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A chemically defined production process for highly attenuated poxviruses

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ABSTRACT

Highly attenuated poxviruses are promising vectors for protective and therapeutic vaccines. These vectors do not replicate in human cells and can therefore be safely given even to immunocompromised recipients. They can accomodate very large inserts and provide strong stimulation of the immune system against the vectored antigen. Disadvantages include that very high numbers of infectious units are required per dose for full efficacy. Because they are difficult to produce, improved cellular substrates and processes are urgently needed to facilitate programs intended to reach a large number of vaccinees. We have developed a fully scalable and very efficient chemically-defined production process for modified vaccinia Ankara (MVA), canarypox (CNPV, strain ALVAC) and fowlpox viruses (FPV) based on a continuous cell line.

1. Introduction

Compared to vaccination with inactivated virions or purified subunits, live vaccines induce a broad response that also involves the cellular compartment of the immune system. For safe imitation of a natural infection the vaccine strains are attenuated, but for certain viruses there is a risk of reversion to pathogenic strains [1] and potential residual virulence for some vaccinees or contact persons of the vaccine recipients [2].

Whereas attenuated strains still proceed toward usually benign infection replication of vectored vaccines derived from “highly attenuated” strains is blocked at the molecular level without involvement of adaptive immunity. Promising members of the highly attenuated vectors are avipoxviruses such as fowlpox virus (FPV, especially strain FP9) and canarypox virus (especially highly attenuated strain ALVAC), and derivatives of mammalian orthopoxviruses such as modified vaccinia Ankara (MVA) [3–8]. With very few exceptions [9–11], replication of these highly attenuated vectors is restricted to avian cells. However, mammalian cells are susceptible to infection and although the virus cannot complete a full infectious cycle a strong immune response is induced against the vectored antigen [12–18]. Even immunocompromised patients may receive these vectors opening the potential for therapeutic vaccines against chronic or latent infectious diseases and certain cancers [19–23].

This increased safety comes at the cost of dose requirement: 10^8 infectious units MVA per vaccination are estimated to be required for efficient stimulation of the immune system [23,24] so that especially for global programs against complex infectious diseases such as HIV or tuberculosis hundreds of million of doses of the host-restricted poxviruses may be required annually. For comparison, lesser-attenuated strains also produced on avian cells include vaccines against measles, mumps and yellow fever; these require only 10^3, 2×10^4 and 5.5×10^4 infectious units per dose, respectively (information from the package inserts of YF-VAX from Sanofi Pasteur and M-M-R II from Merck). The protective dose of the vaccinia strain Dryvax in routine vaccination against smallpox is 2.5×10^5 pfu [25], 400 fold lower than the dose recommended for MVA. Fowlpox virus also is used as a replication competent live attenuated vaccine against field fowlpox and its diphteroid form in chicken, and as a vehicle for immunization with various poultry disease antigens [26–28]. For these applications, 100–1000 EID50 (egg-infectious doses) per animal are applied.

Production of MVA, fowlpox or canarypox vectors depends on avian cells. Currently, vaccine strains adapted to avian hosts are produced only in embryonated chicken eggs or on fibroblasts

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prepared from such eggs, a venerable technology but also associated with certain disadvantages. Because primary cells suffer senescence within few passages they have to be supplied continuously. Differences in timing and preparation may lead to lot variations [29,30]. The embryonated eggs as source for the fibroblasts are from expensive SPF (specific pathogen free) flocks. The SPF status requires elaborate husbandry, and transport of material across country borders complicates logistics and may cause occasional shortages. Even with SPF precautions in place contamination with extraneous agents cannot always be prevented. Because time from collection of the embryonated eggs to production of the vaccine is short testing for extraneous agents is performed on the final bulk [31]. Occasionally, complete vaccine lots have to be discarded when contamination is confirmed by quality testing [32].

Finally, with primary cells it is also not possible to stably express transgenes that may further enhance production of highly attenuated viruses or allow packaging of replication-deficient vectors.

We have generated an immortal avian cell line with complete documentation of derivation and exhaustive characterization for regulatory approval to facilitate many aspects essential to modern vaccine production. To obtain the cell line, we have immortalized primary cells from a muscovy duck embryo with the E1A and E1B genes of human adenovirus 5 on separate expression cassettes [33]. Adenovirus 5 causes common cold and is not associated with tumors [34]. The focused biochemical approach for immortalization with genes from this virus has significant advantages in estimates of potential risks to recipients of biologicals produced on continuous cell lines [35]. Here, we describe a chemically fully defined production process for highly attenuated pox viruses on the avian cell line, compare properties of different pox viruses, and demonstrate first steps toward purification of these vectors. The developed process is robust and suitable for industrial production at large scales without requirement for medium replacement or perfusion procedures.

2. Material and methods

2.1. Cell lines and processes

Derivation of AGE1.CR cell line and rationale behind modification by stable expression of the pIX gene from human adenovirus serotype 5 are described elsewhere [33]. The CR and CR.pIX cell lines were cultivated either in DMEM/F12 (Gibco, USA) containing 5% FCS in adherent phase, or in the chemically defined medium CD-U2 (produced by Biochrom, Germany, or PAA, Austria) in agitated suspension.

Small-scale suspension cultures were performed in vented tubes or flasks in a Multitron (Infors HT, Switzerland) shaking incubator with 5 cm platform amplitude and 180 rpm (tubes) or 150 rpm (flasks) rotation and 8% CO_2 atmosphere.

Cultivation in bioreactors at 1 L scale was performed with the cellferm pro (DASGIP, Germany) parallel reactor system. Bioreactor runs at 50 L scale were performed using the disposable SUB 50 system (Hyclone, USA) with a Bioengineering (Switzerland) digital control unit. Default setup was pH of 7.2 units (controlled with 0.5 M NaOH and via CO_2 supply), oxygen saturation of 50% and stirring energy input of 12 W/m^3. Cultivation in the Wave reactor was performed with the BIOSTAT CultiBag RM (Sartorius, France) programmed for 50% oxygen saturation, pH of 7.2 units, rocking amplitude of 6° and frequency of 12 rocks/min.

Tangential flow filtration (TFF) was performed with hollow fiber cartridges (GE Healthcare Life Sciences, USA) with an average pore size of 0.1 μm and surface area of 850 cm^2. The system parameters were conventional transmembrane pressure of 0.3 bar, flow rate of 10 L/min and shear rate of 6000 s^{-1}. Intended concentration factor was 10-fold.

The concentrated suspension was subjected to sonification and the resulting lysate was directly applied or diluted 5–10 fold with 20 mM Tris, pH 8.0, for loading onto an Äkta Explorer System (GE Healthcare) equipped with an experimental affinity membrane adsorber displaying conjugated heparin molecules (Sartorius, pore size >3 μm, adsorption area 250 cm^2, bed height of 4 mm, bed volume 7 mL). Prior to sample loading, the membrane adsorber was pre-washed with 250 mL elution buffer (20 mM Tris, pH 8.0, 2 M sodium chloride) and subsequently equilibrated with 250 mL adsorption buffer (20 mM Tris, pH 8.0). After loading, the membrane adsorber was washed with 70 mL running buffer equivalent to 10 membrane bed volumes to remove unbound substances.

Adsorbed virus particles were eluted by a step gradient using 20 mM Tris buffer, pH 8.0, containing 2 M sodium chloride as the eluent. To achieve complete elution, the membrane adsorber was flushed with 10 bed volumes of eluent at a flow rate of 10 mL/min. The collected elution volume was about 50 mL. The membrane adsorber was used repeatedly via regeneration after each run with 10 bed volumes of 1 M sodium hydroxide.

The entire process was monitored by UV absorption at 280 nm and 260 nm, by dot-blotting against viral antigens, and by dsDNA concentration measurements using Quant-iT PicoGreen dsDNA Kit (Invitrogen, USA) intercalating dye according to the instructions of the manufacturer.

2.2. Viruses

For production of highly attenuated pox viruses, cells are cultivated in CD-U2 in 50% of the reactor volume (for example, 400 mL in the 1 L DASGIP units or 25 L in the 50 L SUB). Seeding density was 0.8–1 × 10^6 cells/mL. After three days, cell density was 4–6 × 10^6 cells/mL. One volume of CD-VP4 virus production medium (produced by Biochrom) was added and MVA (wild-type ATCCVR-1508 or recombinant), ALVAC-GFP (vCP1540 provided by Sanofi Pasteur, Toronto, Canada), or fowlpox virus vaccine strain HP-B (provided by Lohmann Animal Health) was added directly to the culture. Isolation of virus was performed by three cycles of freeze/thawing or sonification of the infected cell suspension. The cryogenic lysate was centrifuged with 13,000×g for 5 min; cellular debris was discarded and only the clear lysate was further analyzed. Sonification was performed with a Branson S250-D unit powering a 3.2 mm sonifier tip with 10% energy for 45 s for volumes up to 3 mL, or a continuous flow chamber with 100% energy and a flow rate of 0.23 L/min for volumes greater than 400 mL.

Titration of MVA was performed in Vero cells (African green monkey kidney cells; ATCC CCL-81) in a variation of the immunofocus assay by Reed and Münch as described previously [33]. ALVAC-GFP was titrated on CR cells in a microfocus assay using GFP expression as marker for productive infection. 50%-Cell culture infectious doses (CCID50) of FPV were calculated according to Spearman and Kärber with CR.pIX cells as indicator cells and at least 6 replicates per titration. The titration results were confirmed by parallel assays in SPF eggs to correspond to egg infectious doses (EID50), wherein CCID50 was approximately twofold more sensitive.

2.3. Quantitative PCR

Levels of viral and cellular genomic DNA were determined from the same lysates that were also assayed for infectious units. The lysate was treated with guanidine isothiocyanate to extract also DNA protected by viral particles. Total DNA was then obtained from 200 μL aliquots of the lysate by chromatography affinity purification. 10 μL (12.5% of the yield thereof) of this total DNA was subjected to real time PCR analysis using primers that recognize the 128L gene of MVA (bp 120811–120898, IMV membrane protein)

and the E1A transgene in the cells. PCR was performed in 25 μL final volume with 100 nM of each of the four primers and 80 nM of the probes against the two amplicons, 200 μM dNTP mix, and TaqMan Universal PCR Master Mix (Applied Biosystems, USA) to 1× concentration. Cloned 128L and E1A served as parallel standard to calculate absolute or relative (viral genome per host genome) number of molecules in the samples according to the algorithm of Pfaffl [36]. The average PCR efficiency according to this algorithm for MVA was 1.945 and for E1A was 1.975. Runs were performed in the StepOne Plus (Applied Biosystems) unit programmed to 50 °C for 2 min followed by 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s each.

2.4. Immunofluorescence assay

Suspension cells were collected by centrifugation with 200×g for 5 min, resuspended in methanol for fixation, collected again by centrifugation and washed with phosphate buffered saline (PBS). Unspecific binding was blocked with 10% fetal calf serum in PBS for 10 min. The nuclear E1A marker protein for the duck cell lines [33] was visualized with 1:30 diluted primary antibody (BD Pharmingen), 1:40 diluted secondary antibody conjugated to streptavidin (Dianova, Germany) and Texas Red-conjugated biotin (Dianova). All incubation steps were performed for 45 min and followed by washing with PBS. In the last incubation step, 1 μg/mL DAPI (Sigma) was added to counterstain nuclei.

3. Results

3.1. Development of a chemically defined medium

First step toward development of a pox virus production process was adaptation of the CR and CR.pIX cell lines to proliferation in suspension. We screened several commercially available media and found good proliferation in Adenovirus Expression Medium (Invitrogen), Gene Therapy Medium 3 (Sigma) and ProPer1 (Lonza). However, infection of suspension cells with MVA resulted in burst size (ratio of released progeny to input virus) below 100. As poxviruses utilize the cytoskeleton for spreading [37,38] spherical, non-polarized cells may allow inefficient dissemination of virus in cell cultures. We therefore tested virus replication in parallel experiments with suspension media on adherent cultures and with medium for adherent cultures on suspension cells. Burst rates were high in suspension cultures infected in adherent medium but again very low in adherent cultures infected in suspension media (data not shown), indicating that our suspension cultures can support efficient viral replication but that formulation of the suspension media interfered with virus multiplication.

Using extensive optimization matrices, we were unable to develop a medium that allows both passaging of the avian cell lines and production of virus (although a single proliferation and production medium is possible for influenza virus on the CR cell lines [39]). Complete change of medium or perfusion processes for virus production are inconvenient at very large scales. For this reason we focused on developing a fed-batch production process where the proliferation medium is diluted with one volume of production medium at the time of infection. Development of an infection medium was not possible in combination with Gene Therapy Medium 3 but to a lower efficiency with Adenovirus Expression Medium. Surprisingly, the presence of the pIX gene augmented MVA production in this process [33]: in the presence of the pIX protein in small scale experiments we measured 170 pfu/cell and a burst size of 320 at the peak of virus production, 48 h after infection with a MOI of 0.1. However, the process was not robust in scale up to reactor volumes beyond 1 L and yet greater yields were desirable.

We therefore first developed a chemically defined serum-free proliferation medium for the CR and CR.pIX cell lines to facilitate rational improvements of bioreactor processes [40]. Starting from a 1:1 (v/v) mixture of Dulbecco's Modified Eagle's Medium and Ham's F12, formulation for a proliferation medium was derived by component screening and full factorial design of experiment, followed by optimization of concentrations for amino acids, sugars, vitamins, lipids, and trace elements to address the metabolic requirements of the avian cell lines. In a second step we designed an infection medium (CD-VP4) and then further modified the proliferation medium (to obtain CD-U2 used in the experiments described here) toward maximum titers of MVA.

Fig. 1A shows kinetic of accumulation of MVA infectious units and MVA genomic DNA in CD-U2 and CD-VP4. After infection with a MOI of 0.01, MVA eclipses until 8 h p.i. Peak infectious titers are 48 h p.i. and remain stable until 72 h p.i. These titers translate to 800 infectious MVA particles per cell, with peak values of up to 1300 pfu/cell in bioreactors. This high yield may approach the capacity of the host cell and for this reason further increase in titers in the presence of the pIX protein may have become less visible in the chemically defined process.

Kinetics of accumulation of genomic viral DNA is similar to the kinetic for the infectious units. The high congruency in the two curves may indicate that MVA has an efficient replication cycle where defective particles do not accumulate to great numbers also in late stages of the infectious cycle. For levels of genomic DNA we also observed an eclipse with lowest value 2 h p.i. (as opposed to 6 h p.i for infectious titers) and rapid increase in the first 48 h post infection. The eclipse in viral DNA is found frequently to various degrees and may be mediated by innate defenses of the host cell [41]. However, there currently is no indication that such mechanism indeed is directed against vaccinia virus [42].

Medium design for production of poxviruses had to address the requirements of both host cell and virus, a fundamental difference to the less complex medium design for production of influenza virus [39]. A major development step therefore was focused on determining the best concentration for components that we suspected in the commercial media to be important or inhibitory for poxvirus multiplication. That these components can be adjusted appears to be a fortuitous property of the CR cell lines: Fig. 1B and C show results obtained in a comparison of the CD-U2 proliferation medium for the avian cells and a highly similar proliferation medium CD-C2 (that was developed in parallel to CD-U2 but on a Chinese hamster ovary (CHO) cell line). Whereas CR cells can be passaged in both media, CHO (and other tested cell lines) within a single passage do not tolerate CD-U2. Conversely, whereas CD-U2 allows high yields for MVA, FPV and ALVAC-GFP in the CD-VP4 fed-batch process, yields are at least 10-fold lower if CD-C2 medium is substituted for CD-U2.

3.2. Cell and virus parameters

For some viruses, efficient accumulation of second or third generation progeny decreases with increasing cell density or requires very low or very high MOIs. We therefore investigated effect of cell density and MOI on production processes comparing the three poxvirus species. Fig. 2A summarizes series of small-scale experiments (up to 45 mL cultures in shake flasks): for MVA and ALVAC-GFP, cell density at the time of infection appears to be of little influence in the range tested ($1–3 \times 10^6$ cells/mL, suitable for the fed-batch process where infection is initiated at 6×10^6 cells/mL as a maximum cell density where medium is not spent too much). For FPV, however, yields declined visibly with increasing cell densities, an effect also observed in adherent cultures (Fig. 2C).

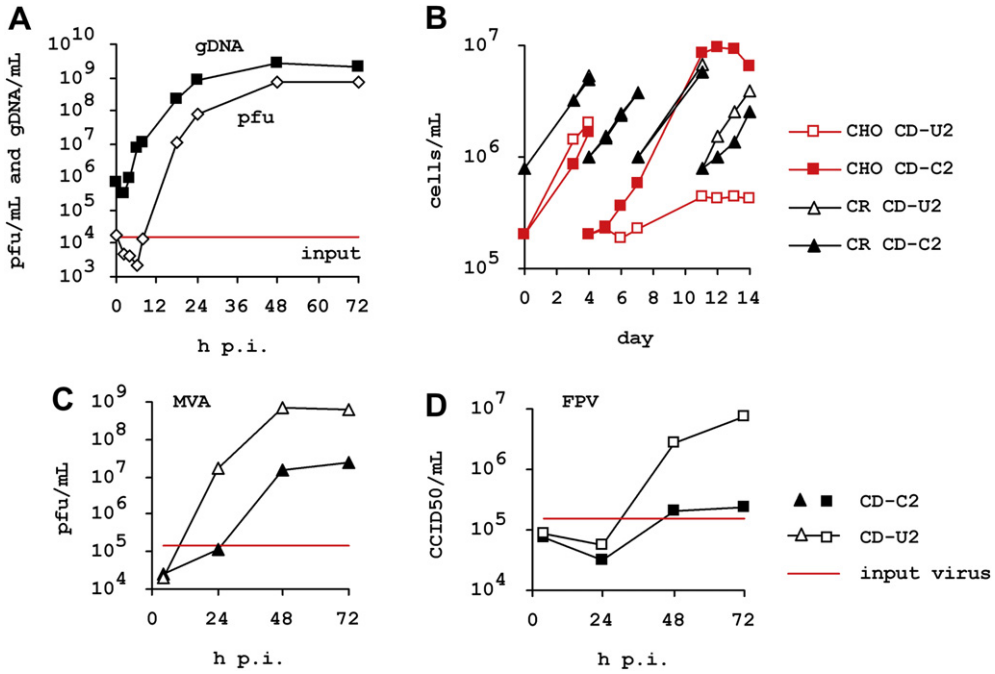

Fig. 1. (A) Replication kinetic for MVA infectious units and genomic DNA (gDNA) in the fed-batch production process with CD-U2 and CD-VP4 chemically defined media. (B) Proliferation of CR and CHO cells in chemically defined media CD-U2 and CD-C2. CR (and CR.pIX) cells can be propagated in CD-U2 and CD-C2 media. CD-C2 medium contains components essential for CHO and other tested cell lines but not important for passaging of CR cells. (C) CD-C2 media components allow passaging of CR cells but interfere with MVA and FPV yields.

Input ratio of virus/cell did not reveal significant differences among the three virus species. Optimal MOI for processes appears to be 0.05 to 0.1 but depending on the value of pharmaceutical grade seed virus MOIs as low as 0.01 also are suitable for bioreactor runs. Spread of CPE in adherent cultures mirrors the measurements of infectious units in the suspension process (for example with FPV in Fig. 2D).

3.3. Bioreactor parameters

Some processes resist scale-up from research and development phase into relevant volumes. In our example, addition of virus production medium induces formation of tight spheres of approximately 50 cells per aggregate. Although we did obtain good burst sizes in experiments with single-cell suspensions aggregate formation appears to be beneficial for reproducibly high yields of poxviruses. We therefore examined whether aggregate induction can be carried into bioreactor runs and obtained good formation and maintenance of infected aggregates (Fig. 3A). Culture appearance mirrors replication kinetic with tight aggregates being present during peak production times 48 h p.i. for MVA and up to day 6 for ALVAC-GFP (Fig. 4B). With increasing CPE aggregates start to dissolve into non-viable single cells.

One aim of the work described here is to facilitate supply with host cells by replacing primary cells from external sources for production of poxvirus vectors. Doubling times for the CR and CR.pIX cell lines is 28–35 h in the current CD-U2 medium primarily designed for compatibility with virus production processes rather than growth rate. Fig. 3B shows an actual seed train toward 50 L scale: starting with a cryopreserved culture a 10 L WAVE bioreactor can be inoculated within 12 days. This WAVE bioreactor can be used to inoculate 50 L volume for virus production at 100 L final volume in the fed-batch process, within 21 days of cryoculture revitalization. Another important aspect concerning supply is properties at higher cell passages. We therefore continuously cultivated the CR and CR.pIX cell lines up to passage 318 and 296, respectively, and tested permissivity of the cultures in parallel infections. For both cell lines permissivity and final yields did not change with passage level, as shown in Fig. 3C for CR. Also, the cellular marker E1A unique to the immortalized duck cells was maintained both in immunofluorescence assay (Fig. 3C) and quantitative PCR (data not shown).

We next investigated production at several scales and reproducibility in independent cultures and with different MVA viruses, FPV and ALVAC-GFP. Fig. 4A shows an overlay of 13 bioreactor experiments with cultures between 1 L (black curves) and 50 L scale (red curves) infected with wild-type or clinically relevant recombinant MVA viruses. MOI was 0.1 or 0.05 in these experiments; for all viruses peak titers were measured 48 h post infection. Lowest titer was 2.95×10^8 pfu/mL for one of the different recombinant MVA species at 50 L scale, maximum titer was 2.60×10^9 for wild-type MVA at 1 L scale.

Fowlpox virus production in the WAVE bioreactor (Fig. 4C) gave 2.37×10^7 CCID50/mL 72 h p.i. Cells were infected at a density of 1.0×10^6/mL and harvested 72 h p.i. to allow further processing of still-intact cells.

Production of ALVAC-GFP requires longer incubation times: peak titers generally were obtained at day 7 p.i. Fig. 4B shows independent representative experiments at 1 L scale with MVA in a parallel bioreactor.

The profile shown in Fig. 4D is consistent with the expected higher metabolic load of MVA-infected host cells. Lactate accumulation accelerates at the end of the eclipse period, whereas in non-infected reference cultures given either one volume of proliferation medium or infection medium lactate accumulation is far less pronounced. In the non-infected reference that received one volume CD-VP4 medium, lactate does not further accumulate 48 h post mock-infection possibly because cell proliferation is inhibited in the tight aggregates. Glucose has to be replenished in infected cultures but not in the two reference cultures. Only ammonium accumulation is similar for infected and non-infected cultures and

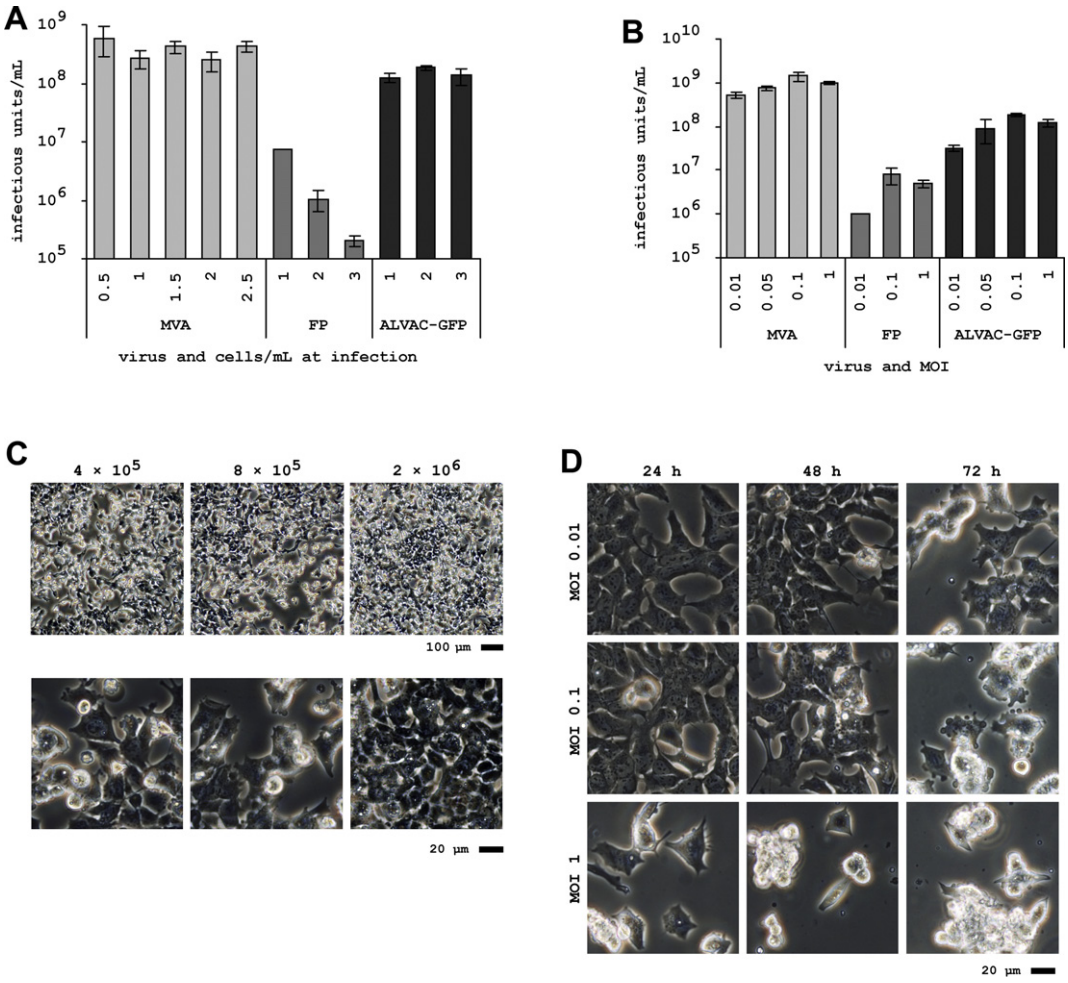

Fig. 2. (A) Determination of influence of cell density at the time of infection for production of MVA, FPV and ALVAC-GFP. (B) Optimization of MOI for production of MVA, FPV and ALVAC-GFP. (C, D) Spread of CPE in adherent CR and CR.pIX cells confirms results obtained in suspension cells: greater extent of CPE at low cell densities (C), and full permissivity also at low MOI (D).

remains at levels below 2 mM throughout the process. Cellular DNA measured by qPCR against the E1A transgene does not accumulate further 24 h post infection and appears to decline at the peak of virus replication 48 h post infection, probably due to degradation in apoptotic cells.

3.4. Downstream processing

Poxvirus morphogenesis is complex and results in three partitions of infectious viruses [43], intracellular mature virus with membranes derived prior to the trans-Golgi network (TGN), intracellular enveloped virus with additional two layers of membranes from the TGN, and extracellular viruses that have left the host via fusion of the outer TGN membrane of the virion. The extracellular viruses may be released into the culture medium or may remain cell-associated.

Culture conditions and individual properties of cell and virus have some influence on the relative amounts of the virus forms [38,44]. In small-scale experiments with MVA we investigated partitioning of infectious units into cell culture supernatant and cytoplasmic or cell-associated space. Cell association was considerable with only 20–30% of the yield in the supernatant 48 h p.i (data not shown). Release of virus increases 72 h p.i. Although total yield remains similar to the levels 48 h p.i, indicating that virus production ceases 48 h p.i. and cell disintegration, possibly with some contribution by induction of apoptosis, allows part of the virions to escape the host cell. Collection of cells infected with MVA, ALVAC-GFP or FPV by centrifugation at the respective expected viral peak levels and resuspension into a smaller volume resulted in titers where increased concentration corresponded to the reduction of volume. Centrifugation also is feasible at large scales but tangential flow filtration (TFF) sometimes is more convenient.

For this reason, between 10 and 50 L of infected cell suspensions were concentrated 10–20 fold using TFF (Fig. 5A and C). Increases in titers generally corresponded to the concentration factor with minimal losses in yield. To confirm that such a dense suspension is amenable to further downstream processing the TFF retentate was sonicated and virus recovery of an aliquot thereof was performed by heparin affinity chromatography. For the chromatogram in Fig. 5B and 1.4×10^{10} pfu in 100 mL were loaded, 3.9×10^{9} pfu eluted in the breakthrough fraction that started to appear at a retention volume of 20 mL, and 7.8×10^{9} pfu were recovered in the elution volume. Concentration of eluted virus was 1.6×10^{8} pfu/mL, equivalent to 56% of the loaded activity. Heparin affinity chromatography was not optimizied further, an excellent application of the method for MVA has already been described in a previous publication [45].

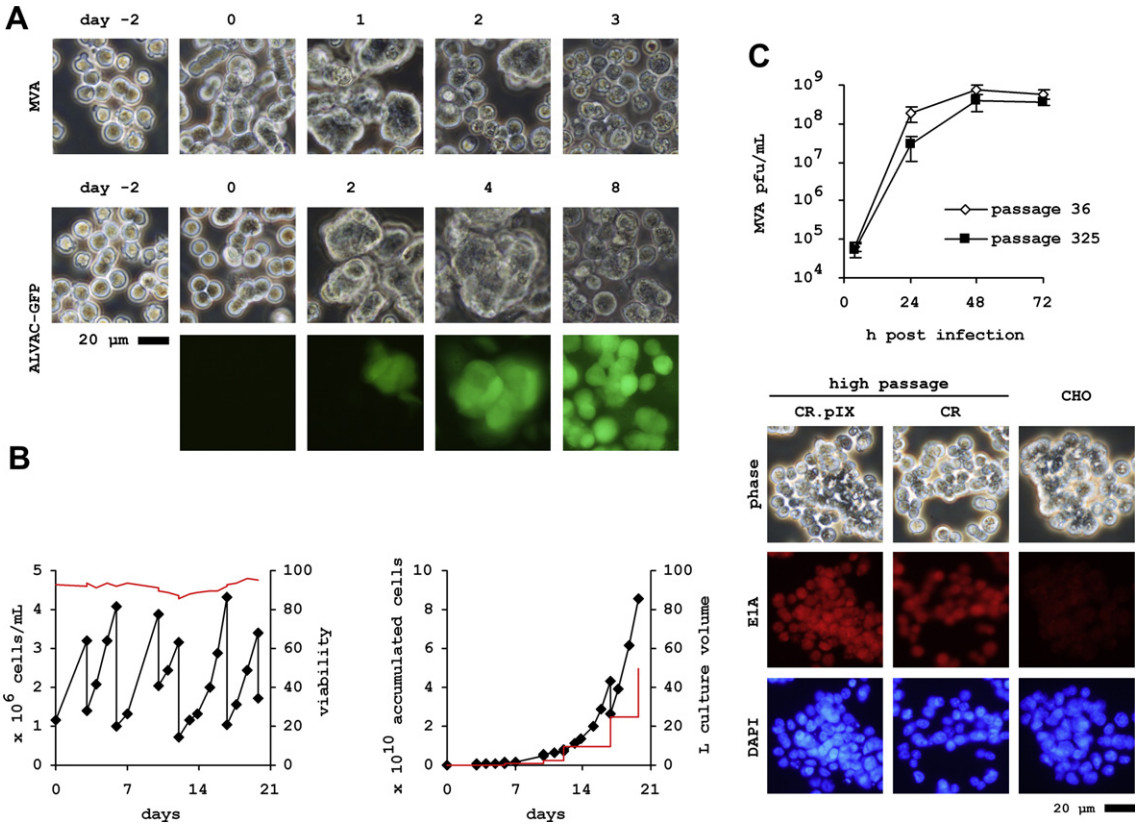

Fig. 3. (A) Induction of cell aggregate formation by addition of CD-VP4 virus production medium. Disintegration of the aggregates coincides with progression of CPE and increases in virus titers, 48 h p.i. for MVA and 8 days p.i. for ALVAC-GFP. (B) Actual seed train in CD-U2 proliferation medium from cryovial to WAVE and 50 L bioreactor. The red curve shows viability in the left chart and culture volume in the right chart. (C) Comparison of cell line properties at high and low passage: unchanged permissivity for MVA in the upper panel (result of two consecutive independent experiments with two parallel cultures each) and continued expression of the cellular E1A marker in the lower panel. CHO cells were shown as negative control.

4. Discussion

Vaccines protect against a surprisingly broad spectrum of infectious diseases with more recent successes directed against human papillomavirus (Cervarix, GlaxoSmithKline, and Gardasil, Merck) and rotavirus (Rotarix, GlaxoSmithKline and RotaTeq, Merck). However, protective or therapeutic immunity still cannot be raised against a number of latent and chronic pathogens, including Mycobacterium tuberculosis, human immunodeficiency virus, hepatitis C virus, and the Plasmodium protists causing malaria. For control of such agents, new approaches for presentation of disease-associated epitopes will be an essential component. The advantages of expressing antigen via viral vectors will have to be balanced against potential risks associated with any live vaccine. The increase in numbers of immunocompromized individuals and the expansion of international mobility demands a very high degree of safety in the vector. This confidence often is not found in older strains, such as the vaccinia virus used in the smallpox eradication program of the 1970s [46].

Modern vectored vaccines [47,48] combine the advantages of live-virus infection with the strong safety profile inherent to the highly attenuated vectors, and thus may provide novel therapeutic or protective approaches. Especially promising vectors are host-restricted poxviruses: they have demonstrated safety in clinical trials (for example, [18–21]) and yet are efficient stimulators of the immune response (for example, [12–16]). However, to provide adequate supply of these vectors can be challenging: they have to be given at high doses because they replicate to very low levels or not at all in the recipient, and because they are highly attenuated they require special host cells for production.

To overcome these limitations, we designed and created a host cell line with focus on compliance with regulatory requirements [33] and have demonstrated here development of an essentially closed production process in a chemically defined medium. The process is a fed-batch procedure scalable to larger reactor volumes and was tested with three disparate [3,49] species of poxviruses: FPV, ALVAC and MVA. The formulation of the media is known thus allowing transfer of the complete production process into geographic regions where the vaccine may be applied.

With exception of FPV, the viruses appear to tolerate a wide range of MOI and cell density at the time of infection. For FPV very low cell densities were important for high yields. There are observations suggesting that ducks are not permissive for FPV and cannot propagate this virus [50,51]. For this reason, veterinary vectored vaccines using FPV have to be given at higher doses to elicit protection in these animals (10^5 TCID50 per dose for ducks, 100–1000 fold higher compared to the dose for chicken). For MVA, productive infection in non-permissive species is prevented late in self-assembly of virions [12,52]. Host restriction for FPV in ducks appears not to be due to an unconditional block at the cellular level as we clearly confirm earlier results obtained with primary cells [53] that continuous duck lines are fully permissive for FPV, both in adherent and suspension cultures.

Especially for MVA, pH control appeared to be essential with yields decreasing rapidly at pH below 7.0 (tested in the DASGIP system and in shake tubes using carbonate pH titration; data not

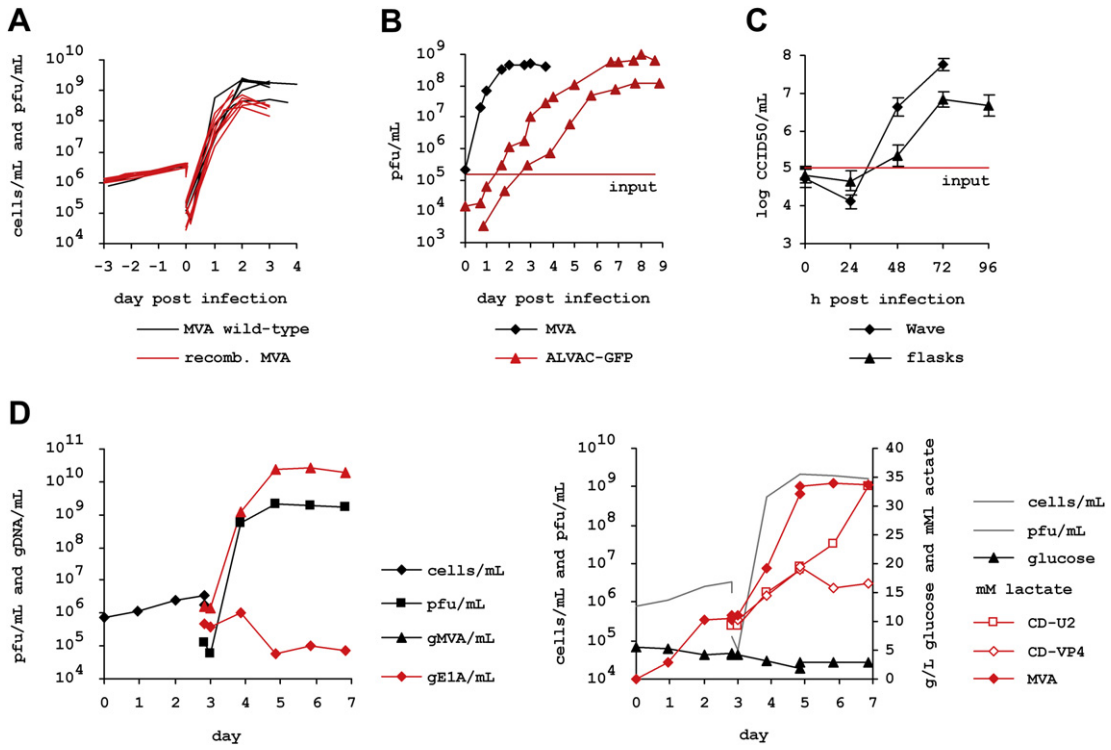

Fig. 4. (A) Overlay of 14 representative bioreactor runs with wild-type MVA and four different recombinant MVAs at volumes between 1 L and 50 L. (B) Two independent ALVAC-GFP production experiments at 1 L scale (red curves) and a parallel wild-type MVA infection (black curve). (C) Production of FPV in the WAVE bioreactor and shake flasks. (D) Virus replication, genomic DNA accumulation (left panel) and metabolic profile of cultures infected with MVA (right panel). Lactate accumulation is shown for an infected bioreactor and two parallel non-infected bioreactors where cultures received one volume of CD-U2 proliferation medium or CD-VP4 virus production medium, respectively.

shown). MVA replicated fast and reached peak titers within 48 h post infection, the culture was in fulminant CPE 48–72 h p.i. FPV replicated only slightly slower and peak titers were obtained within 72 h p.i. In our cell line and medium combination, replication of ALVAC required 6–9 days to reach maximum yield. Lactate accumulation especially in the ALVAC-infected culture was considerable and glucose had to be replenished repeatedly.

To satisfy the energy requirement the virus production process will be further optimized in future studies. Repeated feeding with glucose did not improve metabolic efficiency and, as expected,

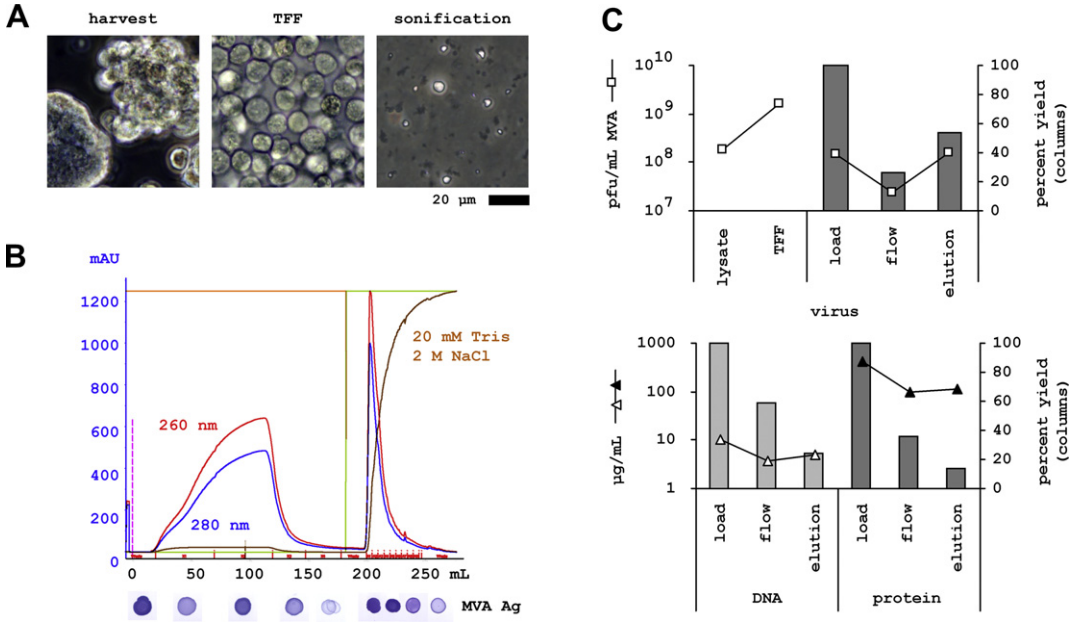

Fig. 5. (A) Concentration of MVA-infected cells by TFF and lysis by sonification, 48 h post infection. (B) Chromatogram of MVA purification by heparin affinity chromatography. Chromatography was monitored by UV-light absorption and immunoblotting of individual fractions against MVA antigens (blue spots below chromatogram). (C) Concentrations of and yields for MVA infectious units, total protein and DNA in the load, flow-through and pooled elution volumes.

lactate accumulation was not reduced. Fructolysis involves different pathways within cells than glycolysis and thus may be less affected by the ongoing infection (if the cell is equipped with appropriate GLUT5 transport molecules for fructose influx). Also, pyruvate may improve energy supply as it is metabolized downstream of glycolysis. However, feeding with either fructose or pyruvate did not boost yields further for MVA or ALVAC in initial experiments. Consistent with the long production phase, yeast or different plant hydrolyzates did improve yields of ALVAC, whereas yields for MVA or FPV were not affected (neither improved nor lowered) by addition of hydrolyzates.

The virions produced in the described chemically defined process are fully suitable for further downstream processing. As a first step we concentrated infected lysates tenfold by centrifugation in small-scale experiments and by tangential flow filtration for volumes beyond 8 L. Several properties combine to make centrifugation or tangential flow filtration (TFF) a convenient concentration step for poxvirus particles: cells even in late stages of the cytopathic effect remain largely intact, a considerable fraction of the poxviruses appear to remain cell-associated [38,44], and the large particle size of pox virions (flattened cylinders with 360 nm at the long axis [54]) allows efficient sedimentation or limits losses in the flow-through. Yields in the retentate after filtration of harvest obtained 48 h p.i. and 72 h p.i. were similar (data not shown) suggesting that cell association and particle size are the main determinants for robust concentration of infectious units out of the cell suspension.

Following TFF, chromatography was initiated with titers approaching 10^{10} pfu/mL in the crude lysate to confirm that in such a dense suspension generated with chemically defined medium heparin-affinity chromatography is possible. Important parameter and various materials for affinity chromatography are described in great detail by Wolff et al. [45]. Here, we demonstrate that also infectious units can be concentrated into a well-defined peak with yields of approximately 55%.

In summary, we describe development of chemically defined media in combination with a process for production of highly attenuated poxviruses. This process is freely scalable from 5 mL to 50 L or beyond and based on a continuous cell line created with intention to meet regulatory guidelines. At small scale, the procedure may facilitate research on and improvement of vector stability [55] (a potential problem with certain clinically relevant inserts), at large scale this development may contribute significantly to ambitious vaccine programs.

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Conflict of interest: IJ and VS have patent protection on the avian cell lines described in this publication.

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