Pall SoloHill® Animal Protein-Free Microcarriers for Dengue Virus Production
Introduction

Microcarrier cultures have a long and successful record for use in the large-scale production of viruses using anchorage-dependent mammalian cells. They have been used in the animal vaccine market for decades as a cost-effective and robust platform. These successes have resulted in an increasing use of microcarrier culture for the production of human vaccines, which is rapidly moving toward animal component-free materials and media. Pall Life Sciences offers a variety of regulatory-friendly animal protein-free (APF) microcarriers which can be used as attachment substrates for adherent cell types in serum-free vaccine production platforms. Here, the compatibility and utility of Pall APF microcarriers in both serum-containing and serum-free cell culture media (SFM) for efficient Vero cell growth and production of dengue virus is demonstrated.

Materials and Methods

Cells, Virus and Media
Vero African green monkey kidney cells from American Type Culture Collection (ATCC;CCL-81; P124) were expanded directly into SFM for four passages prior to use. Wild-type dengue viruses (DEN-2 16681 and attenuated DEN2 IC/VV45R vaccine strains) were received from Center of Disease Control (CDC) and used for inoculation of cultures. Three media were employed in these studies: an optimized SFM formulation from InVitria designated as IM 1, a commercially-available SFM medium; and Gibco* Dulbecco’s Modified Eagle Medium (DMEM; Thermo Fisher Scientific; Cat#12100-046) containing 10% HyClone® fetal bovine serum (FBS) (GE Healthcare Cat# SH30071.03).

Microcarrier, Spinner and Bioreactors
Four types of Pall SoloHill microcarriers (Hillex®II, Plastic, Plastic Plus and ProNectin®F) were used at 5 and 10 cm²/mL microcarrier densities in spinner cultures, and 10 and 13 cm²/mL microcarrier densities in bioreactors. Corning 250 mL (Corning: # 4500-250) reusable glass spinner flasks were used to assess Vero cell growth and Dengue virus production. Processes were transferred to 2 L stirred-tank bioreactor systems.

Harvest enzymes
Cells were harvested with trypsin (Thermo Fisher Scientific, Cat# 15050-065) or recombinant trypsin (Novozymes, Cat# (EC 3.4.21.4).

Spinner culture
Spinner flasks were incubated in humidified incubators at 37 °C, 5% CO₂, and agitation was maintained at 40 rpm and 65 rpm for the non-Hillex II and Hillex II, respectively. Spinner cultures were carried out without medium exchange (unless specified in text) but were supplemented with glucose as needed. A GlucCell® glucose meter (CESCO Bioengineering; Cat# CLS-1322-02) was used to measure glucose concentrations and glucose (Sigma Cat# G8769) was supplemented up to 1.5 g/L when the concentration reached ≤1 g/L. Immobilized cell density on microcarriers was assessed via cell lysis and counting of nuclei.

Bioreactor Culture
Cells expanded in spinner cultures were used to seed 2 L bioreactors. Agitation was initiated at 35 rpm and 60 rpm for non-Hillex II and Hillex II, respectively. Bioreactor cultures were seeded at 2 X 10⁴ cells/cm² or 4 X 10⁴ cells/cm². Temperature was maintained at 37 °C. Bioreactors were equipped with pitched-blade impellers for agitation and 40 µm spin filters for medium removal. Bioreactor cultures were carried out without medium exchange but were supplemented with glucose when the concentration reached less than ≤1 g/L.
Virus infection
Virus infection was carried out when cells reached ~90% cell confluence (~14 × 10⁴ cells/cm²), after 2 to 4 days of cell culture. In spinners, pH of the media was daily adjusted to 7.2 to 7.4 with 1 NaOH during the virus production phase. Dissolved oxygen (DO) in the bioreactors was maintained at 50% air saturation and pH at 7.4 via using a hollow fiber membrane external gas exchange method. Glucose was measured daily and supplemented up to 1.5 g/L (in spinners) and 2 g/L (in bioreactors) when concentrations were ≤1 g/L. Daily 5 mL samples were retrieved from spinners and bioreactors to monitor cell density on microcarriers, access cytopathic effect (CPE) after virus inoculation, and for plaque assays to measure virus infectivity (PFU/mL) up to 12 days post infection.

Results
Cells were harvested from flatware for seeding microcarrier spinner cultures (5 and 10 cm²/mL). Seeding densities were set to optimize attachment of cells to microcarriers ensuring that each microcarrier was populated with cells. In these studies, cells were seeded at a density of 2 or 4 × 10⁴ cells/cm² in an attempt to maximize utilization of the entire surface area within the culture and to obtain uniform attachment at seeding. This cell density maximizes surface area (SA) utilization by ensuring that all microcarriers in suspension are populated with cells and that cells can reach confluence simultaneously, as well as allows for attachment of a predicted cell number on each microcarrier (approximately 16 cells/microcarrier). Figure 1 demonstrates the effect of increasing microcarrier concentration on attachment efficiency in IM 1 SFM. Cell distribution was slightly more uniform, and the number of cells per microcarrier peaked, at the predicted cell number of 10 to 20 cells per microcarrier at the higher microcarrier concentrations. Therefore, a microcarrier concentration of 10 cm²/mL was used to assess cell growth in the next round of spinner experiments.

Figure 1
Vero cell distribution on plastic microcarriers on day 1 in IM 1 SFM. The red data points and arrows in the graphs represent the theoretical number of cells per microcarrier expected when a 2 × 10⁴ cells/cm² seeding density is used. Shaded area depicts expected range of microcarriers occupied.
Cell growth in 2 different SFM was evaluated using four Pall SoloHill APF microcarrier types in spinner cultures (Figure 2). Cell densities on all four microcarrier types tested were equivalent to or greater than densities reached in serum-containing medium (data not shown).

**Figure 2**
*Cell growth on APF microcarrier types in spinners at 10 cm²/mL using different SFM formulations.*

Given the excellent growth in spinner cultures achieved, spinners were used as the expansion platform to harvest cells for seeding 2 L bioreactor cultures. Cell growth on Plastic, Hillex II and ProNectin F microcarriers at a microcarrier concentration of 13 cm²/mL in bioreactors was monitored for up to 9 days after seeding (Figure 3). Cell numbers in these glucose-supplemented cultures reached 2.5 to 3.5 million cells/mL. Maintenance of glucose above 1 g/L was sufficient to achieve excellent cell growth with these three SFM formulations with all microcarrier types tested.

**Figure 3**
*Cell growth on APF microcarrier types in bioreactor cultures containing IM1 SFM. Black arrows represent glucose feed to the culture.*
A mock-infection study was then performed in a bioreactor containing IM 1 medium to evaluate cell behavior under conditions that would be used for viral production in bioreactors (Figure 4). Ten days post culture initiation, cell density increased from a maximum of 3.5 million cells/mL to 6 million cells/mL, likely due to the greater level of control of environmental parameters during culture.

**Figure 4**
Mock infection cell growth curve of the bioreactor culture using 13 cm$^2$/mL ProNectinF microcarrier in IM 1 SFM. Black arrows represent glucose feed to the culture and day 4 medium exchange is indicated by dotted line.

Before examining virus expression at the bioreactor scale, virus production in spinners (Figure 5) was characterized and compared to those achieved in flatware cultures by retrieving samples daily and measuring viral titer in the spent medium. Active wild-type dengue virus was first detected in the medium from microcarrier cultures on day 3 post-infection, and levels peaked on day 4. Viral expression was maintained for 5 additional days but titers were lower at later time points. This could be due to a larger CPE effect earlier in the culture and an associated higher viral production level. In both cases, virus expression began earlier and maximum levels reached were greater than those obtained in flatware controls.

**Figure 5**
Wild-type dengue virus production (WT DEN2 16681) in 10 cm$^2$/mL microcarrier spinners and T-flask IM 1 cultures.
To efficiently generate large amounts of dengue virus, the process must transition smoothly to larger scales. To demonstrate this, dengue virus production was evaluated at a microcarrier concentration of 13 cm$^2$/mL in 1.5 L bioreactor cultures with several microcarrier types and in three types of cell culture medium (Figure 6). All three microcarrier types tested performed equally well when virus expression in IM 1 medium was quantified. Measurable virus expression was detected as early as day 1 post-infection and levels increased to 6.5 to 7.5 Log$_{10}$ PFU/mL per day at later time points (day 4 through 10). Virus was detected throughout the 10 day culture period without medium exchange. The kinetics of virus expression on ProNectin F microcarriers was similar in both serum-free media and maximal level of expression peaked between day 4 and 6 post-infection. Virus expression in serum-containing DMEM was slower than that found in SFM formulations, and peak levels were not reached until day 8.

Figure 6
Wild-type dengue virus (DEN2 16681) production in different medium formulations and on different microcarrier types in IM 1 SFM. Expression was evaluated in 13 cm$^2$/mL bioreactor cultures.

ProNectin F microcarriers were next used in spinner cultures to evaluate expression of an attenuated vaccine strain dengue virus in both the IM 1 SFM and the commercial medium (Figure 7). Virus expression was detectable in both media on day 2 post-infection; expression peaked on day 6 and was maintained at or near peak levels for the remainder of the culture period, demonstrating that ProNectin F microcarriers paired with SFM formulations support high viral expression over a 12 day period.
Conclusions

Pall SoloHill APF microcarriers can be used to expand and harvest Vero cells in SFM and DMEM with 10% FBS. Both media support excellent viral production with Pall SoloHill microcarriers in stirred-tank cultures. The data presented show that wild-type and attenuated dengue viruses were expressed three days earlier and with higher overall titers in microcarrier cultures when compare to flatware. Vero cells achieve densities of >3 million cells/mL without a medium exchange in batch culture, and maintained cell growth for 8 to 9 days. In mock infections, IM 1 medium enables cell densities of up to 6 million cells/mL in bioreactors. This platform and the work described here facilitate utilization of APF conditions and microcarrier cultures for virus production that require little effort toward process optimization. These studies provide a platform for evaluation of expression of other viruses which require Vero or other adherent mammalian cell lines used for the production of human vaccines.

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