Cytotechnology (2005) 49:11–23
DOI 10.1007/s10616-005-5361-z

Evaluation of a serum-free medium for the production of rAAV-2 using HeLa derived producer cells

C. Jenny[1,*], E. Toublanc[2], O. Danos[1] and O-W. Merten[1]
[1]*GENETHON, 1 rue de l'Internationale, 91000 Evry, France;* [2]*INSERM U649, CHU Hotel-Dieu, 30 avenue J. Monnet, 44035 Nantes, France; *Author for correspondence (e-mail: jenny@genethon.fr; phone: +33-0-169-472921; fax : +33-0-160-778698)*

Received 9 September 2005; accepted in revised form 22 November 2005

Key words: Producer cells, rAAV production, Serum free, Suspension culture

Abstract

During the last decade, recombinant AAVs have become of increasing interest for gene therapy. Clinical trials have been conducted following promising *in vivo* evaluations, thus leading laboratories to adapt their production systems for larger and higher quality demands. Classical transfection protocols seem difficult and cumbersome to adapt to a bioreactor scale. The use of stable producer cells appears as an attractive alternative, as this system requires only a single infection step to induce rAAV production. Furthermore, the switch to a serum-free medium is an interesting strategy to increase the biosafety level to satisfy clinical grade requirements for gene therapy products. Here, we have combined both approaches and evaluated different rAAV producer clones in a serum-free medium. We first evaluated the cell growth in a serum-free medium and then did a partial optimisation of the medium composition to obtain vector yields as close as possible to the yields obtained in a classical serum containing medium. Different helper viruses, multiplicity of infection, times of infection and harvest have been compared in small scale cultures in order to determine the optimal settings which were then transferred and evaluated in suspension cultures in spinner flasks. The yields obtained in this system were similar to or at most 2 times lower than those obtained in a serum-containing medium. The scale-up of such a production system as well as the use of high cell density perfusion culture systems will probably lead to considerably higher yields than those obtained in a classical process.

Introduction

Adeno-associated virus is a small dependovirus without any pathogenicity in humans and its simple genomic organisation (2 open reading frames coding for regulation (rep) and structural (cap) functions, respectively) facilitates its use as a gene vector. Recombinant adeno-associated viruses (rAAV) are amongst others the most attractive vectors for gene therapy (Snyder 1999; Zhao et al. 2001). They are able to transduce dividing as well as non dividing cells and are less immunogenic than other viral vectors. They can only replicate after induction with a helper virus (adenovirus or Herpes virus) (Leonard and Berns 1994). Numerous *in vivo* studies have demonstrated their interest as vectors for efficient and long term gene transfer in muscle, liver, brain, eye… and they are currently used for clinical trials (Kay et al. 2000; Aitken et al. 2001).

The perspectives for clinical applications (treatment of haemophilia, cystic fibrosis, myopathies…) imply the capability to produce lots in sufficient quantities and with a high quality to meet clinical requirements.

AAV vectors are commonly produced via transfection of adherently growing 293 cells (Matsushita et al. 1998; Salvetti et al. 1998; Xiao et al. 1998). This protocol is adequate for a pre-clinical research scale and for the screening of a large number of genes of interest. However this production system becomes cumbersome for clinical applications. In order to scale up the production size, the culture surface can be augmented in different ways: (a) the automation of transfection based processes in roller bottles (Liu et al. 2003) or the use of large surfaces such as provided by the CellCube (Brown et al. 1998), (b) microcarriers that support high density growth of adherent cells (Griffiths 2001), and (c) suspension cultures developed in bioreactors. Although a lot of improvements have been realised in order to develop protocols to transfect mammalian cells in suspension in a bioreactor, for the production of recombinant proteins (Meissner et al. 1999; Schlaeger and Christensen 1999; Wurm and Bernard 1999; Durocher et al. 2002), these techniques cannot be easily adapted to the production of viral vectors.

Infection is more easily adaptable to large scale and suspension procedures than transfection. That is why several groups developed stable cells lines. Packaging cell lines harbour rep and cap genes of AAV whose expression is induced following a double infection (wtAd and rAAV/Ad hybrid virus) (Gao et al. 1998; Liu et al. 1999). These cells are used to generate producer cells containing the AAV vector. Viral vectors are then produced following a single infection step with a helper virus. 293 cells are not adequate to generate producer cells as they constitutively express the Ad E1 protein that activates AAV p5 promoter and expression of the large Rep proteins, which were shown to be toxic for the cells (Clark et al. 1995; Chadeuf et al. 2000; Neyns et al. 2001). Thus, stable producer cells are classically based on HeLa cells (Clark et al. 1995; Chadeuf et al. 2000; Liu et al. 2000; Mathews et al. 2002) though 2 cell lines derived from A549 have also been described (Gao et al. 2002; Farson et al. 2004). More sophisticated producer cells have been constructed, with inducible systems, mainly in order to regulate large Rep proteins expression and attenuate the toxicity for the cells (Inoue and Russell 1998; Ogasawara et al. 1999; Okada et al. 2001; Mizukami et al. 2004). Producer cells constitute an interesting alternative to transfection systems (Blouin et al. 2004), they are known to produce similar titers as obtained with 293 transfected cells and many investigators have demonstrated that less rcAAV are generated with stable cell lines (Inoue and Russell 1998). Nevertheless, this option implies the coproduction of wt adenoviral particles with rAAV and is also time consuming, requiring 2 cloning steps for each specific cell line.

An essential requirement for clinical grade vectors is a high quality and safety profile. It thus seems important to develop the use of serum free (SF) media (Cote et al. 1997). Classical media are often complemented with fetal calf serum (FCS), which presents several disadvantages: animal origin, unknown and variable composition, potential source of adventitious agents… As opposed to that, SF media are free, or almost free, of animal derived components, their composition is often completely defined and their use greatly facilitates downstream processing. Moreover SF media are usually specifically developed for certain cell lines and are then really adapted for supporting their growth and this, often in suspension. Thus an SF environment supports the manufacturing of a safer final product under technologically simpler conditions.

For these reasons, we have chosen to develop a protocol to produce rAAV using producer cells cultivated in suspension in a serum free medium. This combination appeared to us as most adequate to meet quality, scalability and easiness of production. Several producer cells were available for this study and the first step has been their adaptation to an adequate serum-free medium. Different parameters like the production medium, helper viruses, multiplicity of infection (MOI), kinetics of infection and harvest, etc… have been studied and optimised in order to obtain vector yields as close as possible to those obtained with reference protocols (tri-transfection of 293 cells and producer cells in a serum complemented medium). Under SF optimised conditions, we obtained almost equivalent production yields, indicating that this approach is viable and is of potential interest for a large scale production.

Materials and methods

Cell lines

Three different producer cell lines were analysed. Two of them were generated in the laboratory of

Ph. Moullier (Nantes, France) (Chadeuf et al. 2000; Tessier et al. 2001). They were obtained following 2 cloning steps: the first one by co-transfecting a pRepCap2 plasmid, which contains the serotype 2 AAV rep-cap genome, and a plasmid containing the neomycin resistance gene into HeLa cells. The selected packaging cell line RC32 obtained was then transfected with either a pCMV-LacZ or a pSMD2-GFP plasmid to generate the stable producer clones: RC32LacZ, RC32GFP. Cells were maintained in DMEM (Cambrex) + 10% FCS (JRH Biosciences). Once adapted to SF medium, the cells were renamed RC32LacZSF and RC32GFPSF.

The third producer clone was generated by the HGTI (Harvard Medical School, Boston) (Mathews et al. 2002). Cells were also derived from HeLa cells, by co-transfecting a pRepCap2 plasmid (with attenuated large Rep proteins expression) and a neomycin resistance plasmid. The integration of the transgene was realised by transducing the packaging cells with a rAAV-LacZ vector. ROS26Z9 were cultivated in DMEM + 10% FCS and were also adapted to SF medium (ROS26Z9SF).

The cells used to determine the infectious titer were 293, cultivated in DMEM + 10% FCS.

Plates or T-flasks were incubated at 37 °C in a incubator (CO_2-5%). Cells in spinner flasks (Techne) were agitated at 30 rpm and maintained at 37 °C.

Adaptation to SF cultures

The producer cells were adapted to the HeLa SF medium (JRH Biosciences) either by direct or sequential weaning (from 10% to 5, 2.5, 1 and 0% FCS). Once adapted, they were passaged twice a week, centrifuged (900 rpm, 5 min) and seeded at 3×10^5 cells/ml, either in static conditions (6-wells plates or T-flasks) or in suspension (spinner flasks).

Viruses

Wild type adenovirus 5 (wtAd) was used to induce rAAV production. It was produced with 293 cells as previously described (Graham and Prevec 1991). Alternatively, wtHSV and ΔPS Ad were also used to infect producer cells, they were kindly provided by Ph. Moullier (Toublanc et al. 2004) and B. Massie (Oualikene et al. 2000), respectively. An E1 deleted adenovirus was used for the vector titration. It was produced in Genethon in 293 cells as described before (Graham and Prevec 1991).

Vector production

Cells were seeded in HeLa SF medium at a density of 5×10^5 cells/ml either in 6-wells plates (2 ml) or in spinner flasks (50 ml). On the following day, they were infected with wtAd (MOI = 35). SF medium was changed for DMEM 24 h post-infection via centrifugation. Cells were harvested 48 h post-infection, pelleted and stored at −20 °C.

In the case of the use of ΔPS Ad, cells were treated as for wtAd.

Production of rAAV based on tri-transfection of 293 cells was realised using standard procedures (Drittanti et al. 2001).

The following additives were tested: non essential amino acids (Gibco), chemically defined lipid concentrate (Gibco), trace element solutions 1 and 3 (ICN), MEM vitamins solution (JRH Biosciences). Their initial concentrations were 100 × and they were added at a final 1 × concentration.

Experiments were realised in duplicate or in triplicate when indicated, in the figures legend. They were reproduced at least twice. Standard deviation was calculated for each experiment and is represented on the graphs as error bars.

Titration

Cell pellets were thawed, resuspended in IMDM (JRH Biosciences) and lysed by 4 freeze/thaw cycles. After a quick centrifugation to pellet cell debris and 30 min inactivation at 56 °C, the crude lysate was loaded on 293 cells in 6-wells plates (4 dilutions), in the presence of ΔE1Ad (MOI = 5). LFU (LacZ Forming Unit) titer was determined following fixation and X-Gal staining 20 h later (Drittanti et al. 2001). TU (Transducing Units) for GFP expressing cells was measured with FACS analysis also 20 h after transduction. This titration method has a variability of 25–30% (n = 17).

Results

Three different producer cell lines were available for this study. However most experiments were realised only with one clone and the main results were subsequently validated with the other clones. Thus the results presented below concern RC32LacZSF yet they are also valid for RC32GFPSF and ROS26Z9.

Cells were easily adapted to growth in serum free (SF) medium

The adaptation to the HeLa SF medium (JRH) was performed directly for the RC32GFP clone and gradually (with sequential weaning steps from 10% to 5, 2.5, 1 and 0% FCS) for the lacZ clones. Growth kinetics have been realised in SF medium in order to check that it was adapted for these cells (Figure 1). The growth rate was slightly lower (generation time = 38 h vs. 30 h) than in serum containing medium. After 4–5 days, cell densities beyond 2×10^6 cells/ml with a viability above 90% were obtained. Cells grew as a unicellular suspension in spinner flasks. We thus considered that the growth was satisfying enough to analyse AAV production.

The low vector yield in SF medium can be increased by using un-supplemented DMEM

The first results in SF medium indicated that this medium was insufficient to support vector production. However, the comparison to normal un-supplemented DMEM gave an interesting aspect (Figure 2a). Depending on the time the medium was changed, we could significantly increase the vector titer. The best condition observed was to infect cells in SF medium and to replace it by DMEM 24 h later. We further investigated whether this positive effect was an artefact due to the centrifugation step or the addition of fresh medium (Figure 2b). When SF medium was renewed 24 h after infection, the vector yield was still very low. When fresh DMEM was added to the SF medium, its effect was not as important as when it was completely replaced. Thus, it was established that the medium had to be changed 24 h post-infection. These results strongly suggested that DMEM was necessary and HeLa SF medium could interfere with its positive effect.

These results were first observed in 6-wells plates, under static conditions. We realised similar experiments in spinner flasks in order to check that this effect was independent of the culture vessel used (Figure 3). We observed a 10-fold increase in the titer when changing the SF medium to DMEM 24 h post-infection. Thus the medium requirements for optimal vector production seemed to be the same for suspension as for static cultures.

No further supplementation is necessary for an optimal DMEM effect

The previous results demonstrated the interest to use DMEM but we had no information about which component was precisely necessary for a

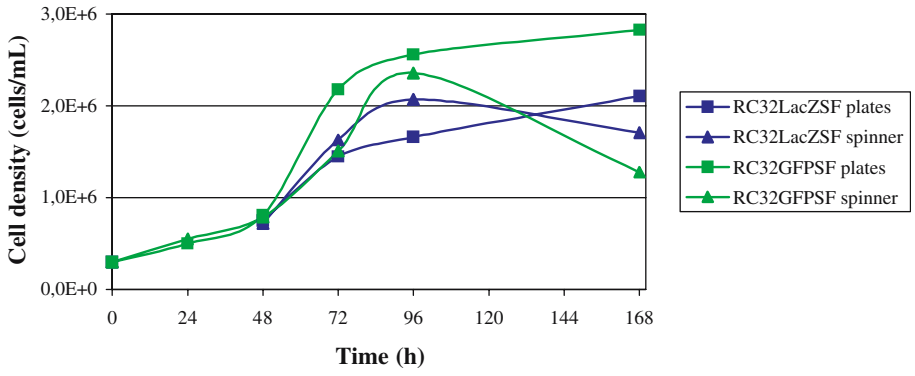

Figure 1. Growth kinetics during 7 days for 2 clones in static conditions in 6-wells plates (■) and in suspension in agitated spinner flasks (▲). Cells were seeded at 3×10^5 cells/ml either in 6-wells plates or in spinner flasks (50 ml). They were counted daily during 1 week.

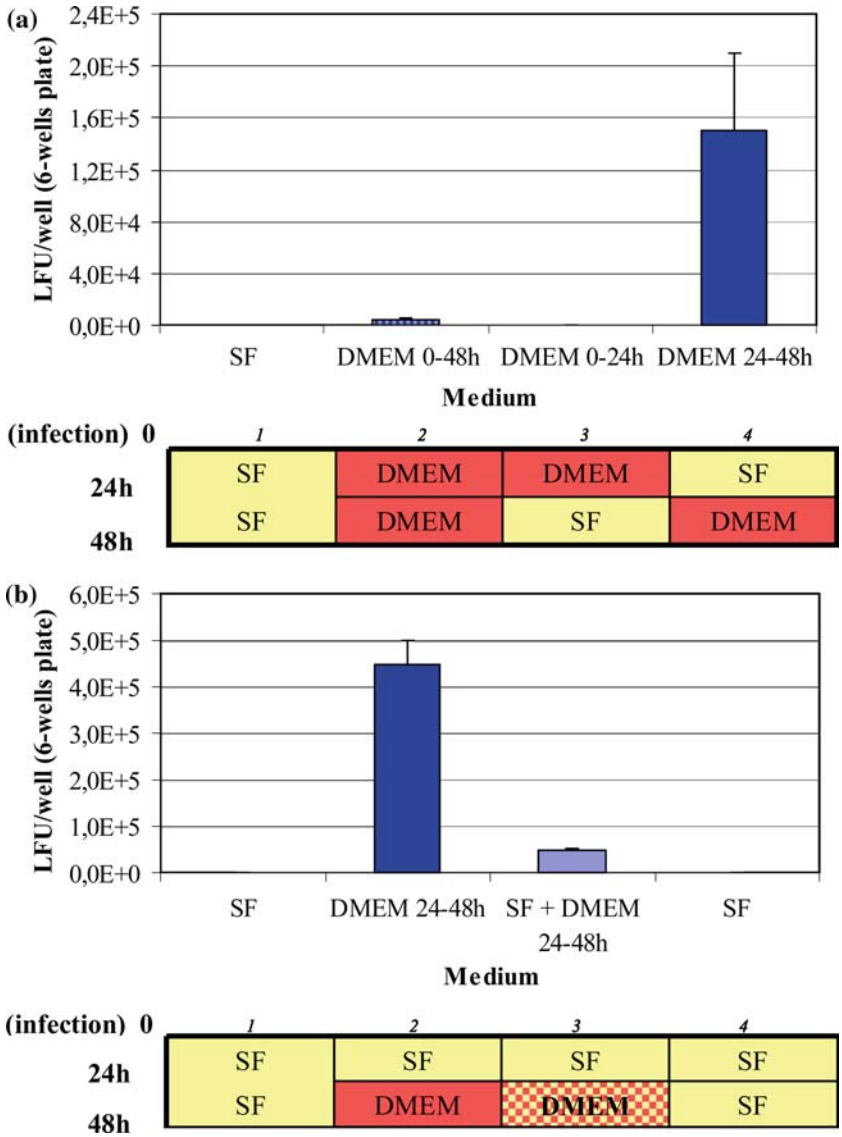

Figure 2. Vector titer of RC32LacZSF in SF medium or in DMEM. Cells were seeded in 6-wells plates in SF medium (5×10^5 cells/ml) and infected on the following day. The medium was changed or not, by centrifugation, for DMEM as described in the table below the graph. (a) The medium was not changed in (1); SF medium was replaced by DMEM at the time of infection (2–3) or 24 h later (4); DMEM was replaced by SF 24 h post-infection (3). (b) The medium was not changed in (1); SF was replaced by DMEM (2) or fresh SF (4) 24 h post-infection; DMEM was added to SF 24h post-infection (3). Each condition was realised in triplicate.

productive infection. We tried to identify which elements were critical for an optimal production and may lack in the SF medium. As it was impossible to know the precise composition of this commercial medium, we decided to investigate different additives: lipids, amino acids, trace elements or vitamins were added to the SF medium or to the DMEM (Figure 4), 24 h post-infection. Concerning the SF medium, no additive was able to increase the efficiency of this medium: the yield was still nearly zero. Regarding the DMEM, none of the supplements added was able to enhance the effect of the DMEM alone. Thus, these elements did not seem to be limiting for the vector production.

To complete the data on cell requirements for rAAV production, we evaluated the influence of the glucose concentration. Actually, in the classical

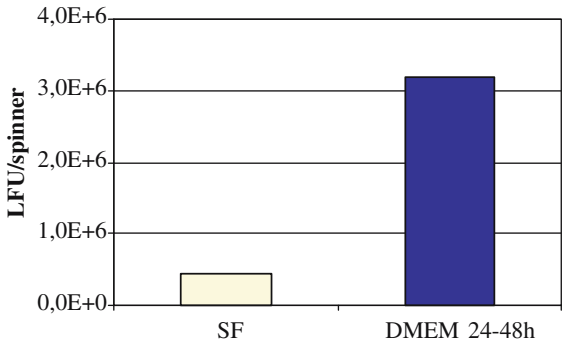

Figure 3. Effect of DMEM in spinner flasks. Cells were seeded in a 50 ml-spinner flask in SF medium (5×10^5 cells/ml) and cultured in suspension. They were infected on the following day. The medium was changed by centrifugation for DMEM 24 h later (DMEM 24–48 h) or not (SF). One spinner was seeded for each condition and the experiment was realised twice.

triple transfection protocol, glucose concentration is reduced (from 4.5 to 2.3 g/l) during the production phase in order to increase the final titer. We compared different glucose concentrations in the DMEM (Figure 5) and observed similar yields in a range of 1–4.5 g/l glucose. At lower glucose concentration, the titer dropped significantly. These results indicate that the glucose was neither limiting nor inhibiting in DMEM but was necessary for vector production.

Finally, we investigated the role of calcium ions. The calcium concentration is classically reduced in serum-free media in order to avoid cell clumping. In the SF medium, it was only 8 mg/l, compared to 264 mg/l in DMEM. This difference could explain the decrease in vector yield. The comparison of SF and DMEM with or without calcium (Figure 6) indicated that SF medium supplemented with $CaCl_2$ (to reach the same concentration as in DMEM) did not provide better results. On the other hand, when $CaCl_2$ was not present in DMEM, the vector titer decreased by 2-logs. These observations suggest that calcium was not a limiting element in the SF medium but was essential for an optimal production.

The use of a defective adenovirus can avoid the production of wtAd

One of the major disadvantages of producer cell lines is the use of wtAd, which means a mixed production of rAAV particles and wt helper virus particles. We decided to evaluate the efficiency of a

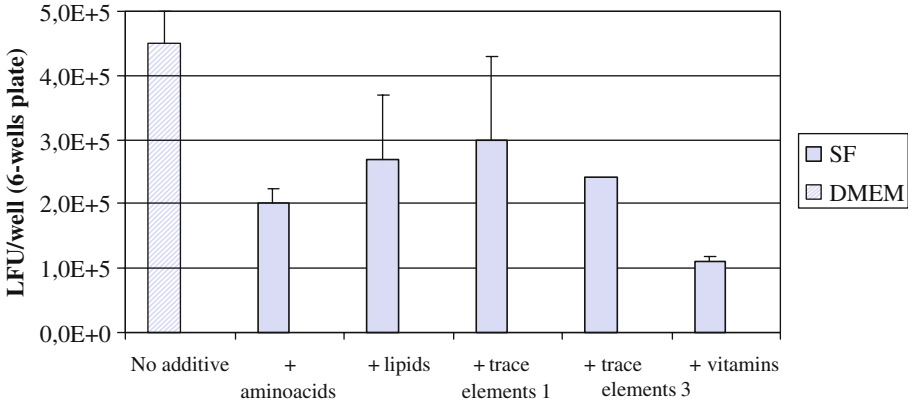

Figure 4. Effect of different supplements on SF and DMEM media. Cells were seeded in 6-wells plates in SF medium (5×10^5 cells/ml) and infected on the following day. *SF*: The SF medium was complemented 24 h post-infection with non essential amino-acids, lipids, trace elements or vitamins (striated bars on the right: they are not apparent as their value is nearly zero). *DMEM*: The SF medium was changed 24 h postinfection for DMEM, alone or supplemented with non essential amino-acids, lipids, trace elements or vitamins (plain bars, on the left). Cells were harvested as described.

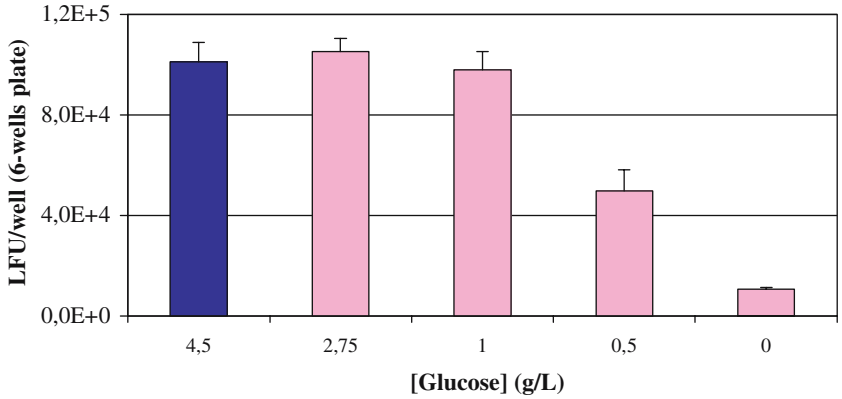

Figure 5. Influence of glucose concentration in DMEM. Cells were seeded in 6-wells plates in SF medium (5×10^5 cells/ml) and infected on the following day. The SF medium was replaced 24 h post-infection by DMEM, with different concentrations of glucose. The glucose concentration range was realised by mixing DMEM with 4.5 g/l glucose and DMEM without glucose. Cells were harvested as described.

replication defective adenovirus: a protease deleted adenovirus (ΔPSAd) (Oualikene et al. 2000). We compared both helper viruses at different MOIs (Figure 7). The optimal MOI for wtAd was 35, as already determined for producer cells cultivated in a serum supplemented medium (Tessier et al. 2001; Mathews et al. 2002). Concerning ΔPSAd, optimal MOI was around 50 and the highest titer obtained was about 2 fold lower when compared to the use of wtAd (Figure 7).

Production and harvest kinetics

AAV production was analysed with respect to the optimal time to infect the cells with wtAd, change the medium and harvest the final pellet (Figure 8). Cells were grown during 5 days and this period comprised all phases of a batch culture, from the initial lag phase, over the exponential growth phase up to the stationary phase. The optimal time of harvest appeared to be independent of the time of infection. We observed that the titer tended to increase while the cells were in an advanced culture phase. This was probably due to the growing biomass. The optimal harvest time was between 48 h and 72 h after infection and 96 h appeared definitively too late for obtaining a reasonable rAAV titer. This decrease can be explained by a loss of vector into the supernatant due to extended cell lysis beyond 72 h post infection (data not shown). This phenomenon is probably limited in

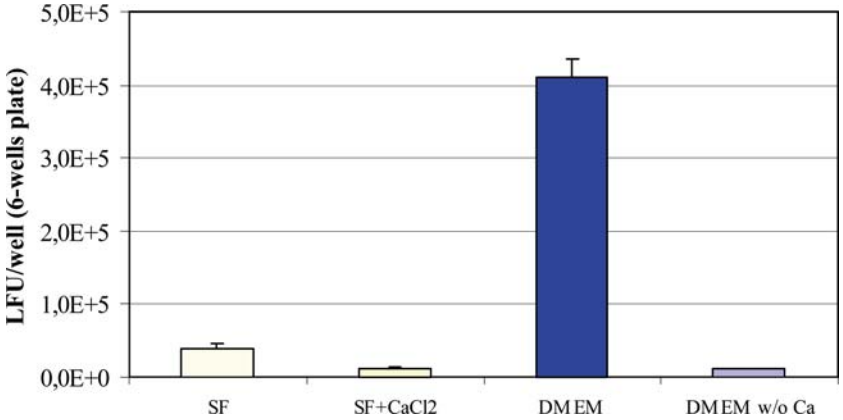

Figure 6. Influence of calcium concentration on vector yield. Cells were seeded in 6-wells plates in SF medium (5×10^5 cells/ml) and infected on the following day. When indicated, the SF medium was replaced 24 h post-infection by DMEM, with (basic concentration = 264 mg/ml) or without calcium. SF + $CaCl_2$: SF medium was supplemented with 264 mg/ml $CaCl_2$, 24 h post infection.

static conditions and enhanced in suspension in spinner flasks. Actually, under suspension conditions, we measured the vector titer in the cell pellet as well as in the supernatant. We observed an increase of the vector titer in the supernatant with the time: 48 h post-infection, the ratio of vector in the supernatant to vector in the cell pellet was zero and increased to about 4% at 72 h. Harvesting beyond 72 h post-infection led to an important ratio of rAAV in the supernatant and, in general, to a loss of vector (data not shown).

The production protocol can be scaled up to a 100 ml spinner and is applicable to several producer clones

The protocol, developed and optimised in 6-wells plates, has been transferred to a spinner scale. Cells were seeded in the same way and the major difference was the agitation conditions as cells grew entirely in suspension. The vector yield, expressed as LFU per ml of culture, was the same under agitated and static conditions (Figure 9), indicating that a scale up is easy and that the cells behave similarly in plate and in spinner cultures.

This optimised protocol has also been applied to other clones in order to verify that it was valid for different producer cells. Another LacZ clone and a GFP producer clone were evaluated using the same optimised protocol (Table 1). As for the RC32LasZSF cells, it was also necessary for these producer cells to switch from the SF medium to DMEM 24 h post infection in order to get to rAAV production. Thus, this phenomenon was not clone nor transgene dependent.

Comparison of the serum-free protocol to a classical serum-complemented process

In order to value the titer of SF clones, we compared the yields with those obtained with the same producer cells cultivated in a serum containing medium. Furthermore, it was interesting to refer to our traditional triple-transfection protocol using 293 cells (Table 2). Concerning the producer cells, we observed that the vector yields were maximally 2–3 fold lower under SF conditions than in classical serum supplemented method. Moreover, regarding the use of serum containing media, the 'reference' method utilising triple-transfection provided similar vector yields as the producer cells.

Discussion

Although, the traditional transfection approaches are widely used for the production of research but also clinical grade rAAV vectors, they are still hampered by a cumbersome and scale limited procedure. Even if transient transfection methods using 293 cells under SF conditions have been successfully transposed to a bioreactor scale for the production of recombinant proteins (Meissner et al. 1999; Schlaeger and Christensen 1999; Wurm

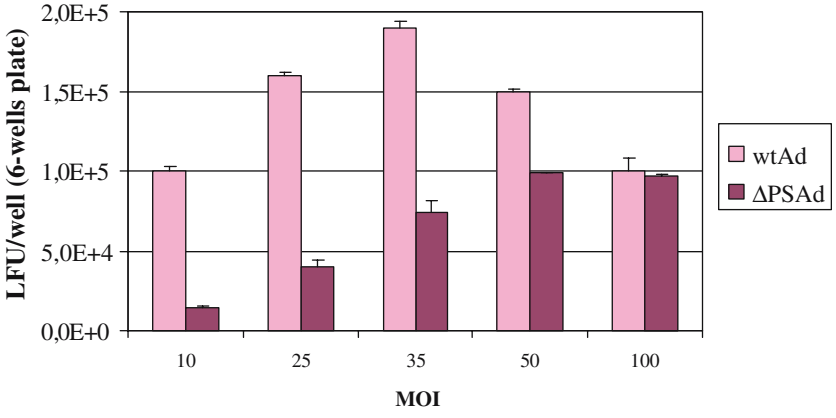

Figure 7. Comparison of wtAd and AdΔPS, as helper viruses. Cells were seeded in 6-wells plates in SF medium (5×10^5 cells/ml) and infected with the respective helper virus on the following day, as described in Materials and methods. MOIs tested were 10, 25, 35, 50 and 100.

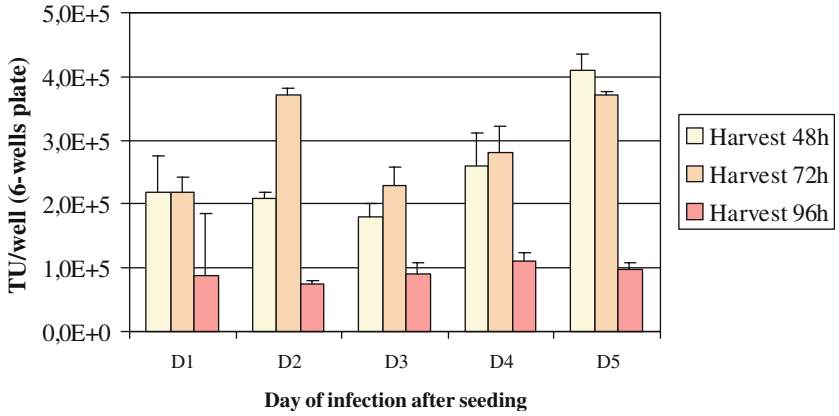

Figure 8. Evaluation of wtAd. RC32GFPSF were seeded in 6-wells plates in SF medium (5×10^5 cells/ml) at day 0. They were infected on day 1, 2, 3, 4 or 5. The medium was changed for DMEM 24 h post-infection. Cells were harvested 48 h or 72 h or 96 h after infection.

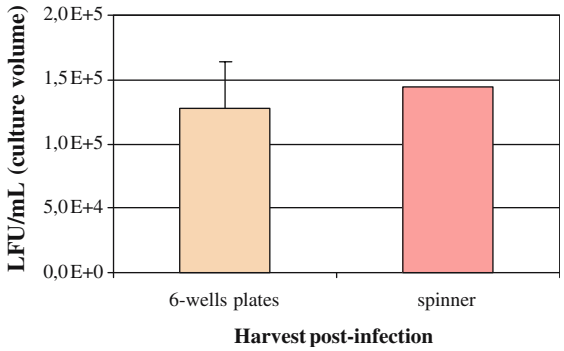

Figure 9. Scale up from 6-wells plates to a 100 ml spinner flasks. Cells were seeded in 6-wells plates and in a 100 ml spinner, in SF medium (5×10^5 cells/ml). They were infected on the following day. The medium was changed for DMEM 24 h postinfection. Cell suspension was harvested 48 h post infection. Results were calculated per volume of culture. Standard deviation is not represented for spinner results because only one spinner was seeded per experiment.

and Bernard 1999; Durocher et al. 2002), they remain difficult to be considered for viral vectors production. In addition, under GMP conditions, for clinical applications, the transient transfection methods require the certification of several components (plasmid DNA, transfection reagents and cell lines) and it is known that these methods are associated with recombination events leading potentially to rcAAV (Inoue and Russell 1998), which is a safety concern. Thus transfection independent methods are of high interest. In order to develop a process able to satisfy clinical grade and scale requirements, we evaluated the growth and production potential of rAAV producer cell lines in a SF medium. On one hand, as already demonstrated (Gao et al. 1998; Liu et al. 1999; Liu et al. 2000; Chadeuf et al. 2000; Gao et al. 2002), the single infection step with an helper virus to induce rAAV production is easier to scale up and on the other hand, the use of serum free medium greatly improves the bio-safety profile of the final product.

Three different producer clones, generated in 2 different labs (Chadeuf et al. 2000; Mathews et al., 2002), were available for the present evaluation

Table 1. Comparison of the vector yield for 3 different producer cell lines in SF medium.

Clone	SF medium	Medium change 24 h post-infection
RC32LacZSF	0 LFU/well	3 10^5 LFU/well
RC32GFPSF	4 10^4 TU/well	5 10^5 TU/well
ROS26Z9SF	3 10^4 LFU/well	5 10^5 LFU/well

Cells were seeded, infected and harvested following the same protocol described in Materials and methods.

Table 2. Comparison of the vector yields for 2 producer cell lines in medium with or without serum and triple-transfected 293 cells.

	LFU/well	Cells/well
293 (tri-transfection)	1E6	6E5
RC32LacZ	7E5	1E6
RC32LacZSF	3E5	1E6
RC32GFP	1E6	3E5
RC32GFPSF	5E5	1E6

Producer cells were seeded, infected and harvested following the same protocol described in Materials and methods. The triple-transfection protocol for 293 cells is also described in Materials and methods.

and showed similar behaviour. They were adapted to a commercial serum free medium (HeLa SF medium from JRH Biosciences), specifically developed for suspension growth of HeLa cells and which was also appropriate for our HeLa derived clones. Cells grew in a single cell suspension in static as well as in agitated cultures, with a satisfying doubling time (38 h). However, the medium was apparently not compatible with acceptable rAAV production.

Nevertheless it was possible to restore vector production by replacing the SF medium by unsupplemented DMEM 24 h after infection. This positive effect was specific for DMEM and not due to the centrifugation step or the addition of fresh medium to the culture. Moreover, the optimal time to change the medium was evaluated at 24 h post-infection.

We hypothesised that the SF medium was limiting for vector production either because of the lack of some essential or the presence of inhibiting components. We investigated a potential deficiency but could not identify any substance (amino acids, vitamins, lipids or trace elements) which was able to improve the SF medium or enhance the DMEM effect. In addition, the comparison with other basal media of known composition did not allow the identification of any critical parameter for viral production (data not shown). Otherwise, we observed that the glucose concentration of the DMEM was adequate in a range of 1–4.5 g/l. On another hand, calcium was demonstrated to be necessary but not sufficient for vector production. Actually, the unknown composition of the SF medium did not allow us to precisely investigate specific medium components and explain why the DMEM was more adequate for vector production than SF medium. We were only able to outline some important elements like glucose or calcium.

We also studied the vector production kinetics in order to define optimal times for adenoviral infection and harvest. The vector titer was not influenced by the culture phases (thus it is independent of the physiological state of the cells) up to 5 days after seeding. This was not observed with similar producer cells in classical serum complemented medium: vector titer is stable for cells cultivated during 24 h before infection but dramatically drops beyond 48 h. As for the classical triple transfection method, the optimal harvest time was 48–72 h after induction of rAAV production. Beyond this time, we observed an enhanced mortality in the culture supernatant due to an advanced cytopathic effect of the wtAd. This resulted in an increased proportion of the vector loss in the supernatant. This phenomenon was more pronounced under agitated conditions, due to shear forces.

One of the limitations for the use of producer cells is the co-production of wt viral particles (Clark et al. 1995). This implies a downstream processing procedure able to remove contaminating viral particles and proteins. It thus seemed preferable to use replication-defective helper viruses. A protease deleted adenovirus (Oualikene et al. 2000) was evaluated and appeared less efficient than wtAd (2-fold). Despite this titer reduction, its use is of potential interest because no infectious adenoviral particles were detectable although viral proteins were present in the crude lysate (data not shown).

Wild type HSV is another efficient helper virus for rAAV production (Weindler and Heilbronn 1991). Some advantages of its use are a quicker production (the production peak is observed around 24 h instead of 48 h post-infection) and a lower optimal MOI (5), when compared to adenovirus (Toublanc et al. 2004). Although under SF conditions, similar kinetics and rAAV yields as under serum-containing conditions were obtained (data not shown), studies with wtHSV were discontinued due to the potential risk linked to contaminating wtHSV particles in the final product (safety concern).

The protocol developed was scaled up to a 100 ml spinner flask. We demonstrated that it was

not specific for the producer clone studied: on one hand, it was applicable to LacZ and GFP producer cells established and cloned under the same conditions (Chadeuf et al. 2000). On the other hand, it was also reproducible for another producer cell line generated in another lab and following another cloning protocol (the main difference relies on the second cloning step which was realised by transfection for RC32-cells (Chadeuf et al. 2000) and transduction of rAAV for ROS26SZ9 cells (Mathews et al. 2002). Moreover, even if the titers seemed rather low, with our titration protocol, we obtained greatly similar yields for the cells cultivated in the SF medium or FCS complemented DMEM. As our major aim was to evaluate rAAV production in a SF medium and establish a protocol transferable to a larger scale, we did not further investigate in order to explain the effect of DMEM on rAAV production. The most important result from this study was to demonstrate the feasibility to produce AAV vectors in a serum-free environment.

However, this is not the first study on the use of SF media for the production of AAV vectors. The first publication concerned a transfection protocol (Smith et al. 2003) which does not seem scaleable. Recently, Farson et al (2004) realised an interesting work with A549 derived producer cells in SF medium, cultivated up to a 15 l scale reactor. Although the scale-up was promising, no information on the SF medium was provided (composition, manufacturer…).

Therefore we consider that the present study is the first one which gives all essential details for establishing the production of rAAV using producer cell lines under serum-free conditions. Despite the demonstrated scale up is limited to a 100 ml spinner flask, it can reasonably be expected to easily transfer the protocol to a small scale bioreactor. The main potential issue concerns the medium change 24 h post-infection to replace HeLa SF medium by the un-supplemented DMEM. Equipments such as the continuous centrifuge "Centritech" (Grönvik et al. 1989) or the ultrasound retention device from Applicon (Trampler et al. 1994) can be considered here. Moreover the use of such a perfusion system is also the base for the development of high density cultures. Actually, the increase of the production titer can be performed either by a classical scale up of the reactor size, or by increasing the culture cell density. A higher cell density would then lead to a higher and improved volumetric productivity.

In conclusion, we have developed an AAV vectors production protocol using producer cells in a SF environment, which is as efficient as the same production system using a serum supplemented medium. Moreover, in our hands the vector production with these stable cell lines is equivalent to our reference protocol based on the triple transfection of 293 cells. In addition, this present infection based protocol has a much more promising scale-up potential than transfection methods. Although only a 100 ml spinner scale was studied for the moment, scale-up to a laboratory scale reactor is straight forward and only a question of some additional optimisation work.

Acknowledgements

This work was performed with the financial support of the Association Française contre les Myopathies (AFM). We thank Dr Philippe Moullier for providing us the RC32LacZ and RC32GFP clones and HSV preps.

References

Aitken M.L., Moss R.B., Waltz D.A., Dovey M.E., Tonelli M.R., McNamara S.C., Gibson R.L., Ramsey B.W., Carter B.J. and Reynolds T.C. 2001. A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease. Hum. Gene Ther. 12: 1907–1916.

Blouin V., Brument N., Toublanc E., Raimbaud I., Moullier P. and Salvetti A. 2004. Improving rAAV production and purification: towards the definition of a scaleable process. J. Gene Med. 6(Suppl 1): S223–S228.

Brown P., Barrett S., Godwin S., Trudinger M., Marschak T., Norboe D., Mcquiston S. and Kurtzman G. 1998. Optimization of production of adeno-associated virus (AAV) for use in gene therapy. Presented at: Cell Culture Engineering VI. San Diego/CA.

Chadeuf G., Favre D., Tessier J., Provost N., Nony P., Kleinschmidt J., Moullier P. and Salvetti A. 2000. Efficient recombinant adeno-associated virus production by a stable rep-cap HeLa cell line correlates with adenovirus-induced amplification of the integrated rep-cap genome. J. Gene Med. 2: 260–268.

Clark K.R., Voulgaropoulou F., Fraley D.M. and Johnson P.R. 1995. Cell lines for the production of recombinant adeno-associated virus. Hum. Gene Ther. 6: 1329–1341.

Cote J., Bourget L., Garnier A. and Kamen A. 1997. Study of adenovirus production in serum-free 293SF suspension

culture by GFP-expression monitoring. Biotechnol. Prog. 13: 709–714.

Drittanti L., Jenny C., Poulard K., Samba A., Manceau P., Soria N., Vincent N., Danos O. and Vega M. 2001. Optimised helper virus-free production of high-quality adeno-associated virus vectors. J. Gene Med. 3: 59–71.

Durocher Y., Perret S. and Kamen A. 2002. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 30: E9.

Farson D., Harding T.C., Tao L., Liu J., Powell S., Vimal V., Yendluri S., Koprivnikar K., Ho K., Twitty C., Husak P., Lin A., Snyder R.O. and Donahue B.A. 2004. Development and characterization of a cell line for large-scale, serum-free production of recombinant adeno-associated viral vectors. J. Gene Med. 6: 1369–1381.

Gao G.P., Lu F., Sanmiguel J.C., Tran P.T., Abbas Z., Lynd K.S., Marsh J., Spinner N.B. and Wilson J.M. 2002. Rep/Cap gene amplification and high-yield production of AAV in an A549 cell line expressing Rep/Cap. Mol. Ther. 5: 644–649.

Gao G.P., Qu G., Faust L.Z., Engdahl R.K., Xiao W., Hughes J.V., Zoltick P.W. and Wilson J.M. 1998. High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. Hum. Gene Ther. 9: 2353–2362.

Graham F.L. and Prevec L. 1991. Manipulation of adenovirus vectors. In: Murray E.J. (ed.), Gene Transfer and Expression Protocols, Vol. 7. Humana Press, Clifton, New Jersey, pp. 109–128.

Griffiths B. 2001. Scale-up of suspension and anchorage-dependent animal cells. Mol. Biotechnol. 17: 225–238.

Grönvik K.-O., Frieburg H. and Malmström U. 1989. Centritech Cell – a new separation device for mammalian cells. In: Spier R.E., Griffiths J.B., Stephenne J. and Crooy P.J. (eds), Advances in Animal Cell Biology and Technology for Bioprocesses. Butterworths, Sevenoaks/U.K, pp. 428–433.

Inoue N. and Russell D.W. 1998. Packaging cells based on inducible gene amplification for the production of adeno-associated virus vectors. J. Virol. 72: 7024–7031.

Kay M.A., Manno C.S., Ragni M.V., Larson P.J., Couto L.B., McClelland A., Glader B., Chew A.J., Tai S.J., Herzog R.W., Arruda V., Johnson F., Scallan C., Skarsgard E., Flake A.W. and High K.A. 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat. Genet. 24: 257–261.

Leonard C.J. and Berns K.I. 1994. Adeno-associated virus type 2: a latent life cycle. Prog. Nucleic Acid Res. Mol. Biol. 48: 29–52.

Liu X., Voulgaropoulou F., Chen R., Johnson P.R. and Clark K.R. 2000. Selective Rep-Cap gene amplification as a mechanism for high-titer recombinant AAV production from stable cell lines. Mol. Ther. 2: 394–403.

Liu X.L., Clark K.R. and Johnson P.R. 1999. Production of recombinant adeno-associated virus vectors using a packaging cell line and a hybrid recombinant adenovirus. Gene Ther. 6: 293–299.

Liu Y.L., Wagner K., Robinson N., Sabatino D., Margaritis P., Xiao W. and Herzog R.W. 2003. Optimized production of high-titer recombinant adeno-associated virus in roller bottles. Biotechniques 34: 184–189.

Mathews L.C., Gray J.T., Gallagher M.R. and Snyder R.O. 2002. Recombinant adeno-associated viral vector production using stable packaging and producer cell lines. Methods Enzymol. 346: 393–413.

Matsushita T., Elliger S., Elliger C., Podsakoff G., Villarreal L., Kurtzman G.J., Iwaki Y. and Colosi P. 1998. Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther. 5: 938–945.

Meissner P., Girard P., Kulangara A., Tsao M.C., Jordan M. and Wurm F.M. 1999. Process development for transient gene expression in mammalian cells at the 3 L scale: 10–50 mg of r-protein in days. In: Bernard A. (ed), Animal cell technology: Products from Cells, Cells as Products. Kluwer Academic Publishers, The Netherlands, pp. 351–357.

Mizukami H., Okada T., Ogasawara Y., Matsushita T., Urabe M., Kume A. and Ozawa K. 2004. Separate control of Rep and Cap expression using mutant and wild-type loxP sequences and improved packaging system for adeno-associated virus vector production. Mol. Biotechnol. 27: 7–14.

Neyns B., Vermeij J., Teugels E., De Rijcke M., Hermonat P. and De Greve J. 2001. Characterization of permanent cell lines that contain the AAV2 rep-cap genes on an Epstein-Barr-virus-based episomal plasmid. Intervirology 44: 255–263.

Ogasawara Y., Mizukami H., Urabe M., Kume A., Kanegae Y., Saito I., Monahan J. and Ozawa K. 1999. Highly regulated expression of adeno-associated virus large Rep proteins in stable 293 cell lines using the Cre/loxP switching system. J. Gen. Virol. 80: 2477–2480.

Okada T., Mizukami H., Urabe M., Nomoto T., Matsushita T., Hanazono Y., Kume A., Tobita K. and Ozawa K. 2001. Development and characterization of an antisense-mediated prepackaging cell line for adeno-associated virus vector production. Biochem. Biophys. Res. Commun. 288: 62–68.

Oualikene W., Lamoureux L., Weber J.M. and Massie B. 2000. Protease-deleted adenovirus vectors and complementing cell lines: potential applications of single-round replication mutants for vaccination and gene therapy. Hum. Gene Ther. 11: 1341–1353.

Salvetti A., Oreve S., Chadeuf G., Favre D., Cherel Y., Champion-Arnaud P., David-Ameline J. and Moullier P. 1998. Factors influencing recombinant adeno-associated virus production. Hum. Gene Ther. 9: 695–706.

Schlaeger E.J. and Christensen K. 1999. Transient gene expression in mammalian cells grown in serum-free suspension culture. Cytotechnology 30: 71–83.

Smith R.H., Ding C. and Kotin R.M. 2003. Serum-free production and column purification of adeno-associated virus type 5. J. Virol. Methods 114: 115–124.

Snyder R.O. 1999. Adeno-associated virus-mediated gene delivery. J. Gene Med. 1: 166–175.

Tessier J., Chadeuf G., Nony P., Avet-Loiseau H., Moullier P. and Salvetti A. 2001. Characterization of adenovirus-induced inverted terminal repeat-independent amplification of integrated adeno-associated virus rep-cap sequences. J. Virol. 75: 375–383.

Toublanc E., Benraiss A., Bonnin D., Blouin V., Brument N., Cartier N., Epstein A.L., Moullier P. and Salvetti A. 2004. Identification of a replication-defective herpes simplex virus for recombinant adeno-associated virus type 2 (rAAV2) particle assembly using stable producer cell lines. J. Gene Med. 6: 555–564.

Trampler F., Sonderhoff S.A., Pui P.W., Kilburn D.G. and Piret J.M. 1994. Acoustic cell filter for high density perfusion culture of hybridoma cells. Biotechnology 12: 281–284.

Weindler F.W. and Heilbronn R. 1991. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. J. Virol. 65: 2476–2483.

Wurm F. and Bernard A. 1999. Large-scale transient expression in mammalian cells for recombinant protein production. Curr. Opin. Biotechnol. 10: 156–159.

Xiao X., Li J. and Samulski R.J. 1998. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J. Virol. 72: 2224–2232.

Zhao N., Liu D.P. and Liang C.C. 2001. Hot topics in adeno-associated virus as a gene transfer vector. Mol. Biotechnol. 19: 229–237.