Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 148 (2008) 253–259
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Sorting vector producer cells for high transgene expression increases retroviral titer

E.L. Beaudoin, A.J. Bais, R.P. Junghans *

Boston University School of Medicine, Department of Surgical Research, North Campus, Roger Williams Medical Center, 825 Chalkstone Avenue, Providence, RI 02908, USA

Received 13 December 2006; received in revised form 11 December 2007; accepted 13 December 2007
Available online 4 February 2008

Abstract

Vector producer cells are derived from helper cell lines expressing viral proteins that have been transduced to express a transgene-carrying retroviral genome. Vector producing cells express two relevant forms of RNA in their cytoplasm: vector RNA (vRNA) that is packaged as the actual gene transfer agent, and messenger RNA (mRNA) from which transgene is translated. Two premises underlie this study: (1) vRNA is limiting for virus production and (2) mRNA is proportional to vRNA. Together, these premises predict that transgene expression in the vector producing cells will be predictive of the viral titer from those cells. In this case, sorting the vector producing cells for high transgene expression should select for more virus production in vector producing cell supernatants. This prediction was supported, with a greater than fivefold benefit in viral titer. This demonstrates a rapid and simple method by which to obtain significantly increased viral titers from the same vector producing cell preparation.

Keywords: Vector producing cell; Retroviral supernatant; Real-time PCR; Viral titer; Cell sorting; Poisson calculation

1. Introduction

One of the challenges for retroviral gene therapy has been the typically low transduction rates obtained with viral supernatants. This has been particularly true with T cells that are more resistant than many other cell types. This difficulty has stimulated the development of procedures (e.g., centrifugation: Annenkov et al., 2002; Del Vecchio et al., 2001; Kuhlcke et al., 2002; Ohkubo et al., 2001; Sanyal and Schuening, 1999, viral preloading: Kuhlcke et al., 2002, static versus flow-through infections: Del Vecchio et al., 2001; Chuck and Palsson, 1996 and additives—e.g., polycations: Del Vecchio et al., 2001 and retronectin: Sanyal and Schuening, 1999; Lamers et al., 2002; Li et al., 2003) to enhance the transduction process. Whereas these processes improve the efficiency of viral entry, a complementary route to improve transductions is to increase the actual titer of the viral supernatants. With increased supernatant potency (i.e., viral titer), higher fractions of cells can be modified per transduction or smaller supernatant volumes can be used in transductions, thus reducing materials and costs. A comprehensive study of time, temperature, and cell density was undertaken that showed vector producer cell culture conditions could yield modest improvements in viral titers (Reeves et al., 2000). In the present report, a further method to increase titers is presented. It is shown that the production of infectious virus parallels transgene expression by the vector producing cells, and that sorting for such expression selects for improved vector producing cells with several-fold higher titers.

2. Materials and methods

2.1. Vector and vector producing cell

The retroviral vector was an MFG vector backbone (gift of R. Mulligan, Children's Hospital, Boston, MA, USA). The transgene is a chimeric immune receptor (CIR) of an anti-CEA (carcinoembryonic antigen) IgCD28TCR (T cell receptor) (called Tandem for two signals) based on a previously published IgTCR (Nolan et al., 1999). Plasmid DNA encoding the CIR was used to transfect a mix of Phoenix ampho- and eco-tropic

* Corresponding author. Tel.: +1 401 456 2507; fax: +1 401 456 4812.
E-mail addresses: rjunghans@rwmc.org, ebeaudoi@rwmc.org (R.P. Junghans).

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.12.008

cells[1] two times (American Type Culture Collection, Manassas, VA, USA [ATCC], CRL-SD3443 ampho/CRL-SD-3514 eco). When Phoenix eco- and ampho-tropic cells are co-cultivated, the vectors produced will infect the host cells of the opposite env-type cell ("ping-pong") thereby greatly enhancing the original transfection. Phoenix cells are resistant to reinfection by viruses of the same env-type (Coffin et al., 1997). After 24 h, the transduced Phoenix-mix cells were sorted once for CIR expression. The viral supernatant from these transiently high-expressing ampho/eco virus-producing cells was used to transduce a PG13 (empty) murine helper cell line (ATCC: CRL-10686) for stable integration and virus particle production. This transduced vector producing cell line (PG13/Tandem) was used for the experiments in this report. The PG13/Tandem culture was fluorescence-activated cell sorting (FACS) sorted three times over a period of 3 weeks for high transgene expression, each time keeping the upper 5% by mean fluorescence intensity (MFI). The cells were expanded between sorts and frozen as sort 0 (presort), 1, 2, and 3 sorted cultures.

2.2. Cell sorting and flow cytometry

Cells were trypsinized and stained by standard procedures. An anti-idiotype antibody (WI2) is reactive with the surface-expressed CIR. WI2 [Immunomedics, Morris Plains, NJ] was used as the primary antibody and detected by secondary goat anti-mouse-PE (GAM-PE) (R-Phycoerythrin [Caltag Labs, Burlingame, CA]). A stained (WI2/GAM-PE) PG13/no vector sample was used for a background control. Cells were analyzed by flow cytometry (using the FACS Calibur [Becton Dickinson]) for transgene expression and estimated for percent transduction and MFI. Data were analyzed with the Cell Quest program.

Vector producing cell sorting was performed after primary staining, as above, at the Rhode Island Hospital Core Research Labs Cell Sorting Facility with the Becton-Dickinson FACSVantage SE cell sorter. Selected cells were in the upper 5% from sorts 1 to 3 (5.3, 4.5 and 5.4%, respectively). The population of cells comprising the upper 5% that is selected, gated, and sorted is based on its having the highest signal on log scale for WI2-PE, thus the highest transgene cell surface expression.

2.3. Testing the viral titers of the PG13/Tandem sorts

PG13/Tandem cells, sorts 0–3, were freshly thawed and placed in culture for 3 days when the cultures were trypsinized and counted. Four 75 cm^2 flasks were seeded with 2.9×10^6 cells, one culture for each sort, and grown at 37 °C, until the cultures reached 85% confluence. Harvest 1 at 37 °C was collected after 24 h when the cultures reached confluence. They were then placed in a 32 °C incubator to slow down cell growth because PG13 cells continue to expand after they reach confluence and for improved viral titer at the lower temperature. Previous experiments have shown that harvests collected after 24-h intervals at 32 °C are more infectious than harvests collected after 8- and 24-h at 37 °C (Beaudoin and Junghans, unpublished). Three more harvests were collected over 3 days at 24-h intervals from each sort: harvests 1–3 at 32 °C. All harvests were filtered through 0.45-μm filters to remove any cells then frozen promptly at −80 °C. Because of higher cell densities over the 3 days past confluence the cultures may reach suboptimal conditions of pH, glucose, and lactate more quickly over the 24-h interval thereby affecting the viral potency of later harvests.

Four viral supernatant harvests from each sort were thawed quickly in a 37 °C water bath then placed on ice. 293T cells (ATCC#: CRL-11268) (0.25×10^6 in 50 μL) were incubated with 0.5 ml of undiluted viral supernatant containing 10 μg/ml protamine sulfate in a 24-well microplate. Several wells of control untransduced 293T cells in growth medium (GM: RPMI-1640 + 10% FCS + 1% antibiotics) plus protamine sulfate were included. Spinfection was performed by centrifugation of the microplate for 1 h at 2500 rpm ($1000 \times g$) after being loaded with cells and viral supernatant. After spinfection, 350 μL viral supernatant were removed from the wells and replaced with 1 mL GM. The cells were then cultured for 48 h to allow for gene expression before conducting flow cytometry to assess transduction efficiency.

2.4. Poisson correction

The Poisson correction (Sokal and Rohlf, 1995) addresses the fact that higher percent transductions will have increasing fractions of cells infected with >1 virus particle wherein the transduced fraction becomes a non-linear representation of infection events that underestimates titer. The mean number of infection events (titer) is given as $\mu = \Sigma nP(n)$, in which n is the number of times a cell is infected and $P(n)$ is the probability of n infections of a cell. The fraction of cells with *any* infection is $x = \Sigma P(n)$, $n > 0$, or $x = 1 - P(0)$. In this assay, x is the same as the fraction transduced. The Poisson terms are

$$P(n) = \exp(-\mu)\frac{\mu^n}{n!}$$

For $n = 0$, $x = 1 - \exp(-\mu)$

The measured transduction fraction (x) is corrected upward to a number that is directly in proportion to titer by a rearrangement of the prior equation:

$$\mu = \ln\left(\frac{1}{1-x}\right)$$

This corrected number, expressed as a percent, is taken to be proportional to titer in infectious units per milliliter. (A titer of 100% on this adjusted scale would correspond to 5×10^5 IU/ml under the conditions of our infection procedure, corresponding to one virus particle infecting one cell, each cell being infected only once.)

2.5. Real-time PCR

Total RNA was isolated from PG13/Tandem cell sorts 0–3 using the RNeasy kit (Qiagen Inc.) and oligo(dT) reverse tran-

[1] A description of how these cell lines were first generated can be found on the Nolan Lab website at www.stanford.edu/group/Nolan/.