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Current status for high titre poxvirus stock preparation in CEF under serum-free medium conditions: implication for vaccine development

Philippe-Alexandre Gilbert, Lacrimioara Comanita, John Barrett, Andrew Peters, Marta Szabat, Grant McFadden and Gregory A. Dekaban*
*BioTherapeutics Research Group, Robarts Research Institute, P.O. Box 5015, 100 Perth Drive, London, ON, Canada, N6A 5K8; *Author for correspondence (e-mail: dekaban@robarts.ca; phone: +1-519-663-5777, ext. 1-34241; fax: +1-519-663-3789)*

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Abstract

In light of the recent detection of BSE in North America and its endemic nature in other regions of the world, there is a real need to employ cell culture conditions that do not require any animal-derived material. Here we report the use of an ultra-low protein serum-free medium (VP-SFM, Invitrogen) for the amplification of poxviruses in primary chicken embryo fibroblasts (CEF). We compared the amplification of four different poxviruses (canarypox, modified Ankara Virus (MVA), vaccinia virus strain Copenhagen and myxoma strain Lausanne) in three different media: DMEM 10%, DMEM 2% and serum-free medium VP-SFM. VP-SFM is a serum-free, ultra-low protein medium containing no proteins or peptides of human or animal origin designed to support the replication of viruses and the production of recombinant proteins and monoclonal antibodies. Our results show that high titre poxvirus stocks can be prepared in VP-SFM equivalent to that prepared in serum containing medium.

Introduction

Animal cell culture media used to support the propagation of viruses often contains animal-derived components and some form of serum. However, the presence of animal-derived material comes with several disadvantages for the pharmaceutical industry producing human biologicals and vaccines. Cost, variability between product batches, the undefined nature of serum components and the presence of animal-derived proteins in the production of recombinant proteins makes downstream processing more complex. An additional major concern with the use of animal source material is that it remains a potential source of pathogenic contaminants such as fungi, bacteria, viruses or prion agents such as the bovine spongiform encephalopathy (BSE) prior. At this time, bovine-derived material for the production of human biologics must come from countries free of BSE of which there are only two, New Zealand and Australia.

Currently, the pharmaceutical industry recommends the elimination of serum, and where possible all animal-derived raw materials, from the culture medium used in the manufacture of human biologicals for therapy or vaccination (Staines and Price 2003). Commercial production and isolation of therapeutics is more efficiently realized when

potential infectious agents, and the large amount of extraneous protein present in serum-containing medium, are eliminated. However, removal of animal serum from the culture medium is not without effect as it provides an important source of nutrients, growth factors and attachment factors (Hodgson 1991; Jayme and Smith 2000). Some decline in cell growth performance (i.e., decreased growth rate or cell viability) following serum withdrawal has been reported (Griffiths and Racher 1994; Zang et al. 1995). Virus amplification can also be hampered by the removal of serum, since cells may not sustain the higher metabolic rate required by productive viral infections (Merten 2002).

The majority of poxviruses used as biotherapeutic vaccine vectors (canarypox, modified vaccinia Ankara (MVA), vaccinia) are amplified on chicken embryo fibroblasts (CEF). Primary CEF are commonly used to amplify different virus strains in academic research laboratories and in the biotechnology industry. These cells are prepared from 8 to 11-days-old chicken embryos and largely support all avian retrovirus subgroups and other avian and mammalian viruses compatible with their metabolism. Large-scale producers of CEF provide the cells in serum-supplemented medium to preserve CEF viability until seeding. As an example, Charles River (MA, USA) uses SPAFAS medium which is basal medium Eagles (BME) containing polymyxinB/Neomycin, 1% fetal calf serum (FCS) and 4% newborn calf serum (NCS). Alternate media can also be used, but they also contain serum. Previously, Charles River investigated the use of serum-free medium for the preparation of CEF cells and they observed lower viability and slower growth (Charles River SPAFAS, CT, USA, pers. commun.). In these conditions, it is, therefore, unlikely that recombinant poxvirus titres obtained in serum-free-medium-prepared CEF will be equivalent or superior to that obtained in the presence of serum. At present, there is no alternative to the production of CEF cells in serum-containing medium. We, therefore, investigated whether eliminating or reducing the serum content during virus amplification affected virus titre, because there are many technical and economical advantages in reducing the serum content in production medium as well as reducing the overall risk of unwanted contaminants.

In this study, we assessed the current production limitation related to the use of serum-free medium for the production of recombinant poxviruses. We compared the amplification and titre performance of four different poxviruses that have been used as platforms for vaccines or gene delivery vectors (canarypox, modified Ankara virus (MVA), vaccinia virus and myxoma) in three different media compositions. We report that an ultra-low protein serum-free medium (VP-SFM, Invitrogen) can be successfully employed for the amplification of poxviruses in primary CEF cells. VP-SFM is a serum-free, ultra-low protein medium containing no proteins or peptides of human or animal origin. The trace proteins in VP-SFM are human recombinant epidermal growth factor (EGF) and insulin.

Materials and methods

Virus, cell and media

ALVAC, a canarypox vector, was kindly provided by Sanofi-Pasteur Canada (Toronto, ON, Canada). We constructed an ALVAC-YFP vector that expresses the enhanced yellow fluorescent protein (EYFP) under the control of a vaccinia virus synthetic early/late promoter (VVsynE/L). The rabbit leporipoxvirus myxoma-GFP expresses the enhanced green fluorescent protein (EGFP) under the control of the VVsynE/L promoter as previously described (Opgenorth et al. 1992; Johnston et al. 2003). The vaccinia virus VV-65 expresses β-galactosidase under the vaccinia early/late promoter p7.5 as previously described (Chakrabarti et al. 1997). MVA, kindly provided as a high titre stock by Transgene (Strasbourg, France), is a non-propagative (in mammalian cells) highly-attenuated vaccinia virus strain developed to immunize patients at high risk for adverse vaccinia infection against smallpox (Mayr 1999). ALVAC-YFP, myxoma-GFP and vaccinia were amplified and purified as described previously (Ford et al. 1992; Earl et al. 1998a, b).

Primary chicken embryo fibroblasts are derived from freshly prepared 8–11-days-old SPAFAS chicken embryos (SPAFAS CEF, Charles River, CT, USA). Cells were received in a suspension of Basal Medium Eagle (BME) containing PolymyxinB/Neomycin, 1% fetal calf serum and 4% newborn calf serum.

Two media formulations were used in this study: VP-SFM and Dulbecco's Modified Eagle Medium (DMEM), the standard media used with CEF for virus amplification. VP-SFM is provided in 1 l liquid form (Fike et al. 2001; Radominski et al. 2001; Price et al. 2002; Staib and Sutter 2003, 2004). No supplements were added to VP-SFM, not even L-glutamine as specified by the manufacturer. DMEM was supplemented with 4 mM L-glutamine, 500 U ml^{-1} penicillin G, 500 g ml^{-1} streptomycin, 0.25 g ml^{-1} fungizone and with either 2 or 10% FBS. All media and reagents were obtained from GIBCO Invitrogen (CA, USA). Heat inactivation of serum was performed at 56 °C for 1 h.

Small scale infection settings

CEF were seeded directly into VP-SFM or DMEM containing either 5 or 10% FBS to a total volume of 5 ml in a T-25 flask. Immediately prior to infection the spent medium was completely replaced with the corresponding pre-warmed medium. The poxviruses described above were stored at −80 °C and thawed on ice. Virus was conditioned before infection by indirect sonication with an Ultrasonicator 3000 (Misonix, NY, USA). Sonication was performed twice for 2 min (settings: power 9, pulse on 0.8 s, pulse off 1.2 s) in cold water containing ice that was replenished between each sonication. The sonicated viruses were aliquoted into the corresponding medium to a concentration of 1×10^8 PFU ml^{-1}. Infection at a multiplicity of 0.1 was performed in T-25 flasks seeded with freshly received CEF at a concentration of 5×10^6 cells per flask.

The optimal time of harvest was determined experimentally for ALVAC-YFP and MVA with time course experiments (Figures 1a, b). An optimal harvest time of 48 h post-infection was chosen for vaccinia virus as suggested by literature data (Earl et al. 1998a, b). Myxoma infection of CEF cells is not described in the literature but based on previous experience amplifying myxoma virus in other cell lines, 96 h post-infection was chosen for the harvest time.

Roller bottle infection settings

CEF were seeded directly into VP-SFM or DMEM containing 10% FBS to a total volume of 200 ml in 850 cm^2 roller bottle (Corning, NY, USA). Before each incubation, the roller bottle was injected with CO_2, sealed and transferred to a non-CO_2 incubator (rotation settings: 0.4 rpm for seeding; 1 rpm for infection). Immediately prior to infection the spent medium was completely replaced with the corresponding pre-warmed medium. Infection at a multiplicity of 0.1 was performed with ALVAC-YFP on freshly received CEF at a concentration of 1.5×10^8 cells per roller bottle. Virus preparation was performed as described in the previous section (see small scale infection settings). Harvest was done at 120 hpi by transferring the roller bottle to a −80 °C freezer until titer evaluation.

Virus titer

Six-well plates were seeded with 2×10^6 CEF cells per well in DMEM-10% or VP-SFM and incubated overnight at 38.5 °C. After assessing for good cell adherence, medium was replaced with appropriate viral dilutions. Viral dilutions were done in DMEM without serum. Infection was performed at 38.5 °C for 1 h. Plates were agitated for 15 min. After 1 h, medium was removed and replaced by a 4 ml 1:1 mixture of 1.2% Seaplaque agarose (Cambrex, NJ, USA) and 2× DMEM 20% FBS. Plates were incubated at 38.5 °C for at least 5 days. Virus plaque foci were detected by Neutral Red staining using a 1:1 mixture of 1.2% Seaplaque agarose, 2× DMEM 20% FBS and a sterile stock solution of 0.1% Neutral red. One milliliter of this preparation was overlaid on the previous agarose layer. Plates were incubated overnight at 38.5 °C before counting viral plaques. YFP- and GFP-positive plaque titres agreed with titres obtained with Neutral red staining. Since only GFP foci, but no plaques, were visible on myxoma virus infection of CEF, the plaque assay was repeated on BGMK cells under the same conditions as described above in order to obtain a comparable plaque count.

Fluorescence microscopy

Fluorescent protein expression was observed using fluorescent microscopy employing an Olympus IX50 inverted microscope (Olympus, NY, USA).

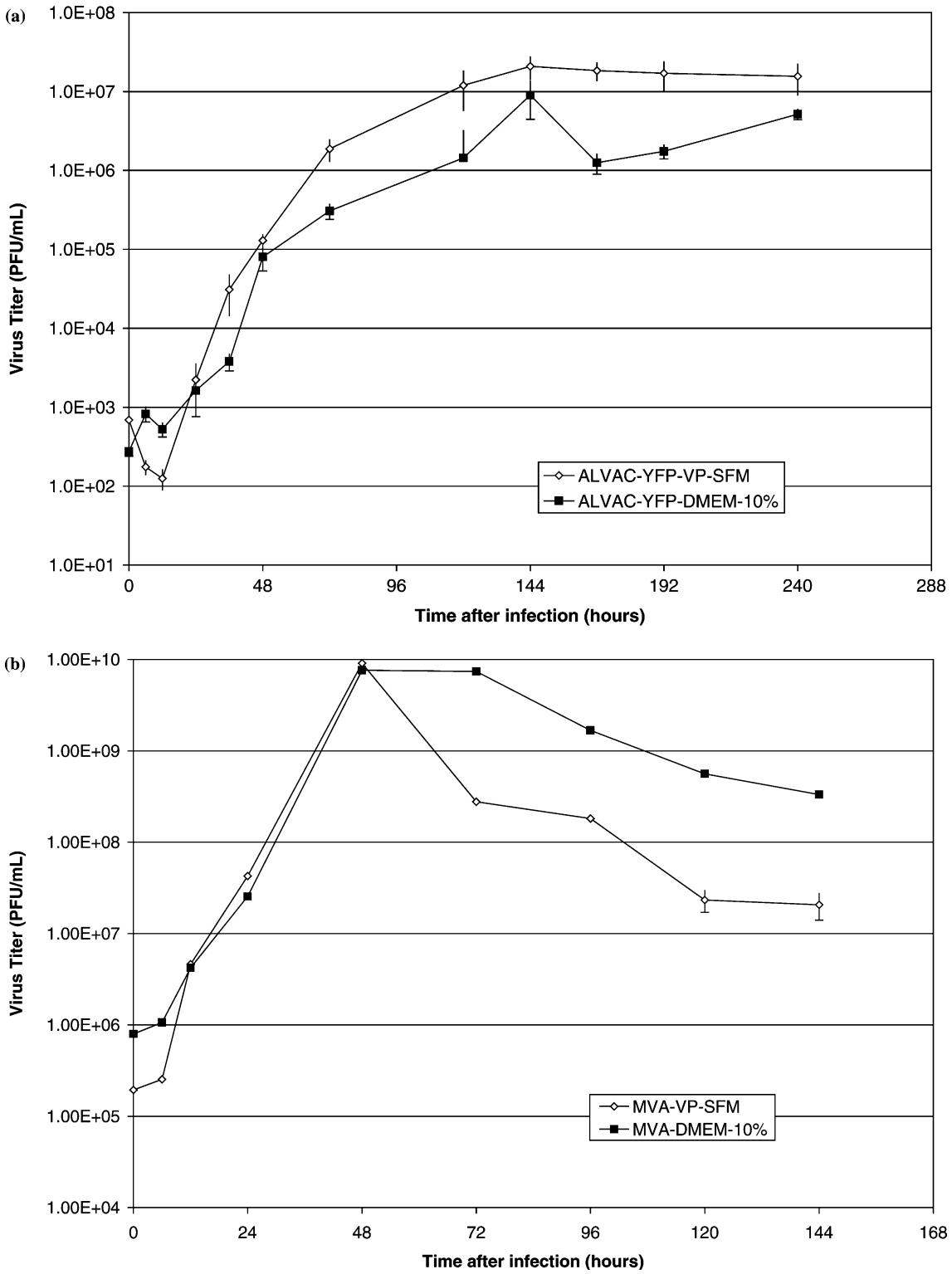

Figure 1. A multi-step growth curve was performed for (a) ALVAC-YFP and (b) MVA in VP-SFM or DMEM-10% to determine the harvest time.

Digital images were captured by a Sony 3CCD Colour Digital Video Camera (Sony, CA, USA). EYFP fluorescence was observed by the use of a filter cube specific for EYFP (U-M41028) (Chroma Technology Corporation, VT, USA). Images were processed with Image Pro Plus Software (Media Cybernetics Inc., Silver Spring, MD, USA) and Adobe Photoshop 6.0 (Adobe Systems Incorporated, CA, USA).

Statistical Analysis

Statistical analysis was performed using the single factor ANOVA function of the data analysis package of Excel 2003 (Microsoft, WA, USA). A value of 0.05 was used for significance level.

Results and discussion

We compared the amplification of four different poxviruses (MVA, ALVAC, vaccinia virus and myxoma virus) in three different media: DMEM 10%, DMEM 2% and serum-free VP-SFM. Our main goal was to quantitatively assess the virus titre produced with each medium. This study permitted us to evaluate the suitability of the serum-free medium, VP-SFM, to support the development of different recombinant poxviruses after initial seeding in a low-serum medium.

We initially monitored the virus titre for ALVAC-YFP and MVA in a multi-step growth curve experiment using either VP-SFM or DMEM-10% to find the optimal harvest time point. Dissimilar virus production kinetics for each medium would result in titre variations at the comparison time point that would not be related to the maximum virus yield attained for each medium. As shown by the multi-step growth curve experiment for ALVAC-YFP and MVA (Figure 1), the curves were similar for the two media tested. The ALVAC-YFP virus was very stable in the serum-free medium as there is no drop in titre for more than 120 h after the infection reached a plateau. The same observation applied to the ALVAC-YFP virus amplified in DMEM-10%. Based on our results, we therefore set an optimal harvest time of 120 h post-infection for ALVAC-YFP.

We also monitored EYFP expression in infected CEF cells with ALVAC-YFP in the different media. As shown in Figure 2, canarypox produced highly concentrated fluorescent foci of infection at 48 h post-infection in VP-SFM. This behavior is different from the infection observed in 2 or 10% FBS medium where the foci were small, spread slowly and in a more diffuse fashion.

The multi-step growth curve for MVA exhibited a similar profile until the optimal harvest time of 48 h was reached. Subsequently, MVA titre decreased more rapidly in serum-free medium than in medium supplemented with FBS at time points after 48 h post-infection. A more obvious and rapidly appearing cytopathic effect was observed after MVA infection, as the infected cells became rounded in shape and non-adherent regardless of the medium used (data not shown). Significant viral titres were found in supernantants of MVA-infected CEF cells indicating that virus was released at later stages of infection (data not shown). This phenomenon was not observed with ALVAC-YFP virus within the time limits studied and may be the result of a less cytopathic infection.

Vaccinia virus induced a cytopathic effect in CEF at 48 h post-infection similar to that observed with MVA (data not shown) regardless of the medium employed. Myxoma infection was the least permissive virus on CEF and did not give rise to typical poxvirus plaques, possibly due to host range differences. Small foci of myxoma-GFP infection could readily be observed based on EGFP expression. Since myxoma-GFP could not form clearly evident large foci or plaques after infection, the myxoma-permissive BGMK cells were instead used to titre the virus obtained from CEF cells.

Figure 3 shows the results of a comparison between the different poxvirus titres obtained in CEF cells using VP-SFM, reduced-serum (FBS 2%) and standard-serum cell culture medium (FBS 10%). The highest titres for MVA (3.28×10^9 PFU ml^{-1}), vaccinia virus (3.58×10^7 PFU ml^{-1}) and canarypox (1.26×10^7 PFU ml^{-1}) were obtained in VP-SFM while the highest titre for myxoma virus (9.46×10^6 PFU ml^{-1}) was obtained in DMEM 10% FBS. One way ANOVA analysis showed that the differences in titre for viruses amplified in the different media were not significant. Moreover, the decrease in FBS concentration from 10 to 2% had no impact on the virus yield under the infection conditions used in this study. We can, therefore, conclude that the

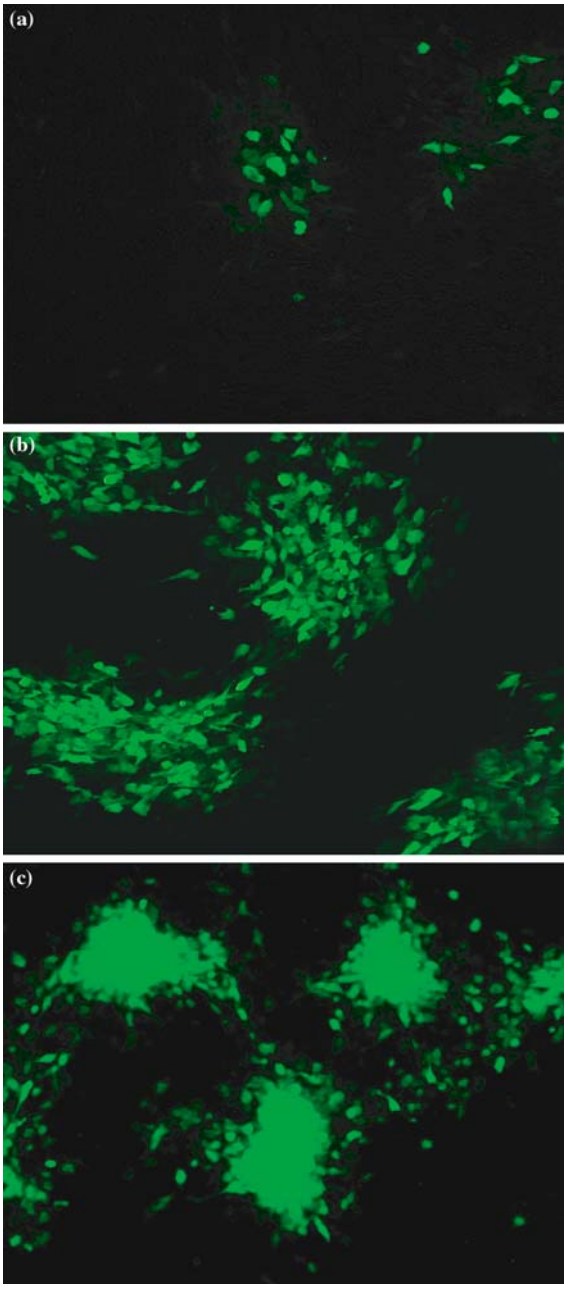

Figure 2. CEF cells infected with ALVAC-YFP at 48 h post-infection. Plaques were observed with a 10× objective on an inverted fluorescence microscope. (a) CEF cells grown in VP-SFM (no FBS), (b) CEF cells grown in DMEM-2%FBS and (c) CEF cells grown in DMEM-10% FBS.

yields from poxvirus amplification using VP-SFM are at least equivalent to those obtained using conventional DMEM plus 2 or 10% serum. Further studies are necessary to look at the behavior of poxviruses in the absence of serum. These results suggest that the presence of FBS alters the propagation of highly replicative poxviruses (e.g., MVA and vaccinia) in CEF cells. We also were able to obtain comparable ALVAC–YFP virus titres by replacing the normal production medium (DMEM 10% FBS) by serum-free medium when cultures were scaled up to roller bottles. ALVAC–YFP

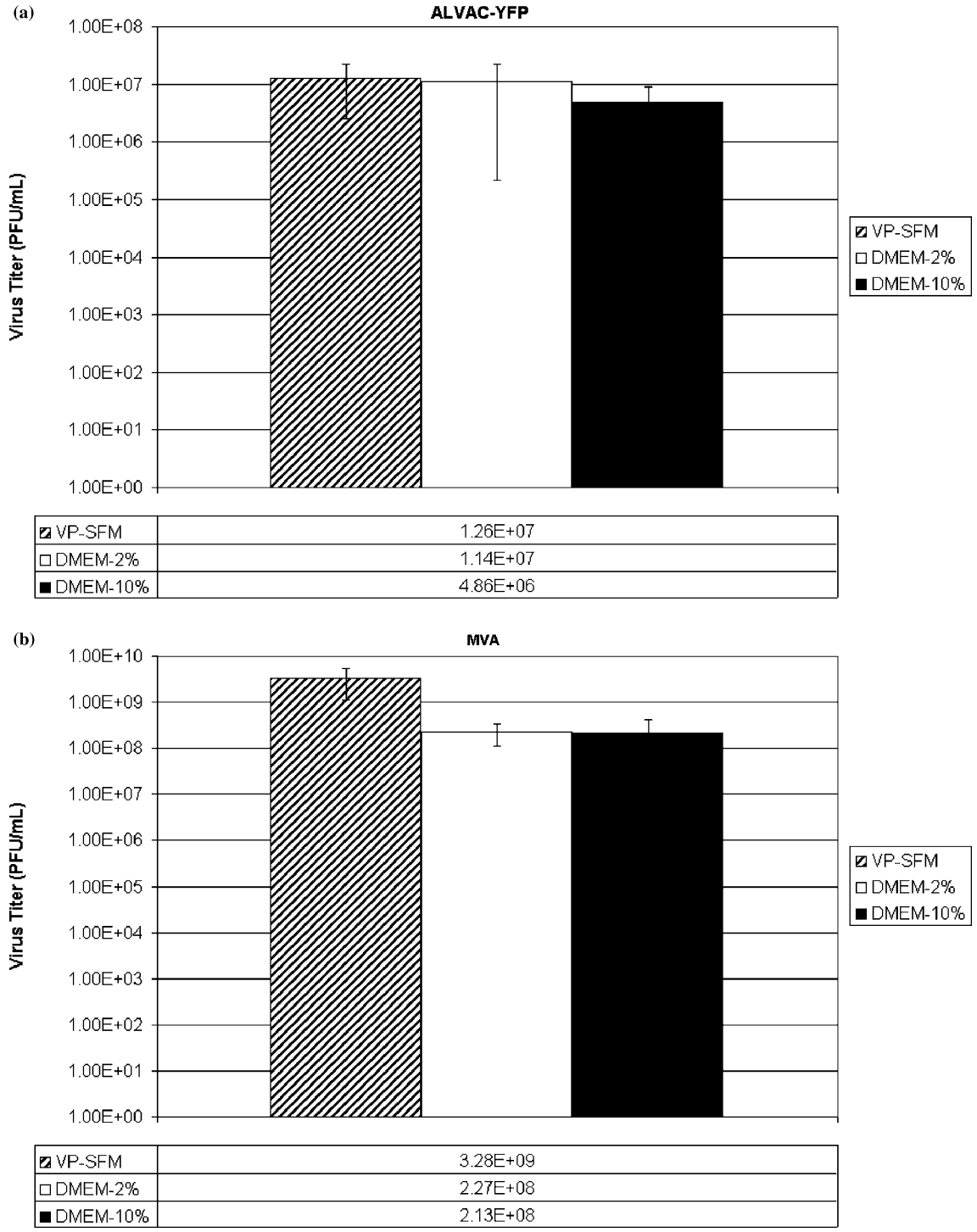

Figure 3. Poxvirus, titres raised in CEF cells using serum-free, reduced-serum (FBS 2%) and standard-serum cell culture medium (FBS 10%) for (a) ALVAC-YFP, (b) MVA, (c) myxoma-GFP, (d) vaccinia. CEF cells were infected at day 0 at a MOI = 0.1, then harvested with the viral supernatants at a time defined either by experiment or literature (ALVAC-YFP = 120 hpi, MVA = 48 hpi, myxoma-GFP = 96 hpi, vaccinia = 48 hpi).

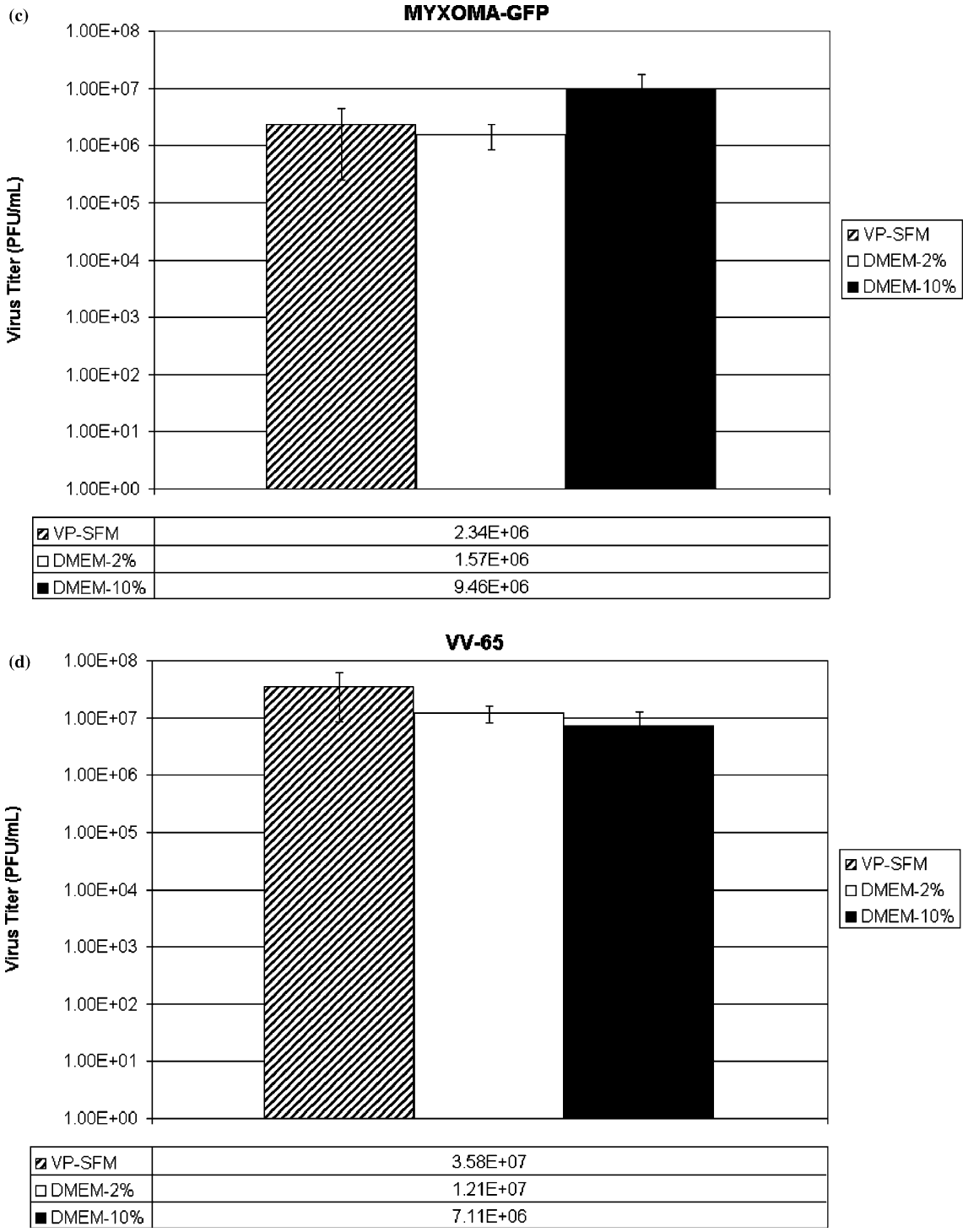

Figure 3. Continued

titers in roller bottle were 1.3×10^9 PFU ml^{-1} for serum free media and 3.01×10^8 PFU ml^{-1} for DMEM 10% FBS. We have also been able to titre poxviruses in VP-SFM and obtained results similar to the one obtained with DMEM plus 10% FBS.

As shown by the titre results (Figure 3c), myxoma-GFP replication was supported in all media. However, the low titre results for myxoma in CEF cells show that host range factors are obviously a major hurdle for myxoma production in this cell line as titres of 10^8 PFU are normally obtained in BGMK cells. Thus, further studies are required to evaluate myxoma infection and replication in permissive cells (e.g. BGMK cells) in serum-free medium conditions. According to the manufacturer, VP-SFM was originally designed specifically for the growth of commonly used mammalian cells such as VERO, COS-7, MDBK and BHK-21. It should therefore be possible to use VP-SFM for adherent mammalian cells such as BGMK or other similar cell lines commonly used for poxvirus production.

Since stock CEF cells are initially prepared by the supplier and suspended in a serum-containing medium, approximately 0.5% of this serum is transferred to the seeding medium when initiating the virus production process. The replacement of medium 24 h after seeding will substantially remove serum from the culture supernatant. The impact of this transfer is probably minimal for FBS-containing medium and corresponds to the industrial standard already in place for seeding CEF cells. However, for the production of viruses in serum-free medium conditions, this implies that CEF cells will initially have been conditioned in a low serum environment (0.5% serum) before infection. Therefore, this process cannot be defined as completely serum-free. However, these conditions are required for ideal for large-scale virus production in roller bottles as some serum proteins are still required for the cells to adhere correctly to plastic surfaces. In a fully defined serum-free process, recombinant fibronectin, a cell adhesion substrate, would have to be considered as an alternative for attachment factor.

In summary, we have demonstrated that the selected poxviruses could be amplified in the serum-free medium, VP-SFM. Virus yield was equivalent for all media formulations tested. Similar results have been obtained for recombinant adenovirus production (Dr. Jack Gauldie, McMaster University, Hamilton, Ontario, pers. commun.). Thus, the use of serum-free media should confer greater safety, simplified downstream processing, and financial savings when generating viral vaccines or biologicals derived from virus-infected cells. Some investigation into the current methods of CEF cell preparation should be undertaken if one desires a truly serum-free process.

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