Effects of Culture Parameters on the Production of Retroviral Vectors by a Human Packaging Cell Line

Sally McTaggart and Mohamed Al-Rubeai*

Animal Cell Technology Group, School of Chemical Engineering, University of Birmingham, Edgbaston B15 2TT, U.K.

The use of retroviral vectors for human gene therapy requires the production of large quantities of high titer vector stocks. Maintaining high titers during the prolonged culture of packaging cells will require that critical parameters be controlled. The aim of this study was to determine which culture parameters critically affect the production/decay of retroviral vectors produced by the human packaging cell line FLYRD18/LNC-hB7. The stability of retroviral vectors released by this cell line was found to be temperature dependent (half-life of 6.9, 11.0, and 64.3 h when incubated at 37, 32, and 0 °C, respectively). Titers increased up to 10-fold when the packaging cells were cultured at 32 °C, compared to 37 °C, despite a decrease in cell yield (cell-specific titers were 20-fold higher). Virus titers were also over 10-fold higher when the packaging cells were cultured in a reduced serum concentration (1%) compared to 5%. Retrovirus production at a range of pH levels revealed a significant decrease in virus titer at pH levels below 6.8 and above 7.2, optimum titers being achieved in cultures at pH 7.2. Dissolved oxygen levels in the range 20−80% did not significantly affect titers under the conditions tested. Finally, a packed bed system containing the packaging cells immobilized on porous microcarriers was shown to sustain the production of active retroviral vectors for over 1 month, in relatively large volumes.

Introduction

Retroviral vectors are currently the most widely used gene transfer system in clinical trials (Gordon and Anderson, 1994; Hodgson, 1995; Morgan, 1995), though other viral vectors are receiving increasingly more attention (Benihoud et al., 1999; Federico, 1999). Retroviral mediated gene transfer offers a number of advantages over other gene transfer vehicles, primarily as a result of the high transduction efficiencies and stability of the integrated DNA in successfully transduced target cells (Mitani and Caskey, 1993; Cosset et al., 1995; Parasrampuria, 1998; Robbin et al., 1998). However, the clinical application of retroviral vectors for gene therapy is limited by the relatively low productivity of retroviral packaging cell lines. A number of gene therapy applications require gene transfer into a large number of cells. Therefore to ensure good transduction efficiencies, a high titer retroviral vector stock is required (Eglitits et al., 1985). It has been estimated that somewhere in the region of 10^7-10^{14} infectious viral particles may be required in a single dose for therapeutic applications (Braas et al., 1996; Lyddiatt and O'Sullivan, 1998), though these figures will obviously depend on the clinical application and site of delivery. It is therefore expected that production of sufficient quantities of retroviral vectors for a large population of potential patients will require significant improvement of the current methods of retroviral vector production for clinical use.

A number of reports exist on the effect of various culture parameters on the production of retroviral vectors from murine packaging cell lines (Kotani et al., 1994; Lee et al., 1996; Shen et al., 1996). However, such studies using human packaging cell lines have received little attention in the literature. The human packaging cell line FLYRD18 (Cosset et al., 1995) was constructed to overcome a number of the potential problems with murine packaging cell lines. Retroviral vectors produced by this cell line are not inactivated by antibodies and complement in human serum (Takeuchi et al., 1996). As with a number of recent packaging cell lines, the *gag-pol* and *env* genes have been inserted on separate expression cassettes and the viral sequences have been minimized to reduce the risk of producing replication-competent retrovirus particles. We have chosen to use this cell line to study the effects of various culture parameters on retroviral vector production and to develop a production method amenable to scale-up, because of the suitability of the FLYRD18 cells for human gene therapy applications (Gerin et al., 1999a).

The stability of retroviral vectors produced by the FLYRD18/LNC-hB7 cell line was assessed at various storage temperatures. In addition, the effect of culture temperature, pH, serum concentration, and dissolved oxygen levels on virus titer were assessed. Finally, cells were successfully immobilized on porous carriers and cultured in a packed bed system, where production of retroviral vectors was intensified during long-term culture and titers were further increased by a reduction in culture temperature.

Materials and Methods

Cell Lines. FLYRD18 packaging cells (derived from HT1080 human fibrosarcoma cell line, Cosset et al., 1995) were used by M. Gilligan and P. F. Searle of the Cancer

* Telephone and Fax: 0121 414 3888. Email: m.al-rubeai@bham.ac.uk.

10.1021/bp000078j CCC: $19.00 © 2000 American Chemical Society and American Institute of Chemical Engineers
Published on Web 09/06/2000

Studies Institute, University of Birmingham, to construct the FLYRD18/LNC-hB7 cell line. This cell line contains the human B7/CD80 gene and the neomycin-resistance gene (Miller and Rosman, 1989; Freeman et al., 1989; Linsley and Ledbetter, 1993; Daly and Gilligan, personal communication) under the control of the internal cytomegalovirus (CMV) promoter.

A2780cp cisplatin-resistant human ovarian carcinoma cells (Khokhar et al., 1992) were used as target cells for infection to measure retroviral vector titers.

Monolayer Cultures. Both the producer and target cell lines were maintained in monolayer cultures using standard tissue culture flasks (T-flasks). Cells were cultured in Dulbecco's Modified Eagles medium and Ham's F-12 nutrient mix (DMEM:F-12, 1:1) plus 5% (v/v) fetal calf serum, (FCS, Life Technologies, U.K.). FLYRD18/LNC-hB7 cells were inoculated at 2×10^4 cells /cm^2 (5×10^4 cells/mL) and passaged once confluent (every 3−4 days) in fresh medium. A2780cp target cells were inoculated at 1×10^4 cells/cm^2 (2.5×10^4 cells/mL) and maintained as above.

Packed Bed Cultures. The packed bed used in this study consists of a cylindrical glass culture chamber with ports for media flow in and out of the chamber and a sampling port for removal of carriers (to check viability of attached cells during culture). Media was circulated through the fixed bed via a peristaltic pump. The entire vessel was packed with 85 mL settled volume (approximately 5.5×10^4 carriers) of ImmobaSil-G porous silicone carriers (Ashby Scientific Ltd., U.K.) before being sterilized in an autoclave at 121 °C for 15 min.

Before inoculation of the packed bed with FLYRD18/LNC-hB7 cells, 100 mL culture medium (DMEM:F-12 + 5% FCS) was recirculated through the packed bed (containing sterile carriers) at 37 °C for 30 min. Meanwhile the cells were released from the T-flasks by trypsin digestion. The rinse medium was removed from the packed bed, and a total of 1.8×10^8 cells were added (suspended in fresh culture medium). The packed bed system was rotated during this inoculation period to aid uniform cell attachment to the carriers. After 2 and 4 h, samples were removed from the packed bed and the cell number was determined by hemocytometer counts (percentage cell attachment was estimated by determining the number of unattached cells remaining in the medium). When over 80% of the cells were attached (4 h post inoculation), the packed bed was connected to a media reservoir containing 400 mL of culture medium (final cell concentration 3.6×10^5 cells/mL), which was recirculated at a flow rate of 8 mL per min. Media samples were taken daily for virus titer and glucose measurements. Culture medium was changed every 1 or 2 days.

Measurement of Virus Titer by Infection Assay. Active virus titers were assessed using an infection assay of the human ovarian carcinoma cell line, A2780cp. In brief, A27080cp cells were inoculated at a density of 10^5 cells per well in 6-well culture plates (Nunc) and cultured in 2 mL of DMEM:F-12 plus 5% FCS (v/v) overnight. The spent medium from each well was replaced with 1 mL of diluted virus supernatant, $1{:}10^2$−$1{:}10^4$, in DMEM:F-12 (no serum) + 8μg/mL Polybrene (Sigma, U.K.). Virus supernatants were filtered through 0.45 μm filters and stored in liquid nitrogen before dilution for this assay. Diluted virus supernatants (two wells for each dilution) were left in the wells at 37 °C for 8 h before addition of 2 mL of DMEM:F-12 + 5% FCS (v/v) + 1.125 mg/mL Geneticin (G418), to give final concentration of 0.75 mg/mL Geneticin per well. A control plate containing no retrovirus supernatant was also cultured under identical conditions, to confirm no colonies would survive in the presence of Geneticin (0.75 mg/mL). All plates were incubated at 37 °C for 10 days, with a complete medium change in each well after 5 days (using fresh DMEM:F12 + 5% (v/v) FCS + 0.75 mg/mL Geneticin). On day 10 the medium was removed and each well was rinsed with 1−2 mL of phosphate buffered saline (PBS). After removal of the PBS, 1 mL of crystal violet solution (0.3% (w/v) crystal violet in 70% (v/v) methanol) was added to each well and left for 15 min. The crystal violet solution was removed, and each well was rinsed with water. The number of stained colonies in each well were counted, and the number of colony forming units (cfu) per mL of neat virus supernatant calculated.

Determination of Vector Stability at Various Temperatures. A stock of retrovirus-containing supernatant, harvested from a monolayer culture of FLYRD18/LNC-hB7 cells, was passed through a 0.45 μm filter and divided into 1 mL aliquots. Replicate samples were stored at 37 or 32 °C or on ice for up to 72 h. At various time points samples were removed from their respective storage conditions and frozen in liquid nitrogen. Control samples were placed in liquid nitrogen immediately after removal from culture and filtering, to determine the percentage of vectors lost during storage at the various temperatures. After all samples had been stored in liquid nitrogen for at least 24 h the active virus titer was measured simultaneously using the infection assay described above.

Culture at Various pH Levels. FLYRD18/LNC-hB7 cells were inoculated into T-flasks as described above and left overnight. The culture medium from each flask was then replaced with media adjusted to pH 6.0, 6.4, 6.6, 7.2, or 7.8 (triplicate flask for each pH level). The cultures were incubated for a further 24 h before removal of the supernatant for measurements of the virus titer and release of the cells by trypsin digestion for determination of cell numbers. Cells were not cultured at the various pH levels for longer than 24 h because of the lack of pH control in monolayer cultures. The pH levels all dropped by 0.4 after 24 h of incubation.

Culture at Various Dissolved Oxygen Levels. A "Maestro" bioreactor (LSL Biolafitte, France) was used to control dissolved oxygen levels. In brief, two flat-bottomed glass vessels were inoculated with 8.5×10^6 FLYRD18/LNC-hB7 cells in a small volume (20 mL) of culture medium (DMEM:F-12 + 5% FCS), and the cells were left to attach to the bottom of each vessel for 4 h. After this time the number of cells remaining in suspension was counted to ensure that over 95% of cells had attached before the media volume was increased to 200 mL and left overnight at 37 °C. The following day both vessels were connected to the controller, after the medium in each was replaced for fresh. The pH control was set at pH 7.2, temperature at 37 °C, and the dissolved oxygen level at 20% in one vessel and 50% in the other. The controlled cultures were run for 2 days before the medium in each vessel was sampled for virus titer measurements and the cells were released by trypsin digestion for determining the final cell number cultured at each dissolved oxygen concentration. The above experiment was then repeated using 50% and 80% dissolved oxygen levels. The use of a 50% dissolved oxygen level in both runs allowed proper comparisons between the two experiments by excluding innoculum conditions as a variable factor.

Reduced Serum Cultures. FLYRD18/LNC-hB7 cells were inoculated into T-flasks, as described above, in 1%

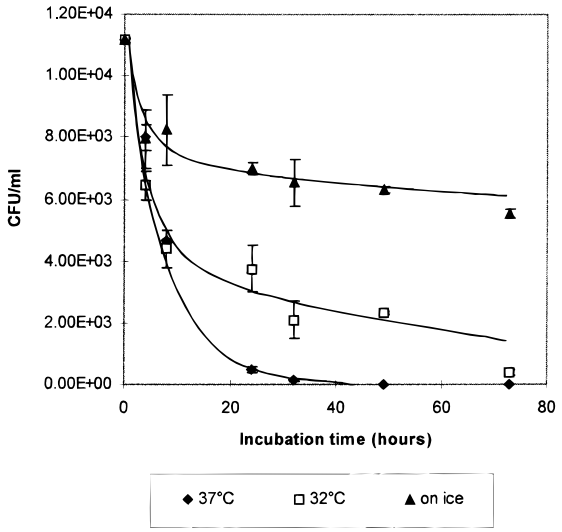

Figure 1. Decay of retroviral vector activity during incubation at various temperatures in DMEM:F-12 + 5% FCS. Error bars represent observed range, $n = 2$. Half-life of virus particles (as determined by exponential curve fit) at 37 and 32 °C and on ice was calculated as 6.9, 11.0, and 64.3 h, respectively.

(v/v) or 5% (v/v) FCS and cultured for 6 days at 37 °C. The culture medium in each flask was replaced for fresh on day 3. On days 2 and 6, samples from each culture supernatant were taken for determination of virus titer. On day 6, the cells were released by trypsin digestion and the cell numbers were counted on a hemocytometer. These data were used to calculate the cell specific titers on day 6.

Results

Retroviral Vector Stability. Incubation of retroviral vectors (produced by FLYRD18/LNC-hB7 cells in DMEM: F-12 + 5% FCS) at various temperatures showed an increase in vector decay rate with increasing storage temperature (Figure 1). Vector decay rate was determined by loss of infectious virus with time (reduction in cfu/mL). Using an exponential curve fit, the half-life of the vector activity during storage at 37 and 32 °C and on ice was calculated to be 6.9, 11.0. and 64.3 h, respectively.

Effect of Culture Temperature on Virus Production in Monolayer. Daily retroviral vector titers in the culture supernatant of FLYRD18/LNC-hB7 cells cultured in monolayers at 32 °C were compared to those from 37 °C cultures (Figure 2). After 8 days the cells were detached by trypsin digestion and counted.

After day 2, virus titers were significantly higher in the 32 °C cultures compared to the 37 °C cultures (up to 10-fold increase by day 7). Cell numbers on day 8 of culture reached 6.3×10^5 cells/mL at 32 °C and 1.3×10^6 cells/mL at 37 °C (mean specific growth rate (μ) over 8 days was 7.6×10^{-3} h^{-1} and 9.0×10^{-3} h^{-1}, respectively). As a result of the higher virus titer and lower cell concentration in the 32 °C cultures, the specific virus productivity (cfu/cell/mL) was over 20-fold higher than that of the 37 °C cultures on day 8.

Effect of pH on Virus Production in Monolayer Culture. The highest vector titer was measured in the supernatants from packaging cells cultured at pH 7.2. The titer at this pH was taken as 100%, and all other titers were calculated as a percentage of this (Figure 3). Since it is unlikely, under normal culture conditions, for the pH of the culture medium to increase, the effect of pH below 7.2 was of more concern than that above pH

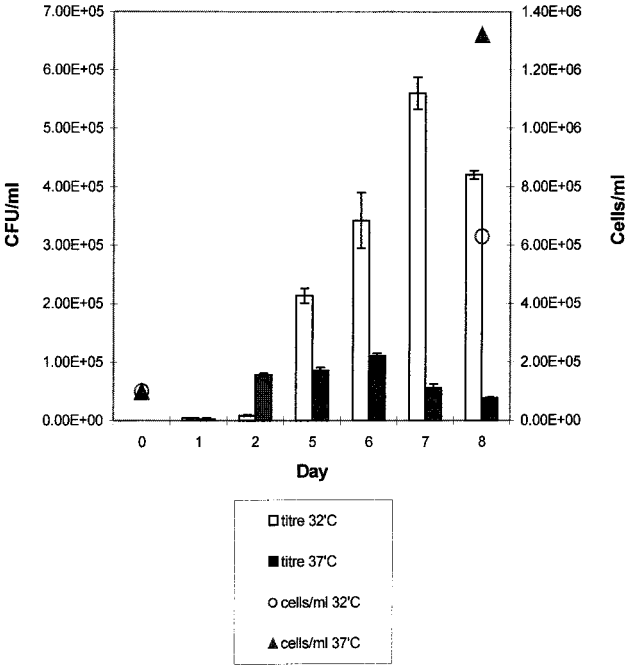

Figure 2. Virus titer and cell number during monolayer culture of human packaging cell line at 32 and 37 °C. Error bars represent observed range, $n = 2$.

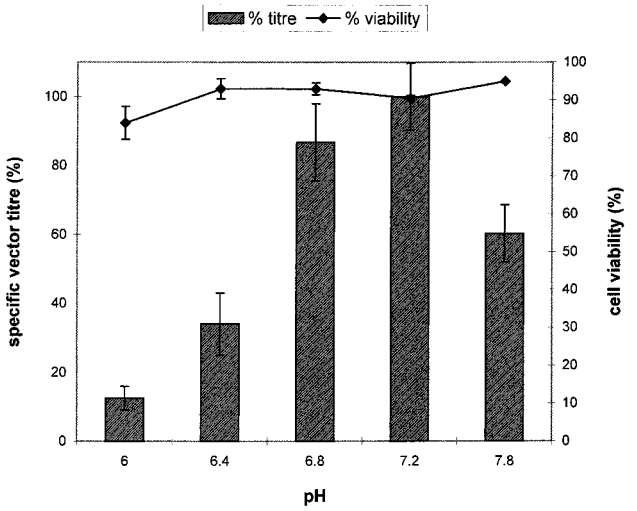

Figure 3. Specific virus titer (cfu/cell/mL) as a percentage of that measured at pH 7.2. Samples measured after 24 h of culture at various pH levels. Error bars represent standard deviation, $n = 3$.

7.2. Figure 3 illustrates the effect of such low pH values on virus production and cell viability. The viability remained above 90% at all pH levels tested except pH 6 (85% viability). However, the specific virus titer (cfu/cell/mL) decreased at all pH levels tested below pH 7.2 (12.5%, 34.0%, and 87% at pH 6.0, 6.4, and 6.8, respectively).

Effect of Dissolved Oxygen Levels on Virus Production in Monolayer Culture. No significant differences in cell numbers or vector titers were observed in cultures maintained for 2 days at 20%, 50% or 80% dissolved oxygen levels (Figure 4). Hence oxygen levels were not limiting to cell growth or virus production under the conditions employed in this study. Cell numbers increased almost 3-fold during the 2 days in culture.

Effect of Serum Concentration on Virus Titer. Virus titers were higher in the 1% FCS cultures compared to the 5% FCS cultures (2-fold and 11-fold higher

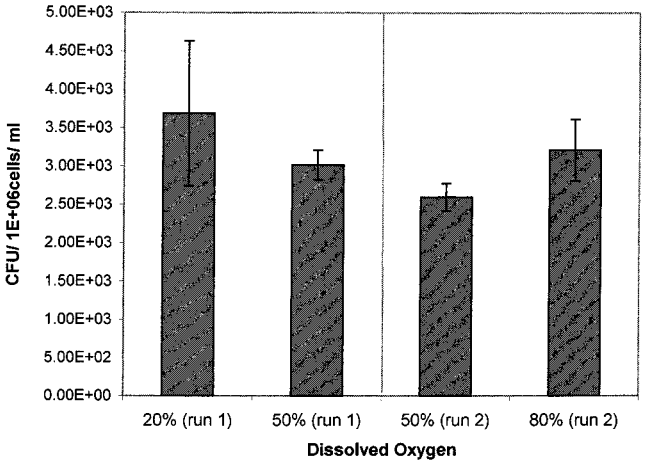

Figure 4. Virus titer, per 1×10^6 cells, after 2 days of culture at various dissolved oxygen levels. Error bars represent observed range, $n = 2$.

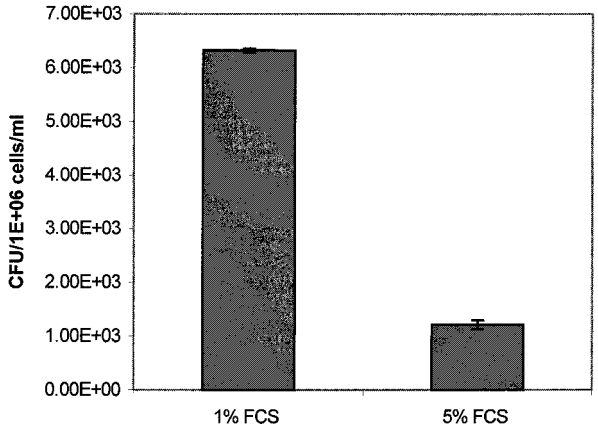

Figure 5. Virus titer, per 1×10^6 cells, in monolayer cultures at 1% or 5% FCS (day 6). Error bars represent observed range, $n = 2$.

on days 2 and 6, respectively). The titers on day 6 were divided by the corresponding total cell number in each culture, to compare the specific titers at each serum concentration (Figure 5). By day 6 the specific titer in 1% FCS was over 10-fold higher than that in 5% FCS.

Packed Bed Cultures at 32 and 37 °C. FLYRD18/LNC-hB7 cells were immobilized on ImmobaSil-G carriers and cultured in a packed bed for up to 10 days at 32 or 37 °C (Figure 6). Initially, the temperature effect was not manifested, but after day 4 the virus titer measurements from the 32 °C packed bed were significantly higher than titers from the 37 °C packed bed culture supernatants.

A further packed bed reactor was set up to determine whether retroviral vector production could be sustained for a prolonged culture period when the cells were immobilized on porous carriers (Figure 7). For the first three weeks of culture the temperature of the packed bed was maintained at 37 °C and then decreased to 32 °C and maintained for a further week. At 37 °C the virus titers fluctuated daily but did not significantly increase after day 9. After 24 h of incubation at the reduced culture temperature (day 31) the virus titers began to increase and continued to do so until the last day of culture. The average daily titer achieved in the packed bed system at 32 °C was more than 3-fold that at 37 °C. Cell numbers were assumed not to be increasing during this culture period, as indicated by constant glucose uptake rates (data not shown). Furthermore, cell growth rates are known to be reduced at 32 °C compared to 37 °C from earlier measurements in monolayer cultures at both these temperatures (Figure 2).

Discussion

Culture Parameters. The negative relationship between temperature and decay rate of retroviral vectors, reported in this study for vectors produced by FLYRD18 human packaging cells, correlates with previous reports on vectors produced by murine packaging cell lines (Lee et al., 1996; Kaptein et al., 1997). The relatively short half-life at 37 °C also falls within the range reported for a number of murine packaging cell vectors (9, 5.5, and 8 h reported by Lee et al., (1996), Shen et al., (1996), and Kaptein et al., (1997), respectively). The dramatically increased stability of the vector particles when stored on ice may prove useful in retaining vector stability during downstream processing of vector supernatants for subsequent gene therapy applications. The culture temperature of packaging cells must, however, be kept at levels suitable for maintaining normal, or near normal, metabolic activity of mammalian cells. An open perfusion system where the culture supernatant can be harvested on ice, while the cells are cultured at a higher temperature, may enable the accumulation of higher vector titers as a result of their increased stability before harvesting. Preliminary studies, however, did not show the significant increase in titers expected by such an approach (data not shown), possibly because of the removal of "conditioned" medium from the FLYRD18/LNC-hB7 cells in an open system.

The virus titers achieved from monolayer cultures of FLYRD18/LNC-hB7 cells at 32 and 37 °C were compared in order to assess whether the benefits of increased virus stability at 32 °C outweighed any loss in titer due to decreased cell metabolic activity. Despite a decrease in cell growth rate and hence a reduced total cell number in the 32 °C cultures, a 10-fold increase in virus titer was obtained compared to the 37 °C cultures. The finding that the actual titer *per cell* was up to 20-fold higher at 32 °C compared to 37 °C exceeds that found for a number of murine cell lines. Kaptein et al. (1997) reported a 12-fold and 16-fold increase in titer per cell for two different packaging cell lines cultured at 32 °C compared to 37 °C. Lee et al. (1996) showed an increase in retroviral vector titer produced by ΨCRIP cells of only 2-fold more than that produced at 37 °C (differences in specific titers were not given). Furthermore, Kotani et al. (1994) reported a 5- to 15-fold increase in vector titer produced by PA317cells at 32 °C. Hence it would appear that the increase in titer due to reduced culture temperatures varies between different cell lines, whether murine or human. This is possibly due to different half-life values of the vectors produced by these cells. The reduced stability of these vectors at higher temperatures (37 °C) has been hypothesized to be due to the inherent instability of the RNA genome or loss of reverse transcriptase activity (Palsson and Andreadis, 1997). Furthermore, since the studies reported here have been performed in serum-containing medium, it is possible that the increased activity of serum antiproteases at 37 °C compared to 32 °C also has an effect on the virus titer. Such inhibitory factors could act intracellularly, resulting in a reduced titer (Gerin et al., 1999a).

A wealth of literature exists on the effects of pH on mammalian cell growth; however, nothing is known about the effects of pH on retrovirus production. Schmid et al. (1990) have reported the beneficial effects of low pH levels (pH 6.7) on monoclonal antibody production by

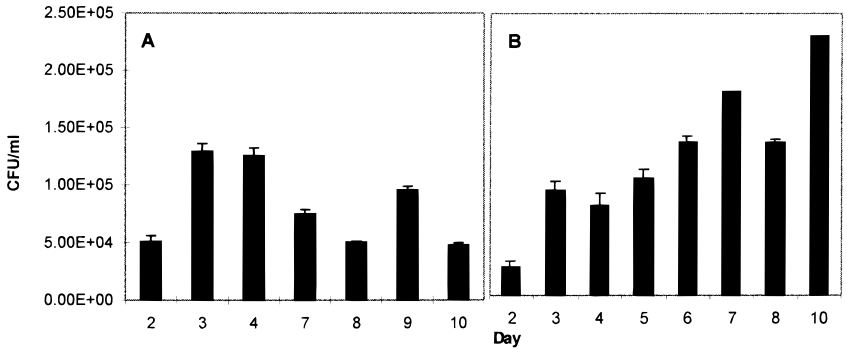

Figure 6. Daily virus titers during culture of FLYRD18/LNC-hB7 cells immobilized on ImmobaSil-G microcarriers in a perfused packed bed system at 37 °C (A) or 32 °C (B). Error bars represent observed range, $n = 2$.

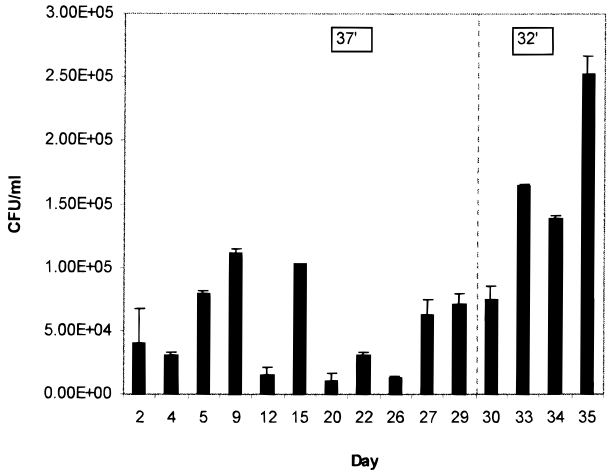

Figure 7. Daily virus titers during culture of FLYRD18/LNC-hB7 cells immobilized on ImmobaSil-G microcarriers in a perfused packed bed system. Three weeks culture at 37 °C and one week culture at 32 °C. Error bars represent observed range, $n = 2$.

hybridoma cells. In contrast, Harbor et al. (1989) showed a negative effect of low pH levels on monoclonal antibody production. These findings illustrate the variability of optimum pH values for growth and production of various cell lines. This study indicated that an optimum pH production was obtained at pH 7.2 and a significant decrease resulted from higher or lower pH levels. This observation has implications where the packaging cells are cultured under batch, fed-batch, or semicontinuous culture conditions where the pH is not normally controlled. In high cell density cultures the pH of the culture medium can drop as low as pH 6.5 over a 24 h period. Hence where daily medium changes are employed for vector harvesting, it is unlikely that the optimum titer will be achieved. The use of a culture system where the pH is continuously controlled is therefore recommended for optimized continuous production of retroviral vectors.

Dissolved oxygen levels have been shown to have a significant effect on the virus production and cell growth of Sf-9 cells infected with Ac-NPV baculovirus. Klöppinger et al. (1990) reported a 50% decrease in virus production when the dissolved oxygen level of the culture was reduced from 40% to 20%, thus illustrating the importance of dissolved oxygen levels in certain culture systems. Furthermore, high oxygen levels (typically over 100% air saturation) have been shown to be detrimental to cell growth (Emery et al., 1995). This paper contains the first report, to the authors' knowledge, on the effects of dissolved oxygen levels on the production of retroviral vectors. Surprisingly, we found oxygen levels between 20% and 80% air saturation did not affect the vector titer under the conditions employed in this study. Lower oxygen levels were not tested but indeed should be assessed if low oxygen levels are expected in any culture system used for vector production, as any oxygen limitation or deprivation could reduce cell viability by inducing apoptosis (Mercille and Massie, 1994). In the packed bed system used in this study, dissolved oxygen levels did not fall below 60% (data not shown), and hence dissolved oxygen levels are not believed to be limiting in these cultures.

A reduced serum concentration (1%) was shown to increase virus titers during the culture of FLYRD18/LNC-hB7 cells in monolayer for 6 days. These data agree with that of Gerin et al. (1999a, 1999b), who reported a dose-dependent negative effect of serum on the virus titer of this packaging cell line during short-term culture (17 h). A study to compare titers after the incubation of supernatants with and without serum showed this negative effect of serum not to be due to extracellular inactivation of virus particles (Gerin et al., 1999a). Rather, the effect is suggested to be the result of an intracellular action of antiproteases in the serum (Gerin et al., 1999a). The FLYRD18/LNC-hB7 cell line, like murine packaging cell lines (Choi et al., 1997; Shen et al., 1996), requires serum for prolonged culture. However, lower concentrations of serum, or complete serum-free medium, should be more advantageous for the production of supernatants with increased virus titers and reduced protein content.

While monolayer culture of packaging cell lines is the technique of choice for most manufacturers of retroviral vectors, this method is very labor intensive in large-scale production. Alternative culture systems for the production of retroviral vectors that are more amenable to scale-up should therefore be investigated. The anchorage-dependence of the FLYRD18/LNC-hB7 cell line prohibits the use of typical suspension cultures, more suited for large-volume cultures. Packed bed systems have been used for the large-scale culture of a number of anchorage-dependent mammalian cells (Looby and Griffiths, 1990). These systems offer a number of advantages, including low surface shear, high cell density and productivity, ease of separation of product from cells, prolonged production times, and potential for radial scale-up. Disadvantages include the poor homogeneity of the systems (though this may have less impact on products harvested from the supernatant, such as retroviral vectors), channel blockage, and little potential for linear scale-up. The use of a packed bed system where the cells are immobilized on porous carriers (ImmobaSil) for the production of retroviral vectors was shown to be an efficient and economical method for prolonged cultivation of the human packaging cell line, FLYRD18. Earlier studies showed titers har-

vested from the packed bed were equivalent to those in monolayer cultures using identical cell concentrations (data not shown). The packed bed cultures reported here maintained vector production for over 1 month, where culture supernatants were harvested daily. A reduction in the culture temperature of the packed bed, from 37 to 32 °C, was found to increase vector titers, as observed in monolayer culture.

Titers. The retroviral vector titers quoted in any study, measured using an infection assay, should not be taken as absolute values. Because of the inherent variability in the number of colony forming units derived from target cells cultured with vector-containing supernatants, the values given (cfu/mL) can only be taken as arbitrary units. The ability of a vector to successfully infect a target cell, such that the cell will go on to express the selectable marker gene, is dependent on a number of parameters, including metabolic state of the target cell culture, concentration of the vector stock, target cell density, ratio of vector particles to target cells, infection medium, time of exposure of target cells to virus (Palsson and Andreadis, 1997), and type of target cells. Therefore the titers quoted in this study can only be used as an indication of increased or decreased virus production/stability as a result of various culture conditions. Actual titers may well be higher than those measured by the infection assay (e.g., we have found increased titer by increasing target cell density, data not shown). Where retroviral vector stocks are produced for clinical applications, the actual titers quoted should be those measured using a method applicable to the protocol used for transduction of patient cells.

In this study certain steps were taken to avoid variability in measured titers, due to factors such as those quoted above (e.g., use of the same target cell density, target cells inoculated for the same length of time before exposure to virus, and the use of the same virus exposure time). However, there will always be variation in the values obtained for the same virus concentration due to interassay variability. For this reason an internal control was included for each infection assay in this study. This control consists of a stock of retroviral vector supernatant divided into a number of aliquots and stored in liquid nitrogen. One aliquot was thawed and measured along with the samples in each infection assay. Past experience had shown that in over 20 separate assays the variation in measured titer of this control is never greater that 2-fold (data not shown). Therefore, where the titers from samples measured in separate assays were compared, any difference between the control titer in individual assays was used to "normalize" the data to account for any inter-assay variation.

The measurement of vector titers, using methods such as those described above, aid in the assessment of conditions optimal for vector production. However, the transduction efficiency of a retroviral vector preparation is as important as the vector titer where clinical applications are intended. The typical transduction efficiency of a retroviral vector supernatant produced by the FLYRD18/LNC-hB7 cell line in DMEM:F-12 was found to be approximately 60% using early passage primary ovarian cancer target cells (OvCaMG79) and 30% using the A2780cp target cell line (Gerin et al., 1999a).

Conclusion

This study reveals a number of factors that affect the titer of retroviral vectors produced for gene therapy. A reduced temperature (32 °C) in FLYRD18/LNC-hB7 cell cultures led to an increased vector titer as a result of the increased stability of these particles at this lower temperature. Furthermore, pH levels during culture critically affected vector titers, optimal levels being pH 7.2. Conversely, dissolved oxygen levels between 20% and 80% air saturation did not significantly affect virus production by this cell line. Lower serum levels were advantageous during vector production as a result of the inhibitory effect of serum on vector synthesis/maturation. Finally, the use of a fixed bed system packed with cells immobilized on porous carriers (ImmobaSil) was demonstrated to allow the prolonged production of vector particles in large quantities.

Acknowledgment

This work was supported by Ashby Scientific Ltd. (ASL). The authors gratefully acknowledge Peter Searle and Moira Gilligan (Institute for Cancer Studies, University of Birmingham) for the use of their cell lines.

References and Notes

Benihoud, K.; Yeh, P.; Perricaudet, M. Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol.* **1999**, *10*, 440−447.

Braas, G.; Searle, P. F.; Slater, N. K. H.; Lydiatt, A. Strategies for the isolation and purification of retroviral vectors for human gene therapy. *Bioseparation* **1996**, *6*, 211−228.

Choi, J. H.; Jun, S. C.; Lee, G. H. Effect of serum concentration on retroviral vector production from the amphotropic ψCRIP murine producer cells. *Bioprocess Eng.* **1996**, *17*, 349−353.

Cosset, F. L.; Takeuchi, Y.; Battini, J. L.; Weiss, R. A.; Collins, M. K. L. High-titre packaging cells producing recombinant retroviruses resistant to human serum. *J. Virol.* **1995**, *69*(12), 7430−7436.

Eglitis, M. A.; Kantoff, P.; Gilboa, E.; Anderson, W. F. Gene expression in mice after high efficiency retroviral-mediated gene transfer. *Science* **1985**, *230*, 1395−1398.

Emery, A. N.; Jan, D. C.-H.; Al-Rubeai, M. Oxygenation of intensive cell-culture system. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 1028−1033.

Federico, M. Lentiviruses as gene delivery vectors. *Curr. Opin. Biotechnol.* **1999**, *10*, 448−453.

Freeman, G. J.; Freedman, A. S.; Segil, J. M.; Lee, G.; Whitman, J. F.; Nadler, L. M. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* **1989**, *143*, 2714−2722.

Gerin, P. A.; Gilligan, M. G.; Searle, P. F.; Al-Rubeai, M. Improved titres of retroviral vectors from the human FLYRD18 packaging cell line in serum- and protein-free medium. *Hum. Gene Ther.* **1999a**, *10*, 1965−1974.

Gerin, P. A.; Searle, P. F.; Al-Rubeai, M. Production of retroviral vectors for gene therapy with the human packaging cell line FLYRD18. *Biotechnol. Prog.* **1999b**, *15*, 941−948.

Gordon, E. M.; Anderson, W. F. Gene therapy using retroviral vectors. *Curr. Opin. Biotechnol.* **1994**, *5*, 611−616.

Harbor, C.; Low, K. S.; Marquis, C. P.; Barford, J. P. *Biotechnol. Tech.* **1989**, *3*, 73−78.

Hodgson, C. The vector void in gene therapy. *Bio/Technology* **1995**, *13*, 222−225.

Kaptein, L. C. M.; Geijer, A. E.; Valerio, D.; van Beusechem, V. W. Optimised conditions for the production of recombinant amphotropic retroviral vector preparations. *Gene Ther.* **1997**, *4*, 172−176.

Khokhar, A. R.; Xu, Q. Y.; Newman, R. A.; Kido, Y.; Siddik, Z. H. Synthesis, characterisation and anti-tumour activity of new chloroethylamine platinum complexes. *J. Inorg. Biochem.* **1992**, *45*, 211−219.

Kotani, H.; Newton, P.; Chiang, Y. L.; Otto, E.; Weaver, L.; Blease, R. M.; Anderson, W. F.; McGarrity, G. J. Improved methods of retroviral vector transduction and production for gene therapy. *Hum. Gene Ther.* **1994**, *5*, 19−28.

Klöppinger, M.; Fertig, G.; Fraune, E.; Miltenhurger, H. G. Multistage production of *Autographa californica* nuclear

polyhedrosis virus in insect cell cultures. *Cytotechnology* **1990**, *4*, 271−278.

Lee, S.-G.; Kim, S.; Robbins, P. D.; Kim, B.-G. Optimization of environmental factors for the production and handling of recombinant retrovirus. *Appl. Microbiol. Biotechnol.* **1996**, *45*, 477−483.

Linsley, P. S.; Ledbetter, J. A. The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol.* **1993**, *11*, 191−212.

Lyddiatt, A.; O'Sullivan, D. Biochemical recovery and purification of gene therapy vectors. *Curr. Opin. Biotechnol.* **1998**, *9*, 177−185.

Mercille, S.; Massie, B. Induction of apoptosis in oxygen-deprived cultures of hybridoma cells. *Cytotechnology* **1994**, *15*, 117.

Miller, A. D.; Rosman, G. J. Improved retroviral vectors for gene transfer and expression. *Biotechniques* **1989**, *7*, 980−988.

Mitani, K.; Caskey, C. T. Delivering therapeutic genes—matching approach and application. *Trends Biotechnol*. **1993**, *11*, 162−166.

Morgan, R. A. Retroviral vectors in human gene therapy. In *Viruses in Human Gene Therapy*; Vos, Jean-Michel H., Ed.; Carolina Academic Press: Durham, NC, 1995; pp 77−107.

Palsson, B. O.; Andreadis, S. The physicochemical factors that govern retovirus-mediated gene transfer. *Exp. Hematol.* **1997**, *25*, 94−102.

Parasrampuria, D. A. Therapeutic delivery issues in gene therapy, Part 1: Vectors. *BioPharm* **1998**, March, 38−45.

Robbins, P. D.; Hideaki, T.; Ghivizzani, S. C. Viral vectors for gene therapy. *Trends Biotechnol.* **1998**, *16*, 35−40.

Schmid, G.; Blanch, H. W.; Wilke, R. W. Hybridoma growth, metabolism, and product formation in HEPES-buffered medium: II. Effect of pH. *Biotechnol. Lett.* **1990**, *12*, 633−638.

Shen, B. Q.; Clarke, M. F.; Palsson, B. O. Kinetics of retroviral production from the amphotropic CRIP murine producer cell line. *Cytotechnology* **1996**, *22*, 185−195.

Tacheuchi, Y.; Porter, C. D.; Strahan, K. M.; Preece, A. F.; Gustafsson, K.; Cosset, F. L.; Weiss, R.A. Sensitization of cells and retroviruses to human serum by (α-1-3) galactosyltransferase. *Nature (London)* **1996**, *379*, 85−88.

Accepted for publication July 5, 2000.

BP000078J