Production of Retroviral Vectors for Gene Therapy with the Human Packaging Cell Line FLYRD18

Patrick A. Gerin,[†] Peter F. Searle,[‡] and Mohamed Al-Rubeai*[,†]

School of Chemical Engineering, and Institute for Cancer Studies, University of Birmingham, Edgbaston, Birmingham B15 2TT, England

The development of gene therapy is hampered by the difficulty of producing large stocks of retroviral vectors at high titer. This study aimed to improve culture conditions and to intensify the production of retroviruses by FLYRD18, a packaging cell line derived from the HT1080 human fibrosarcoma line. Batch virus production proved to be feasible in unsupplemented basal medium and provided significantly higher titers and productivities than medium supplemented with 10% serum. For longer-term production, however, AIM-V complete serum-free medium and basal medium supplemented with 2% serum gave superior results. Serum supplementation should nevertheless be optimized to take into account the presence of inhibitors of viral production. In monolayer cultures with 0.2 mL/cm^2, the cell concentration was increased up to 2×10^6 cells/mL without loss of cell productivity. A semicontinuous production process, which enables the collection of larger amounts of viruses from the same culture, has also been successfully used. Suspension culture processes were prevented by the anchorage dependency of the FLYRD18 cell line. Microcarrier cultures were able to produce viruses but will require further investigation and optimization for their performance to become competitive with monolayer cultures. In the course of this study, more than a 10-fold increase of titer has been achieved.

Introduction

Gene therapy is a developing therapeutic technology which promises to revolutionize the practice of medicine. Its full-scale application will nevertheless be hampered by the need for large amounts of vectors at reasonable cost. Such vectors are required to ensure the transportation, insertion, and expression of "therapeutic" genetic material into the patient cells, either in-vivo or ex-vivo. At present, recombinant retroviral vectors are being employed in most of the clinical trials. Production of retroviral vectors is ensured by specific "packaging" cell lines, that is, cell lines genetically modified to synthesize replication defective retroviral vectors with the required host range. The FLYRD18 packaging cell line has recently been developed by introducing the Moloney Murine Leukaemia (MoMLv) virus *gag-pol* gene and the cat endogenous virus RD114 *env* gene into HT1080 human fibrosarcoma cells (Cosset et al., 1995). The *gag-pol* gene codes for the various capsid proteins and viral enzymes, while the *env* gene codes for the envelope protein, which determines host range specificity (Coffin, 1990; Nermut and Hockley, 1996). The FLYRD18 packaging cell line offers the advantage of producing a high titer of retroviral vectors resistant to inactivation by anti-Gal-α(1−3)Gal antibodies and complement present in human serum (Takeuchi et al., 1996). The risk of producing replication competent retroviruses has also been reduced by minimizing the viral sequences and by using separate expression cassettes for the *gag-pol* and *env* genes. Onodera et al. (1998) have shown that FLYRD18-derived vectors provide a higher level of adenosine deaminase expression in human cells, as compared to vectors derived from murine cells. In addition, FLYRD18 cells are closely related to FLYA cells (they differ only by the *env* gene), which have been shown to produce retroviral particles far less contaminated by endogenous viral sequences than murine packaging cells (Patience et al., 1998). Thus, the FLYRD18 cell line offers great potential for the development of human gene therapy.

Up to now, the production of retroviral vectors by the FLYRD18 cell line has been performed mainly at the scale of T-flasks with titers in the range 10^5-10^7 infectious virus per milliliter (Cosset et al., 1995; Onodera et al., 1998). The application of gene therapy is currently estimated to require $10^{11}-10^{20}$ infectious particles per patient (Braas et al., 1996), that is, the production, handling, and processing of a few tens of liters to a few tens of cubic meters of virus suspension to treat each patient. With respect to the number of potential patients, there is a great challenge in scaling-up and intensifying the production of retroviral vectors at the bioreactor scale.

Most animal cell lines require supplementation of their culture medium with serum. Serum is an expensive raw material, and its presence in the culture medium where the viral vectors are secreted will increase the complexity, duration, and cost of downstream processing (purification, concentration, and recovery). Longer purification processes can lead to reduced efficiency due to the short retroviral half-life. Serum also presents a major risk of introducing biochemical and biological contaminants (proteins, hormones, viruses, prions, etc.). The development of serum-free processes would thus improve the efficiency of the production of retroviral vectors and make

* Corresponding author. Tel: 44-121 414 3888. Fax: 44-121 414 5324. E-mail: m.al-rubeai@bham.ac.uk.
[†] School of Chemical Engineering.
[‡] Institute for Cancer Studies.

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the validation of their safety much easier. Widely used murine packaging cell lines have been shown to require the presence of serum (Choi et al., 1997; Shen et al., 1996) or the development of specifically designed culture media (Glimm et al., 1998; Seppen et al., 1997). In previous work, we have demonstrated the feasibility of producing retroviral vectors with the FLYRD18 cell line in basal medium, in the absence of serum, with a significant improvement of the virus titer and virus/total protein ratio (Gerin et al., submitted for publication). The aim of this paper is to better identify the other requirements of the FLYRD18 packaging cell to identify the culture parameters most critical for transposing the production process from T-flask to bioreactor scale and to provide a rational background for further process optimization.

Materials and Methods

Chemicals and Medium. All cultures were performed in Dulbecco's Modified Eagle's Medium F-12 Ham nutrient mixture (DMEM/F12 1:1 mixture, Gibco), further referred to as Basal Medium (BM). When indicated, BM was supplemented with 10% heat-inactivated foetal calf serum (FCS). The virus production was also tested in RPMI1640, CHO-S-SFM-II, Hybridoma-SFM, and AIM-V culture media, all from Gibco. BM was sometimes supplemented with 1% insulin/transferrin/sodium selenite/ethanolamine mixture (Gibco), further indicated as ITS and providing the medium with 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 ng/mL sodium selenite, and 2.0 μg/mL ethanolamine.

Cell Lines. The FLYRD18/LNC-*hB7* packaging cell clone was derived from the HT1080/FLYRD18 line described by Cosset et al. (1995). For this purpose, the FLYRD18 cells were transduced with pLNCX-based vectors (Miller and Rosman, 1989) containing the neomycin resistance gene and the human B7/CD80 gene (Freeman et al. 1989; Linsley and Ledbetter, 1993; Daly and Gilligan, unpublished results) under the control of the internal human Cytomegalovirus (CMV) promoter. The A2780cp cisplatin-resistant human ovarian carcinoma cell line (Khokhar et al., 1992) was used as a target cell line for assessing virus titer. Human cells were used for virus titration, as the RD114 envelope does not allow virus entry into murine cells. The use of human cells is also more relevant to clinical applications.

Production of Retroviral Vectors in T-Flasks. All cultures were performed at 37 °C. Unless otherwise stated, the packaging cells were inoculated at a density of 2.0×10^4 cells/cm^2 in 25 cm^2 T-flasks (Nunc) and grown for 30 h in 10 mL of BM supplemented with 10% FCS (BM + 10% FCS). At this stage, they were nearly confluent. For batch experiments, the virus production phase was initiated by totally replacing the used medium with 10 mL of fresh medium, unless otherwise indicated, and the virus-containing supernatant was collected after 15−17 h incubation. For semicontinuous production, the virus production phase was initiated by totally replacing the used medium with 5 mL of fresh medium. The virus-containing supernatant was then harvested every day and totally replaced with 5 mL of fresh medium, unless otherwise indicated. Immediately after harvest, the retroviral vectors were separated from cells and cell debris by filtration through 0.45 μm pore size filters. When titration was not performed directly at the time of virus collection, 1 mL aliquots of the filtered supernatants were stored in liquid nitrogen and thawed at 37 °C immediately prior to titration. During virus production experiments, the intermediate cell growth was determined in independent flasks run in parallel.

Microcarrier Cultures. Cultures were performed at 37 °C in 100 mL Duran bottles containing a 30 mm magnetic stirrer bar suspended 5 mm above the bottom surface and containing 30 mg dry weight of either Cultispher-G (Percell Biolytica) or Cytopore-1 (Pharmacia). The packaging cells were inoculated in 5 mL of BM + 10% FCS and manually agitated for few seconds every 30−45 min for 3 h. The medium volume was then adjusted to 14 mL, and the stirring speed set at 20 rpm. After overnight incubation, stirring was set at 60−80 rpm, just enough to maintain the microcarriers in suspension. Twelve out of the 14 mL of the virus-containing supernatant were harvested daily and replaced with fresh medium. The harvested supernatant was handled for virus titration as indicated above.

Titration of Retroviral Vectors (Infectivity Assay). Determination of vector titers involved infection of A2780cp target cells with the vector-containing supernatants and selection with Geneticin (Gibco). Under this selection procedure, only cells infected by the vector and expressing the neomycin resistance gene survived and formed colonies. Under the standard conditions, A2780cp cells were inoculated at a density of 1.0×10^4 cells/cm^2 in 6-well plates (Nunc) and incubated overnight in 2 mL of BM + 10% FCS. The spent medium was then replaced by 1.0 mL of virus supernatant diluted 1:10^2 to 1:10^4 in BM containing 8 μg/mL Polybrene (Sigma). After 6−10 h of incubation, 2 mL of BM + 10% FCS with 1125 μg/mL Geneticin was added to each well. This medium was replaced by 2 mL of fresh BM + 10% FCS with 750 μg/mL Geneticin after 5 days. The number of colonies which resisted Geneticin were counted 11 days post-infection after fixing and staining the cells in 70% aqueous methanol + 0.3% Crystal Violet. All virus production experiments were performed at least in duplicate, and every supernatant was titrated in duplicate. Only titers measured at the same time in the same experiment are presented together on one graph. Unless otherwise stated, error bars represent the range of titers measured for a given supernatant.

Results

1. Batch Virus Production in Monolayer Culture.

1.1. Influence of Culture Medium. In a previous study, we demonstrated that virus production is feasible in unsupplemented basal medium (Gerin et al., in press). To further investigate the adequacy of nutrient supply during the virus production phase, various commercial serum-free media were tested and compared to DMEM/F12 supplemented with 10% FCS. Figure 1 shows the titers and productivities supported by these media sorted by order of increasing complexity with respect to their content in proteins and growth factors. According to the supplier's specifications, DMEM/F12 and RPMI1640 are free of proteins and growth factors; Hybridoma-SFM contains 20 mg/L proteins consisting only of insulin and transferrin; CHO-S-SFM-II is a complete serum-free medium with less than 100 mg/L total protein; AIM-V is a proprietary formulation but contains sufficient growth factors and proteins, including 2500 mg/L albumin, to support the growth of many different cell types. Significantly higher virus titers were obtained under all serum-free conditions, as compared with BM supplemented with 10% serum. However, none of the tested serum-free media led to better titers or cell-specific production than BM or BM supplemented with insulin/transferrin mixture (BM + ITS). Media with increasing content of proteins and growth factors led to lower cell-specific

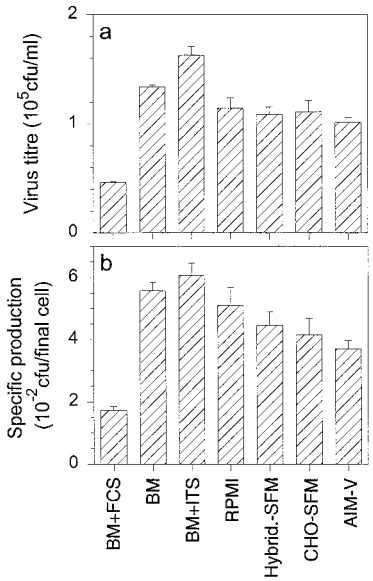

Figure 1. Batch virus production by monolayer cultures of FLYRD18/LNC-*hB7* packaging cells incubated for 17 h in various commercial serum-free media: DME/F12 (BM) + 10% FCS; DME/F12; DME/F12 + ITS; RPMI1640; Hybridoma-SFM; CHO-S-SFM-II; AIM-V. (a) Virus titer and (b) cell-specific production.

production. BM (DMEM/F12) was selected for further experiments.

1.2. Influence of Medium Volume. The influence of the volume of culture medium available to the cell during the virus production phase was investigated. The cells were grown under the standard conditions to form a monolayer in T-flask. The volume of basal medium during the production phase was then adjusted to get 0.1−0.6 mL/cm^2 of the cell monolayer. Figure 2 shows the virus titer and cell-specific production reported as a function of the cell concentration per unit of medium volume. Reducing the medium volume increased the virus titer proportionally to the cell concentration while affecting neither the cell-specific production nor the total production. The culture medium became orange indicating acidic pH only at the highest cell concentration. Supplementing the medium with 10% FCS had a negative effect on production at all cell concentrations.

1.3. Influence of Cell Density. As another way of intensifying the production process, we investigated the influence of the inoculated cell density per unit surface area on the virus production in BM and in BM + 10% FCS. Figure 3 shows that the virus titer increased with the number of cells per unit area. In the absence of serum, the cell-specific production was not markedly influenced by the cell density. In the presence of FCS, the cell-specific production increased with cell density but remained lower than that under serum-free conditions.

2. Semicontinuous Virus Production in Monolayer Culture. ***2.1. Unsupplemented BM.*** The feasibility of repeating virus production phases with cells maintained in the absence of serum was tested. Cells initially grown in BM + 10% FCS were incubated overnight in fresh BM. The virus-containing supernatant was then harvested every day and totally replaced by fresh BM. Figure 4, parts a and b, shows the initial and final cell density for a 6-day experiment, the virus titer, and the glucose, glutamine, lactate, and ammonia concentrations in the supernatant harvested every day. The daily virus production increased initially but decreased after the third day, while the cells continued to multiply

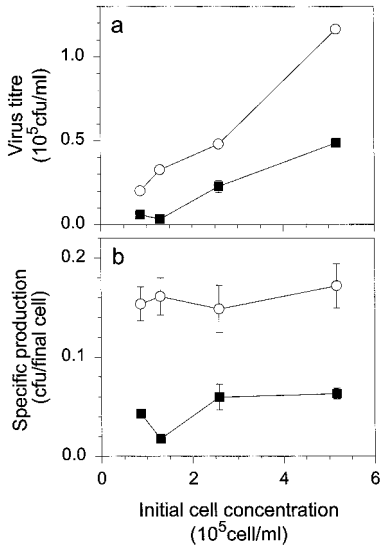

Figure 2. Batch virus production by cell monolayers prepared in parallel and subsequently incubated for 17 h with volumes of BM (open symbol) or BM + 10% FCS (closed symbol) decreasing from 0.6 to 0.1 mL/cm^2. Results are reported as a function of the cell concentration per volume of culture medium.

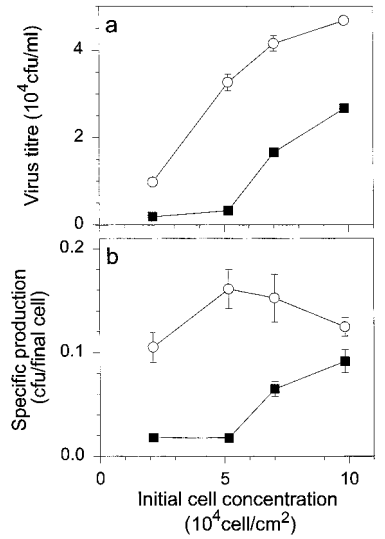

Figure 3. Batch virus production in monolayer culture: influence of cell concentration per unit surface area at the beginning of the production phase on the virus titer (a) and cell-specific production (b) for cells incubated for 17 h in BM (open symbol) and BM + 10% FCS (closed symbol).

as indicated by the final cell density (Figure 4a), by observations under the microscope during the experiment, and by the increasing amount of nutrients utilized every day (Figure 4b). Increasing numbers of dead cells were found in suspension, but their concentration remained below 10^4 cells/mL, that is, less than 1% of the total attached cell concentration.

2.2. Supplemented Media. Figure 4, parts c and d, shows a parallel experiment performed in BM supplemented with ITS. As compared with BM (Figure 4a), insulin/transferrin supplementation initially enhanced the virus production but was not able to enhance the longer-term production. To test whether the decreasing virus production was the result of nutrient and/or growth factor depletion, we measured 6-day virus production kinetics in Hybridoma-SFM, CHO-S-SFM-II, AIM-V, and BM + 2% FCS. AIM-V and BM + 2% FCS supported both cell growth and increasing virus production over the

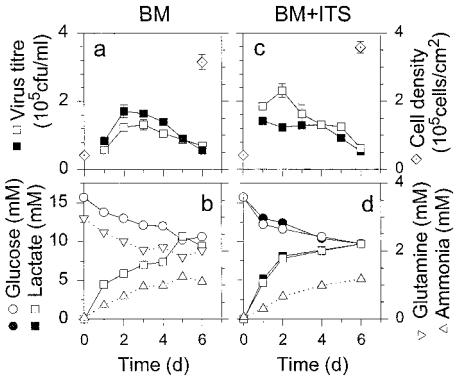

Figure 4. Semicontinuous virus production by monolayer cultures in BM (left) or BM + ITS (right) with daily harvest of the virus-containing supernatant and replacement with 0.2 mL/cm^2 fresh medium: top, virus titer reached daily, initial and final cell concentration; and bottom, residual glucose and glutamine, lactate, and ammonia remained daily in the culture medium. Open and closed symbols refer to duplicate experiments.

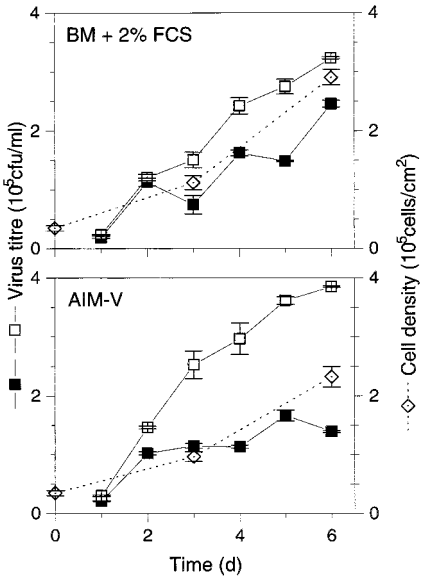

Figure 5. Semicontinuous virus production by monolayer cultures with 0.2 mL/cm^2 BM + 2% FCS (top) and AIM-V (bottom): virus titer in the supernatant harvested daily (squares) and attached cell concentration (lozenges). Open and closed symbols refer to duplicate experiments.

6-day experiment (Figure 5), while virus production declined after 2 days in the other media (not shown). The final cell concentrations reached under these conditions were 1.1×10^6 and 1.5×10^6 cells/mL in AIM-V and BM + 2% FCS, respectively.

3. Semicontinuous Virus Production at Higher Cell Density. ***3.1. Suspension and Microcarrier Cultures.*** Suspension and microcarrier cultures were tested as a possible way to further intensify and scale-up the production process. Preliminary attempts to grow FLYRD18 cells in suspension were performed in BM + 10% FCS and in BM with and without Pluronic F-68, but in all cases, the cultures were dead within a few days. Various microcarriers were tested: Cytodex-1 (Pharmacia) and Biosilon (Nunc) nonporous microcarriers; and ImmobaSil-FS (Integra Biosciences) silicon rubber macroporous microcarriers, either as 10 mm discs or as 1−2 mm beads. The cells attached efficiently within a few hours to all microcarriers tested, but only cells inoculated in Cultispher-G and Cytopore-1 microcarriers were able to survive and grow when stirring was later set at a minimum value to maintain the microcarriers in suspension.

The performances of Cultispher-G gelatin and Cyopore-1 cellulose porous microcarriers are compared in Figure 6 for semicontinuous cultures in AIM-V medium inoculated at 5×10^5 cells/mL medium and 2.5×10^5-cells/mg microcarrier dry weight. Culture with Cultispher-G produced higher virus titers than cultures with Cytopore-1, while their rates of glucose consumption were similar. The daily virus production decreased slowly with time, while all residual glucose and glutamine concentrations remained above 3 and 0.5 mM, respectively. The superiority of Cultispher-G over Cytopore-1, despite similar glucose consumption rates, was also observed in BM + 2% FCS inoculated at a lower density (2.0×10^5 cells/mL, not shown). In this case, however, virus production increased with time.

3.2. Monolayer Cultures. Figure 6 also shows the performance of monolayer cultures inoculated with 5×10^5 cells/mL, corresponding to 5×10^4 cells/cm^2, and run in parallel with the microcarrier cultures. The monolayer cultures produced about 3- and 10-fold higher virus titers than cultures with Cultispher-G and Cytpore-1, respectively, while they consumed glucose at a slower rate (Figure 6b). The rate of glucose consumption was also directly proportional to the attached cell concentration.

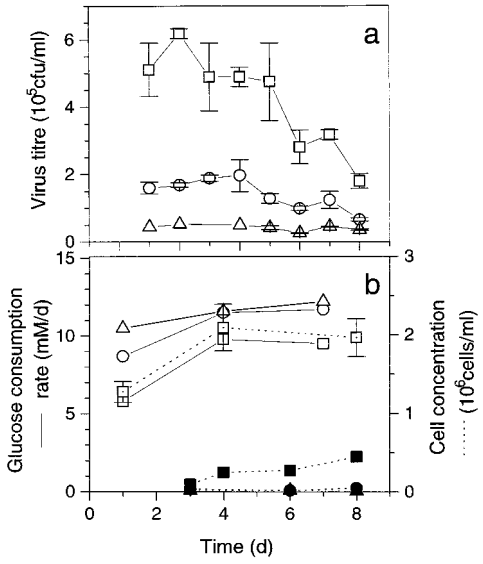

Figure 6. Semicontinuous virus production in AIM-V serum-free medium at high cell density: comparison of static monolayer cultures (squares) and suspended cultures with Cultispher-G (circles) and Cytopore-1 (triangles) microcarriers for virus titer (a), glucose consumption rate (b, open symbols), and dead cells found in suspension (b, closed symbols). The concentration of attached cells in the monolayer cultures is plotted as dotted lines. Symbols and error bars represent, respectively, the mean and range of observations made for duplicate supernatants.

The proportion of dead cells released every day into the supernatant, with respect to the attached cell population, increased from 11% on day 4 to 23% on day 8, while the attached cell concentration and viability remained constant. As for the microcarrier cultures, the daily virus production decreased with time, while all residual glucose and glutamine concentrations remained above 5 and 0.6 mM, respectively.

To further investigate the lower titer observed in agitated cultures, as compared to the static monolayers (Figure 6), we tested the influence of agitation conditions on virus stability. Aliquots of a virus supernatant were

incubated for 2 h at 37 °C either static, orbitaly shaken at 150 rpm, or stirred with a magnetic stirrer bar at 80 rpm, in the presence or absence of 0.1% Pluronic F-68. The final virus titer was not significantly affected by any of the conditions tested (not shown).

Discussion

1. Cell Requirements. Developing, intensifying, and scaling-up the production of retroviral vectors on a rational basis requires the prior identification of the factors that can critically affect cell and bioreactor productivities. In particular, developing serum-free processes with the FLYRD18 cells must take into account the nutritional and growth factor requirements of this cell line. Serum-free conditions would be beneficial for biosafety, downstream-processing, and economic aspects of the vector production process. Figures 1, 2, and 3 indicate that serum (FCS) has also a negative effect on the production of retroviral vectors. In a more detailed investigation, we found that this negative effect was specific to the FLYRD18 cell line and can be attributed to the presence of protease inhibitors in serum (Gerin et al., in press). While a dominating negative effect of serum has been identified, the data collected to date do not exclude the possibility that the production of retroviral vectors by the FLYRD18 cells is nutritionally limited or requires the supply of specific growth factors or proteins.

Growth Factors. As compared to basal media (DMEM/F12, RPMI1640), serum-free media with increasing complexity in their content of proteins and growth factors had no beneficial effect on batch virus production (Figure 1). Non-supplemented BM supported an increasing virus production for 2 days (Figure 4), thus indicating that FLYRD18 cells can tolerate growth factor deprivation for this period of time. However, the virus production decreased in BM after 2 days (Figure 4), while AIM-V and BM + 2% FCS supported a longer-term increase of production (Figure 5). This indicates that FYRD18 cells require some components, probably growth factors or proteins, present in AIM-V and serum but neither in the Insulin-Transferrin supplement nor in Hybridoma-SFM or CHO-S-SFM-II media. Surprisingly, these components are required for virus production but not for cell growth. Indeed, Figure 4 shows that cells continued to multiply while the virus production declined. Identification of the exact nature of the required components would allow the design of a low-protein serum-free formulation, enabling both a long-term high-titer virus production and an easy virus purification and/or concentration.

For the experiment presented in Figure 5, a serum concentration of 2% was selected on the basis of our previous results (Gerin et al., in press) in order to provide the cells with enough of the required components while minimizing the serum inhibitory effect on virus production. This serum concentration could be further optimized. For the ΨCRIP murine packaging cell line, reduction of the FCS concentration from 10% to 1% resulted in a decreased virus production within 3 days (Choi et al., 1997). In contrast with the FLYRD18 cells, which were able to grow in the absence of serum (Figure 4), growth of ΨCRIP cells was greatly affected by serum removal.

Nutrients. Productivity was not adversely affected by reducing the volume of basal medium (Figure 2) or by increasing the cell concentration up to 10^6 cells/mL (Figure 5), that is, by reducing the amount of nutrients available per cell. This suggests that, under most of the conditions tested here, virus production was not limited by nutritional deprivation. Absence of nutritional limitation is further supported by Figure 4b,d, where neither the major nutrients nor their byproducts reached limiting or toxic concentrations, despite cell concentrations up to 1.5×10^6 cells/mL. In Figure 6, cells were not able to grow beyond 2×10^6 cells/mL, and the virus titer decreased with time while at least 30% of the initial glucose and glutamine were left in the medium. The dissolved oxygen concentration was not measured, but calculation of the oxygen required to completely oxidize the consumed glucose to CO_2 showed that it would be at most 15% of the oxygen present in the flask. Conversion of glucose to lactic acid, as observed, would require significantly less oxygen. Therefore, oxygen is not expected to be a limiting factor. The cell density was also well below the saturation density of $1-2 \times 10^6$ cells/cm^2 established by Rasheed et al. (1974). Under our conditions, deprivation of some nutrient other than glucose, glutamine, and oxygen may have limited virus production and cell growth. Identification of the exact nature of the limiting factor would enable the design of more appropriate culture conditions.

Environmental Factors. The FLYRD18 cells failed to grow as single cells in suspension. We observed that they were also unable to grow on native T-flask surfaces in basal medium but grew under the same conditions when present in aggregates or when the plastic surface was precoated with serum proteins or collagen (unpublished). All of these results suggest that FLYRD18 cells are anchorage-dependent. They attached quickly to various microcarriers, but their survival appeared to be very sensitive to the agitation conditions. These cells might be sensitive to shear stress. At the end of the experiment described in Figure 6, however, many viable cells were present at the external surface of the Cultispher-G microcarriers, that is, survived exposure to shear stress.

On the basis of the glucose consumption rates, more cells were present in microcarrier cultures than in monolayer cultures but produced less virus (Figure 6). The lower productivity in microcarrier cultures cannot be attributed to virus inactivation by shear forces, as the virus proved to be stable under agitation. Hence, the lower titers probably result from a lower rate of virus production. Productivity also depended on the type of porous microcarrier used, with Cultispher-G being more favorable to virus production than Cytopore-1 (Figure 6). A first hypothesis is that the production of retroviral vectors by FLYRD18 cells is sensitive to the local conditions prevailing in the cell microenvironment. An accumulation of cells in the pores of the microcarriers is suggested by the high glucose consumption rate and the absence of freely suspended active cells (Figure 6b). Cells packed in the microcarrier pores can restrict the transfer of nutrients and maintain most of the inner cell population in a poor nutritional state, inadequate for virus production. They can also restrict the diffusion of the virus particles from the inner parts of the microcarriers toward the free supernatant. Cultispher-G (gelatin) and Cytopore-1 (cellulose) have different surface properties which can lead to different cell spreading/aggregation behavior and different geometric arrangements in the pores, with consequences also on the transfer of nutrients and release of virus particles. Such differences may explain the effect of microcarrier type on the virus production.

A second and complementary hypothesis is that the production of retroviral vectors requires cell mitosis, as observed for wild type Moloney Murine Leukemia virus (Balazs and Caldarella, 1981). In monolayer cultures, the glucose consumption rate initially increased from day to

day and more cells were released every day in the supernatant, indicating that cells continued to divide actively (Figure 6b). On the basis of the increase of glucose consumption between day 1 and 4, the cells divided more actively in the monolayer cultures than in Cultispher-G cultures and more in the latter than in Cytopore-1 cultures. The virus titers initially followed a parallel trend (Figure 6a). The plateau in glucose consumption rate suggests a reduction of the cell division rate after day 4, which is also coincident with the decrease in virus production. As suggested by the first hypothesis, the different rates of cell division can result from a different rate of nutrient transfer, the cells in the monolayer cultures being well exposed to the culture medium, while nutrients need to diffuse along a long and narrow pathway to reach the cells deeply packed in microcarrier pores.

2. Process Intensification. Cell-specific productivities presented here were in the order of 0.2 infectious virus particle per cell per day (Figures 2, 3, 5, and 6). They are in the same range as those observed with murine packaging cells (deduced from Choi et al. (1997) and Gerin et al. (submitted)). Taking into account the suboptimal but convenient conditions used for the infectivity assay (see below), this figure can be multiplied by at least 1 order of magnitude. Nevertheless, cell productivities of 2 or even 20 infectious particles per cell per day are quite low and clearly point out the challenges underlying intensification of the production of retroviral vectors for gene therapy applications. As virus production seems not to be limited by the supply of nutrients, proteins, or growth factors under most of the conditions tested, the low productivities might be intrinsic to the cell metabolism or limited at the level of viral genes or transgene expression.

A first aspect of process intensification investigated here was to increase the cell concentration in monolayer cultures. Cell concentrations up to 2×10^6 cells/mL were reached with a concomitant increase in virus titer and no loss of cell-specific production. While titers can only be compared with caution from experiment to experiment (see below), virus titers moved from the range 5×10^4 cfu/mL at the start of this study to titers in the range close to 5×10^5 cfu/mL (Figure 6).

A second aspect of process intensification is to continuously or repeatedly collect virus particles produced by the same culture over a prolonged period of time. Figures 5 and 6 prove that such prolonged production processes are feasible for at least a few days. When the cultures were initiated at low cell densities (10^5 cells/mL, Figure 5, and 2×10^5 cells/mL, not shown), virus titers and cell densities increased with time. At higher cell concentrations, however, the latter stabilized at around 2×10^6 cells/mL and virus production decreased slowly with time (Figure 6). If this decline really resulted from nutritional limitations, optimizing the feeding rate and medium composition will enable a further increase of the virus production. Due to the short retrovirus half-life (Shen et al., 1996; Lee et al., 1996; Andreadis and Palsson, 1997; Gerin et al., in press), quick collection and refrigeration of the virus-containing supernatants should be taken into account when designing feeding or perfusion protocols.

A third aspect of process intensification investigated here was to transpose the process from flat monolayer cultures (T-flask) to suspension cultures. The latter are better suited to increasing the cell concentration beyond the limit of 2×10^6 cells/mL reached in monolayer cultures. They are also better suited to process scale-up and intensification in bioreactors. Anchorage dependency of the FLYRD18 cell line and the poor results obtained with microaggregate cultures (not shown) restricted this approach to microcarrier cultures. Unfortunately, the virus titers produced by the latter remained significantly lower than for monolayer cultures (Figure 6). Better understanding and optimization of the factors limiting the production in microcarriers would provide much better prospects for the development, scale-up, and intensification of competitive processes using bioreactors with suspended microcarriers or perfused-packed beds.

3. Technological Implications. Monolayer culture processes can be chosen as the most satisfactory way to produce high titer retroviral stocks. The production process developed in T-flask can easily be transposed to CellCube (Corning Costar), Cell Factory (Nunc), and roller bottle processes. Depending on the biosafety issues and on the requirements for further virus purification or concentration, one of the two following options can then be selected: (i) virus production in unsupplemented BM to harvest a "clean" supernatant with a high virus/adventitious protein ratio (only 1 to 2 virus batches can be collected from the same culture); (ii) the production of more virus batches from the same culture, at the expense of a more complex medium (in the current state of the art, AIM-V or BM with 2% serum supplementation) and a higher content of adventitious proteins which will complicate downstream processing.

4. Restrictions on the Performances of the Virus Titration Assay. Like other bioassays, the infectivity assay used for measuring virus titers has a lower accuracy and precision than can be expected from most chemical or biochemical assays used for bioprocess engineering purposes. For example, we found that titers measured for supernatants produced in parallel but analyzed on 2 different occasions varied by a factor of 2−3 between one occasion and the other. Titers were nevertheless consistent within each infection experiment. More generally, the titer of supernatants produced and analyzed independently but using the same protocol was found to vary over a range of 1 order of magnitude (unpublished).

The inherently lower accuracy and precision of the infectivity assay can easily be understood when considering all of the factors that can affect the measured titer (Andreadis and Palsson, 1996; Le Doux, 1996; Hanenberg et al., 1996; Lee et al., 1996; Andreadis et al., 1997; Palsson and Andreadis, 1997): virus inactivation during freezing and thawing; target cell history and plating efficiency; interaction of the viruses with Polybrene and serum components in the infection medium or at the flask wall; diffusionally limited transport of the viral particles to the target cell surface; level of expression of receptors at the target cell surface; inhibition of infection by various components such as proteoglycans or particles such as free envelope proteins or inactive viruses which compete for the receptor molecules at the target cell surface; position in the cell cycle and susceptibility to infection of the target cell; extracellular and intracellular half-life of the virus; and quality of gene integration and level of transient or permanent expression, culture, and selection of the infected cells for more than 10 days. In the absence of more accurate methods to measure the virus titers, only titers measured simultaneously in the same experiment can be compared with each other. Results plotted together in any figure of this paper meet these requirements. This restriction should be kept in mind when comparing the titers and cell productivities obtained in different studies and even in various independent experi-

ments within the same study, unless a well characterized virus supernatant is included in all infectivity assays as a reference. Major progress in this field can be expected from the development of more appropriate methods.

The titers presented here are not among the highest presented in the literature. The human cells used here as target cells might be less susceptible to infection than cell lines more commonly used for measuring murine virus titers. Also, higher titers can be obtained by using retroviral vectors of simpler genome structure such as the MFG vector, which lacks the internal promoter and selectable marker present in the LNCX-type vector (Jaffee et al., 1993). The point of this study was not to get the best possible titer in each experiment but to compare the effect of culture conditions on virus production by using a convenient assay. While the protocol used here is similar to those used by other researchers (Shen et al., 1996; Choi et al., 1997), we found that the measured titer increased 20-fold when the target cell density was increased 10-fold and the 6-well plates were shaken during the infection period (unpublished results). The increased titer indicates that the titers measured under the standard conditions are underestimated by at least 1 order of magnitude due to inefficient delivery of the viral particles to the target cells.

Conclusion

The FLYRD18 human packaging cell line proved to be anchorage-dependent and able to efficiently produce retroviral vectors for 1−2 days in basal medium, in the absence of any supplementation with growth factors or proteins. This offers interesting prospects for further virus purification and concentration, as well as for the biosafety of gene therapy. Longer-term production processes were also developed. In this case, FLYRD18 cells required some growth factors or proteins present in AIM-V medium and in serum. These components were required for virus production but not for cell growth. Serum supplementation must be carefully considered, as some factors present in serum also have an inhibitory effect on virus production.

In this study, the production of retroviral vectors in monolayer cultures has been brought from serum-supplemented medium to serum-free medium, to cell densities up to 2×10^6 cells/mL with at least a 10-fold increase in virus titer, and from a batch to a longer-term semicontinuous mode enabling a proportional increase in total production. Suspended microcarrier cultures also proved to be feasible, but further investigation and optimization will be required for their performances to become competitive with monolayer cultures. Further improvements of virus production will also rely on the identification of the growth factors present in serum and AIM-V, which are required for long-term virus production, as well as on an optimization of the culture medium composition and of the feeding protocol to enable higher cell densities.

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