelleted for 1 h at 20,000 rpm in a Beckman SW40 rotor. The pellet was resuspended in a proprietary formulation.

2.3. Disc centrifuge

The Disc centrifuge (CPS Instruments, Prairieville, LA) was operated at maximum speed (24,000 rpm). Linear gradient from 5% (w/w) tartrate + 15% (w/w) glycerol to 15% (w/w) tartrate was established in the instrument by adding 11 incremental steps of the gradient and allowing the gradient to smooth out by diffusion for approximately 30 min. PVC standard beads (CPS Instruments, Prairieville, LA), 377 nm in diameter, were used to calibrate the instrument. 200 μL of appropriately diluted samples were introduced during the measurement. The CPSV95 software (CPS Instruments, Prairieville, LA) calculates particle size from the sedimentation velocity and converts changes in laser intensity caused by passing particles to a mass distribution using Mie theory. Sedimentation velocity depends on particle size, particle density, gradient density, and gradient viscosity. Since we did not know these parameters exactly we have set them up empirically in the software so that the virion peak would have a diameter of 200 nm, an average size for HCMV virions [13]. This approach permitted us to obtain the general size range of HCMV particles in the sample. As reported in the literature [12], the apparent size difference between virions and NIEPs, as measured by Disc centrifuge, is a result of different density and not the actual size.

2.4. Cryo transmission electron microscopy (cryo-TEM)

Electron microscopy was performed using an FEI Tecnai T12 electron microscope, operating at 120 keV equipped with an FEI Eagle 4k × 4k CCD camera. Before analysis the samples were dialyzed into TN buffer with 0.05% Poloxamer 188. 3 μL drop of the sample was applied on a cleaned grid, blotted away with filter paper, and immediately vitrified in liquid ethane. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below −170 °C.

Please cite this article in press as: Vlasak J, et al. Use of flow cytometry for characterization of human cytomegalovirus vaccine particles. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.03.067