SERUM-FREE MEDIA FOR PROPAGATION OF DENGUE TYPE 2 VIRUS IN VERO CELLS

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Abstract. Dengue is a widely distributed epidemic disease caused by dengue virus, of which there are four serotypes. Development of a tetravalent live-attenuated vaccine has an inherent problem arising from a risk of developing severe dengue following infection of vaccinated individuals displaying seronegativity to the dengue virus, and thus, an alternative vaccination strategy, such as an inactivated virus vaccine, is preferred. As a means towards realizing the latter goal, effect of serum-free (SF) media on growth of dengue virus in Vero cells was investigated by comparing Dengue type 2 virus (DENV-2) yield and protein expression in Vero cells cultured in five types of SF media, namely, BMPro, OptiPro, ProVero, Stem-α, and VP-SFM, and in minimal essential medium supplemented with 2% fetal bovine serum (MEM-2%FBS). BMPro, OptiPro and ProVero sustained higher dengue virus titers than Stem-α, VP-SFM or MEM-2%FBS. No mutations in E gene nucleotide sequences of DENV-2 propagated in OptiPro, VP-SFM or MEM-2%FBS. DENV-2 E protein in Vero cells cultured in OptiPro and ProVero was expressed in higher amounts compared to that in cells cultured in VP-SFM. Taken together, these findings suggest among the five SF media tested, BMPro, OptiPro and ProVero supported more efficient growth of DENV-2. SF media are advantageous in propagating viruses for development of an inactivated dengue virus vaccine.

Keywords: dengue virus, inactivated viral vaccine, serum-free media, Vero cell

INTRODUCTION

Dengue virus (DENV), a member of family *Flaviviridae*, is an enveloped, single-stranded RNA virus, categorized into four serotypes (DENV-1 to DENV-4) based on differences in virus envelope (E) protein sequence (Lindenbach *et al*, 2013; Pierson and Diamond, 2013). Dengue is caused by DENV transmitted to humans via bites of infected mosquitoes primarily in sub- and tropical regions. Dengue has spread across 128 countries (WHO, 2018), and as many as 390 million people become infected annually (Bhatt *et al*, 2013).

In December 2015, the first dengue vaccine (CYD-TDV, a live-attenuated and chimeric tetravalent vaccine) received

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license based on three trials carried out in Latin America and Asia-Pacific region (Sabchareon *et al*, 2012; Capeding *et al*, 2014; Villar *et al*, 2015; Sanofi Pasteur, 2018). However, the major drawback of CYD-TDV that it is not recommended in individuals without prior infection because previously unexposed individuals are at a higher risk of developing severe dengue following vaccination (Sridhar *et al*, 2018). Therefore, there is considerable scope for developing improved dengue vaccines. A potential alternative is the production of an inactivated vaccine containing the four virus serotypes, which is less likely to exhibit interference among serotypes (Fernandez *et al*, 2015).

African green monkey *Cercopithecus aethiops* kidney epithelial Vero cell line is widely used for virus propagation in development of inactivated vaccines (Barrett *et al*, 2009) and for production and propagation of an original recombinant virus used to develop CYD-TDV (Guirakhoo *et al*, 2000; Guirakhoo *et al*, 2001; Guirakhoo *et al*, 2004). Conditions for DENV propagation have recently been extensively investigated using Vero cells cultured in VP-SFM, a representative serum-free (SF) medium (Abbate *et al*, 2019). However, despite accumulating evidence, the effects of different SF media on DENV growth remain unclear. In the present study, Vero cells cultured in seven types of SF media were examined for growth and protein expression of DENV-2 E protein to identify the optimal SF media type(s) for virus propagation.

MATERIALS AND METHODS

Cells and DENV maintenance

Vero cells (ATCC CCL-81; Sumitomo Dainippon Pharma, Osaka, Japan) were maintained in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) (MEM-10%FBS) at 37°C. Prior to experiments, aliquots of cells were cultured for at least six days at 37°C in MEM MEM-2%FBS or in the following SF media: BMPro-V Medium (BMPro) (Cell Science and Technology Institute Inc, Sendai, Japan), COSMEDIUM 013 (Cosmedium) (Cosmo Bio Co Ltd, Tokyo, Japan), EX-CELL VPRO (Ex-Cell) (SAFC Biosciences Inc, Lenexa, KS), OptiPRO™ SFM (OptiPro) (Invitrogen, Carlsbad, CA), ProVero™ 1 Serum-free Medium (ProVero) (Lonza, Basel, Switzerland), STEMα-VERO (Stem-α) (Stem Alpha, Saint-Genis l'Argentière, France), and VP-SFM (Invitrogen, Carlsbad, CA). Dengue type 2 virus (DENV-2) strain D2/Hu/INDIA/09-74 (GenBank/DDBJ accession no. LC367234), kindly provided by Dr Tomohiko Takasaki (National Institute of Infectious Diseases, Tokyo, Japan), was propagated in Vero cells for seven days at 37°C and used as virus stock.

Cell counting

Vero cells cultured in 12-well plates (Thermo Fisher Scientific, Waltham, MA) were trypsinized, harvested and counted using a trypan blue-exclusion method (King *et al*, 1959) employing a TC10™ automated cell counter (Bio-Rad, Hercules, CA).

Plaque titration

Vero cells maintained in MEM-10%FBS were seeded in 12-well plates (Thermo Fisher Scientific, Waltham, MA) (1×10^5 cells/well) and incubated for 24 hours at 37°C. Then cells were infected with aliquots of 10-fold serial dilutions of virus samples (100 μl/well), incubated for 1 hour, followed by addition of one ml aliquot of MEM-2% FBS containing

1% methylcellulose and incubated for a further five days prior to fixing with methanol and staining with crystal violet.

Virus infection

Vero cells (5×10^5 cells) maintained in SF media or MEM-2%FBS were seeded in a well of a 12-well plate (Thermo Fisher Scientific, Waltham, MA), incubated with respective media for 24 hours at 37°C. Then cells were washed with PBS once, and infected with DENV-2 (1 PFU/100 μl MEM/well) for one hour at 37°C. After removal of the virus, cells were incubated with respective media for seven days at 37°C. The percent efficiency of attachment was determined using the following formula:

Percent efficiency of attachment

$$= \frac{\text{PFU used for infection} - \text{PFU remaining in supernatant}}{\text{PFU used for infection}} \times 100$$

where PFU is referred to plaque forming unit, representing the number of infectious particles in the sample and is calculated based on the assumption that each plaque formed is representative of one infectious virus particle.

Western blot analysis

At day 5 post-infection, infected Vero cells were harvested, lysed in 500 μl of buffer (25 mM Tris-HCl pH 7.6 containing 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) on ice for 15 minutes, centrifuged at 20,000 *g* for 15 minutes at 4°C, and protein concentration in supernatant was determined using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Lysate (30 μg protein) was subjected to 10% SDS-PAGE, followed by western blot analysis using an anti-DENV-2 E primary polyclonal antibody (Thermo Fisher Scientific, Waltham, MA) and a goat HRP-conjugated secondary anti-rabbit IgG (GE Healthcare, Little Chalfont, UK), and immunoreactive band was detected using a SuperSignal® West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA), documented with a ChemiDox XRS Plus system (Bio-Rad, Hercules, CA) and quantified with a ImageJ software (https://imagej.nih.gov/ij/).

Nucleotide sequencing

Virus RNA was extracted using RNeasy Mini kit (Invitrogen, Carlsbad, CA) from DENV-2 stock and DENV-2 grown in OptiPro, VP-SFM and MEM-2%FBS for seven days. RNA was reverse transcribed using a ReverTra Ace kit (TOYOBO, Osaka, Japan), followed by PCR amplification of E gene using a primer pair as previously described (Deval *et al*, 2020) and amplicon (1,485 bp) was sequenced (Fasmac Co Ltd, Kanagawa, Japan). (Protocols and primer sequences will be provided upon request to corresponding author).

Statistical analysis

Multiple-group differences were analyzed using a one-way analysis of variance (ANOVA). Tukey's test was performed as a post-hoc test of ANOVA to determine statistical differences between groups, with p-value <0.050 considered significant.

RESULTS

Growth of Vero cells in SF media

Vero cells (5×10^5 cells) were incubated in BMPro, Cosmedium, Ex-Cell, OptiPro, ProVero, Stem-α, VP-SFM, or MEM-2%FBS for eight days at 37°C and cells counted on days 1, 2, 4, 6, and 8 days. Except for Cosmedium and Ex-Cell media (data not shown), Vero cells were able to grow in the remaining five SF media (BMPro, OptiPro, ProVero, Stem-α, and VP-SFM) as well as in MEM-2%FBS, but at varying rates and optimal growth (Fig 1). As there are no statistically significant differences in the numbers of cells determined on day 1 of culture, cells on this day were harvested for use in subsequent experiments.

Vero cell attachment by DENV-2

Attachment constitutes the initial step in a virus life cycle. Vero cells (5×10^5) harvested as described above were treated in the five SF media and MEM-10%FBS with DENV-2 at a multiplicity of infection (MOI) of 0.1 and 1.0 for one hour at 37°C, then the numbers of viruses remaining in supernatant were determined via a plaque assay, and percent efficiency of attachment was determined using the formula previously described in MATERIALS AND METHODS. At an MOI of 0.1 and 1, there are no respective significant differences in efficiency (± SD, n = 3) of attachment among BMPro (76.5 ± 9.3 and 92.0 ± 4.1%), OptiPro (79.6 ± 3.1 and 93.5 ± 2.8%), ProVero (78.2 ± 4.4 and 91.7 ± 6.1%), Stem-α (80.6 ± 9.2 and 91.3 ± 2.6%), VP-SFM (79.0 ± 9.4 and 94.1 ± 1.5%), and MEM-10%FBS (82.5 ± 8.5 and 92.3 ± 3.2%) (data not shown).

Virus propagation in Vero cells cultured in SF media

Vero cells (5×10^5 cells) harvested as described above were treated with DENV-2 (1 PFU) for 1 hour and incubated in the five SF media and MEM-2%FBS for

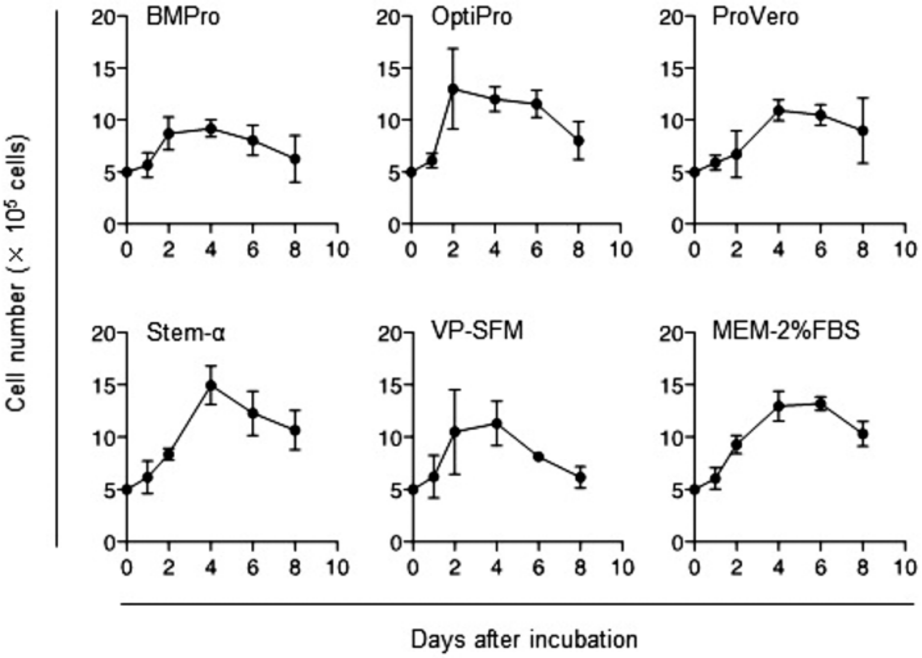

Fig 1-Growth of Vero cells in serum-free and minimal essential (MEM) media.

Vero cells (5×10^5 cells) were cultured in BMPro, OptiPro, ProVero, Stem-α, VP-SFM, and MEM supplemented with 2% fetal bovine serum (MEM-2%FBS) media at 37°C for 8 days. Cells were counted using a dye exclusion method. Data from three independent experiments are presented as mean ± standard deviation.

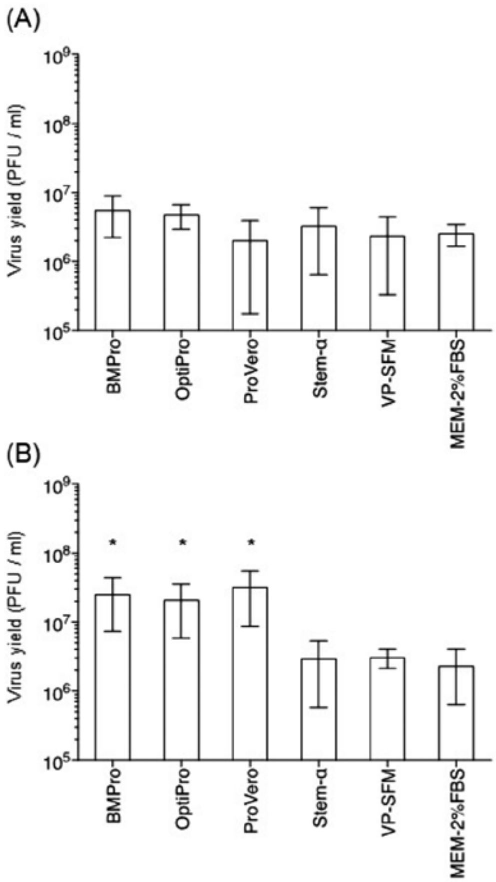

Fig 2-Growth of dengue virus serotype 2 (DENV-2) in Vero cells cultured in serum-free and minimal essential (MEM) media.

Vero cells (5×10^5 cells), cultured in BMPro, OptiPro, ProVero, Stem-α, VP-SFM, and MEM supplemented with 2% fetal bovine serum media (MEM-2%FBS) for one day at 37°C, were infected with DENV-2 (1 PFU) and cultured for a further seven days. Virus amounts in culture supernatants were determined using a plaque assay on day 5 (A) and day 7 (B) post-infection. Data of three independent experiments are presented as mean ± standard deviation.

*p-value <0.050 compared to cells cultured in MEM-2%FBS, ANOVA followed by Tukey's multiple comparisons test for post-hoc analysis; PFU: plaque forming unit.

seven days at 37°C. Infectious viruses in culture supernatant were determined using a plaque assay. At day 5 post-infection (5 dpi), no statistical differences were observed in virus numbers in supernatants among the six media (Fig 2A). At 7 dpi, virus yields in supernatant of cells from cells cultured in BMPro, OptiPro and ProVero, but not in Stem-α and VP-SFM, are significantly higher (13 folds) than that obtained from cells grown in MEM-2%FBS (Fig 2B). Virus yields from cells cultured in the former three SF media were comparable as were yields from the latter two SF media and MEM-2%FBS.

Expression of DENV-2 E protein in infected Vero cells

In order to conform the results of DENV-2 yields obtained above, levels of DENV-2 E protein in infected Vero cells at 7 dpi were compared among cells cultured in OptiPro, ProVero and VP-SFM media using western blotting of 30 μg of protein separated by 10% SDS-PAGE. As expected, relative amounts of immunoreactive DENV-2 E protein from cells cultured in OptiPro and ProVero were higher than in VP-SFM media, but interestingly yield from ProVero media was 3 folds higher than from OptiPro media type (Fig 3).

Nucleotide sequences of DENV-2 genome propagated in Vero cells grown in different SF media

In order to determine whether the differences in ability of DENV-2 to propagate in Vero cells were related to differences in virus genome sequences generated during routine sub-culturing in virus maintenance, sequences of amplicons (1,485 nucleotides) were compared among DENV-2 isolated from stock sample and from infected Vero cells cultured in OptiPro, VP-SFM and MEM-

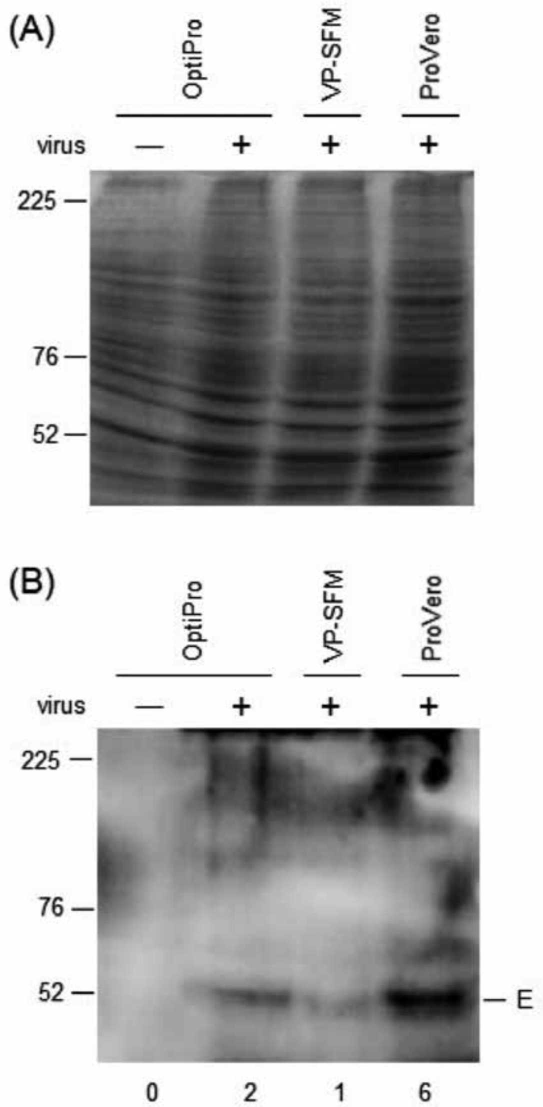

Fig 3-Expression of DENV-2 E protein in infected Vero cells cultured in serum-free media.

Vero cells were infected with DENV-2 and cultured in OptiPro, ProVero and VP-SFM media as described in legend to Fig 2. On day 5 post-infection, cells were lysed and 30 μg of lysate were separated by 10% SDS-PAGE followed by western blotting using an anti-DENV-2 E primary polyclonal antibody and a goat HRP-conjugated secondary anti-rabbit IgG. Immunoreactive protein was detected using a SuperSignal® West Femto Chemiluminescent substrate, documented using a ChemiDox XRS Plus system and quantified with an ImageJ software. Molecular size markers (kDa) are indication on left side of gel or membrane. Number at bottom of lane indicates amount of DENV-2 E protein relative to that from cells cultured in VP-SFM media.

(A): SDS polyacrylamide gel stained with Coomassie brilliant blue dye; (B): Western blot; -: non-infected; +: infected; E: DENV-2 E protein (53 kDa).

2%FBS media at 7 dpi, but no differences were observed among all four samples (data not shown).

DISCUSSION

The study demonstrates DENV-2 grew more efficiently in Vero cells cultured in BMPro, OptiPro and ProVero media compared to that in cells cultured in Stem-α, VP-SFM and (control) MEM-2%FBS media as evidenced by virus yield in cell supernatants and levels of DENV-2 E protein in infected cells. These findings could not be attributed to differences in host cell growth, virus attachment to cells or replication rate due to mutations in DENV-2 genome, but rather that the composition of the SF media affected viral replication.

Although at 5 dpi viral yields from Vero cells grown in all test SF and control media were similar, level of DENV-2 E protein from cells grown in ProVero media was highest followed by that from cells grown in OptiPro relative to cells grown in PV-SFM (the only three SF media tested). How this phenomenon leads by 7 dpi to higher (but comparable) viral yields from infected cells cultured in OptiPro and ProVero media than from cells cultured in PV-SFM media remains to be elucidated. Several studies have attempted to optimize virus growth in cells cultured in SF media (Butler *et al*, 2000; Genzel *et al*, 2006; Rourou *et al*, 2007; Abbate *et al*, 2019).

SF medium has several advantages over serum-containing medium for virus propagation. Serum supplementation in culture media increases cost of media, has risk of microbial contamination, varies in quality among batches and contributes to difficulties in downstream virus purification (Rourou *et al*, 2007). Thus, it is not surprising that SF media have been used for propagating several types of viruses for therapeutic use in humans (Fishbein *et al*, 1993; Merten, 1999; Butler *et al*, 2000; Frazatti-Gallina *et al*, 2004; Genzel *et al*, 2006; Rourou *et al*, 2007; Toriniwa and Komiya, 2007; Hu *et al*, 2011).

In conclusion, the present results indicate proper choice of serum-free media is essential in furthering development of potential inactivated dengue virus vaccines.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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