Retroviral Infection Is Limited by Brownian Motion

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ABSTRACT

Replication-defective retroviruses are frequently used as gene carriers for gene transfer into target cells. Here we show that the short half-lives of retroviruses limit the distance that they can effectively travel in solution by Brownian motion, and thus the possibility of successful gene transfer. This physicochemical limitation can be overcome, and effective contact between the retroviral gene carrier and the target cell can be obtained, by using net convective flow of retrovirus-containing medium through a layer of target cells. Using model cell lines (NIH-3T3 and CV-1), it was shown that gene transfer rates can be increased by more than an order of magnitude using the same concentration infection medium. High transduction rates could be obtained even in the absence of polycations, such as Polybrene, which heretofore have been required to achieve reasonable transduction rates. This development may play an important role in realizing human gene therapy.

OVERVIEW SUMMARY

Retroviruses have short half-lives and therefore can only travel a limited distance by random Brownian motion in infection medium before deactivating. This distance is only a few hundred microns, and this constraint is shown to limit gene transfer rates. This limitation can be overcome by slow flow of the infection medium vertically through the target cell bed and gene transfer rates can be substantially increased.

INTRODUCTION

The ability to introduce DNA into human cells is the basis for the burgeoning field of gene therapy (for reviews, see Anderson, 1984; Miller, 1990, 1992a; Brenner, 1993; Mulligan, 1993). Replication-defective retroviruses have received much attention as vehicles to carry the foreign DNA into many human cell types (Cepko et al., 1984; Miller and Buttimore, 1986; Markowitz et al., 1988; Larrick and Burck, 1991). The process of retroviral infection involves many steps (Dubois-Dalcq et al., 1984): the initial step is that viruses must make contact with the cell; this is followed by specific binding of virus onto a cell-surface protein; the virus is then internalized; the viral RNA is reverse-transcribed into DNA; the double-stranded DNA enters the nucleus; and finally the retrovirus derived DNA is integrated into the target cell’s genome. The last step of DNA integration is believed to require cell division (Springett et al., 1989; Miller et al., 1990; Roe et al., 1993). The overall success of the infection, as determined by expression of the delivered gene, thus depends on the probability of success of each step of the series.

In this communication, we analyze the first step of this series of events and focus on the factors that determine the frequency of contact between the virus and the target cell. The initial encounter of the virus with the target cell is governed by a predictable physicochemical process—random Brownian motion.

ANALYSIS OF THE KINETICS OF VIRUS-CELL ENCOUNTER

Typically, infection is carried out in a system where a liquid layer containing the retrovirus is placed on top of a bed of target cells. The physics of this infection system may be described by three processes occurring simultaneously (Fig. 1): (i) Brownian motion of the retrovirus, (ii) decay of the retrovirus, and (iii) adsorption, or capture, of the retrovirus by the target cell. A retrovirus is a colloidal particle with a density similar to that of tissue culture medium, 1.16–1.18 g/ml (Lowy, 1985). Its root mean square displacement \( \sqrt{ } \) by Brownian motion over time \( t \) can be described by (Einstein, 1905):

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FIG. 1. Static transduction. The schematic shows how a retrovirus' distance from the target cell bed affects the probability of it being adsorbed. The virus close to the bed, at $d_1$, has a high probability of being adsorbed, whereas the virus further away, at $d_2$, decays over the same time period and cannot reach the target cell. Each retrovirus travels an average distance $l$ by Brownian motion over the time period of interest.

$$l = \sqrt{2Dt}$$

(1)

where $D$ is the diffusion coefficient. The numerical value of the diffusion constant for a retrovirus can be estimated from the Stokes–Einstein equation (e.g., see Cussler, 1984) to be approximately $6.5 \times 10^{-6}$ cm$^2$/sec using a viral diameter of 100 nm (Dubois-Dalcq et al., 1984), a temperature of 37°C, and a medium viscosity of 0.007 g/cm-sec.

Retroviral half-lives ($t_{1/2}$) are generally short (Levin and Rosenak, 1976; Sanes et al., 1986; Layne et al., 1989; Paul et al., 1993; Kotani et al., 1994). We have measured the half-life for a murine amphotropic retrovirus produced by the pCRIP packaging cell line (Danos and Mulligan, 1988) to be about 5-8 hr at 37°C (Chuck, 1995). Using the appropriate numerical values for the diffusion coefficient and half-life in Equation (1), we estimate the distance that an average retroviral particle can travel within one half-life ($l_{1/2}$) to be 480-610 μm. Since tissue culture procedures typically use liquid depths of 2-5 mm, the majority of retroviruses in the infection medium, i.e., those above a level of 480-610 μm, will not be able to reach the cell bed within one half-life.

Based on the physics of the viral capture process, the probability of capture is expected to be inversely proportional to the particle's distance from the surface (of capture) (Berg, 1983)—the target cell bed—and the time of capture proportional to the square of this distance (Equation 1). Thus, only those particles closest to the target cells will be captured efficiently and within the time span of the retroviral half-life. Were it not for the short half-life, all the retrovirus particles would reach the target cell surface given an infinite period of time.

The probability of a retrovirus particle adsorbing to a target cell will to a first approximation follow mass action kinetics, where the rate of capture is proportional to the densities of the colliding entities:

$$rate \text{ of adsorption} = k C_v C_t$$

(2)

where $C_v$ is the virus density (number of viruses/volume) close to the target cells, $C_t$ is the target bed cell density (number of cells/area), and $k$ is a second-order rate constant characterizing the adsorption event. Thus, the number of cells transduced is expected to be proportional to both the initial target cell density ($C_{T0}$) and initial retroviral concentration ($C_{V0}$) for fixed transduction times. This prediction is experimentally verified below.

The importance of high target cell density and retroviral concentration for obtaining greater numbers of transduced cells has been emphasized in the literature (Belmont et al., 1988; Bodine et al., 1990; Lynch and Miller, 1991; Hughes et al., 1992; Buchschacher, 1993; Cassel et al., 1993; Rettinger et al., 1993; Kotani et al., 1994). However, it remains that individual retroviruses located at increasing distances from the target cell bed have decreasing probability of ever reaching the target cells, if their movement is due only to Brownian motion. Thus, the number of cells transduced is not expected to increase with additional numbers of viruses located at increasing distances. Again, this prediction is experimentally verified below, where increasing distances are achieved by increasing volumes of virus solution overlaying a target cell bed.

How can the limitations of time and distance for capture imposed by random Brownian motion be overcome? If the motion of the retrovirus is directed toward the target cells, the frequency of virus–cell encounters would increase. The virus can be directed, or carried in this way, by fluid flow. Fluid flow is usually implemented by agitation, or mixing of the target cell culture. However, microhydrodynamics are such that laminar hydrodynamic boundary layers form close to solid surfaces—such as cell growth surfaces in standard cell culture plasticware—even in well-mixed flows. These boundary
layers have flows that are parallel to the solid surface, and their thickness (Schlichting, 1955; Levich, 1962) will be on the order of the penetration distance of the virus, as defined above ($l_p$). Thus, even with bulk fluid agitation, the final encounter of the virus and the target cell is governed by Brownian motion.

This hydrodynamic limitation can be overcome by first seeding the target cells onto a porous surface and then flowing a virus solution directly through the target cell bed. In this way, net fluid flow can be induced over distances shorter than those defined by the viral penetration distance and retroviruses located initially far away from the target cell bed are brought close to the target cells within a controllable length of time. By using a convective fluid flow distributed down to cellular dimensions to drive the virus toward the target cells, we do not rely on Brownian motion to deliver the virus to the target cells, but the fluid motion itself. In the experiments presented below, two model cell lines are used, CV-1 and NIH-3T3, to show increases in transduction efficiencies with this "flow-through" gene transfer technique. Its performance is compared with the traditional method in which there is no net fluid movement, what is termed here as "static transduction."

**MATERIALS AND METHODS**

**Cell culture**

Target cell lines, CV-1 and NIH-3T3, were seeded at 3,000 cells/cm² (unless otherwise stated) in either six-well plates or on collagen membranes 1 day prior to infection. The collagen membranes (Transwell-COL cell culture inserts from Costar, Cambridge, MA) were of 0.4 μm pore size and 24.5 mm diameter. The retrovirus packaging cell line (produced by transfecting a pMFG vector containing a lacZ gene into ΨCRIP) was kindly provided by Dr. James Wilson (construction of a similar vector is described in Wilson et al., 1988). Producer cells were thawed every 6 weeks and grown in 10-ml tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ). Both the target and producer cell lines were grown with 10% calf serum supplement (GIBCO, Grand Island, NY) in DMEM and were cultured at 37°C and 5% CO₂.

**Retrovirus supernatant**

Medium that was conditioned for 24 hr by a confluent monolayer of producer cells was filtered through 0.4-μm pore-sized filters (low protein binding Sterile Acrodisc, Gelman, Ann Arbor, MI). Virus medium harvested from producer cell cultures was assigned a relative retroviral concentration value of 1.0. This rather arbitrary assignment was necessary due to the batch-to-batch variation of retroviral titer that occurs with retroviruses from different producer cell lines (Miller, 1992b; Paul et al., 1993), where the viral productivity of a producer cell declines with each succeeding passage. Variable retroviral concentrations were made by diluting the virus supernatant with growth medium. A relative retroviral concentration of 0.1 was used for infecting cultures that would later be assayed (for lacZ expression) by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining. Similarly, viral concentrations of 0.5 were used for infecting cultures that would be assayed by flow cytometry. Polybrene (Aldrich, Milwaukee, WI) was added to 4 μg/ml (unless otherwise stated). Negative controls (mock infections) were prepared by adding Polybrene (at the same levels as in the retrovirus supernatant) to growth medium. These controls were carried out using both the static and flow-through transduction procedures.

**Static transductions**

Static transductions were carried out on cell culture inserts in parallel with flowthrough transductions, unless stated otherwise. The substrates used in this work (i.e., tissue culture plastic or Transwell-COL) did not affect either cell growth or transduction efficiency (Chuck, 1995). Static transductions were carried out as follows: medium was removed from target cell cultures and replaced by 2 ml of virus solution. The cultures were then incubated for the determined transduction time, after which the retrovirus solution was removed and fresh growth medium was added. Cultures were assayed for lacZ expression 3-4 days later.

**Flow-through transductions**

Virus medium was gravity flowed through the seeded collagen membranes at an average flowrate of ~1 ml/hr for the transduction period (up to 10 hr) at 37°C. A schematic of this setup is depicted in Fig. 3A. Flow-through transductions were always conducted in parallel with static transductions, using the same preparation of virus solution and target cell seedings. After the transduction period, the virus medium was removed from the reservoir above the target cell bed and fresh growth medium was added. Cultures were left to incubate (without media flow) for 3-4 days until the time of assay.

**X-Gal staining procedure**

Each cell culture well or insert was washed twice with 2 ml of Hank's buffered saline solution (HBSS, GIBCO) and fixed with 1.5 ml of 2% (vol/vol) formaldehyde (Sigma) and 0.2% (vol/vol) of glutaraldehyde (Sigma) for 5 min. Following fixation, the cells were washed once more in HBSS before adding 1.5 ml of staining solution. The staining solution consisted of 50 μl of [20 mg/ml X-Gal powder dissolved in N,N-dimethylformamide (DMF, Sigma)] per milliliter of 5 mM K₃Fe(CN)₆ (Sigma), 5 mM K₄Fe(CN)₆·3H₂O (Sigma), and 2 mM MgCl₂ (Sigma) in phosphate-buffered saline (PBS). The samples were incubated for 1–4 days at 37°C to allow any blue cell color to develop, and numbers of colony-forming units (CFU, at 2–8 cells/colony) in each well were counted.

**Flow cytometry**

Each Transwell-COL was washed three times with 2 ml of HBSS and the cells removed by 1 ml of trypsin (GIBCO) exposure. Fresh growth medium was then added to resuspend and wash the cells. Reagents from the FluorReporter lacZ gene detection kit (Molecular Probes, Eugene, OR) were used to prepare and stain the cells. The cells were incubated in a 37°C water bath for 5 min and then loaded with substrate by hypotonic shock as follows: 50 μl of 2 mM fluorescein di-β-D-galactopyranoside (PDG) was added to each tube at 37°C and left to incubate for 1–4 days at 37°C to allow any blue cell color to develop, and numbers of colony-forming units (CFU, per milliliter). The samples were then incubated for 1–4 days at 37°C to allow any blue cell color to develop, and numbers of colony-forming units (CFU, at 2–8 cells/colony) in each well were counted.
incubate for 90 sec. The tubes were then immersed in ice, and 450 μl of ice-cold PBS with human IgG (Sigma) and 1 mg/ml propidium iodide (PI) was added. A Coulter EPICS flow cytometer was used to determine percentage of cells transduced, as indicated by positive green fluorescence (Chuck and Palsson, 1991).

RESULTS

The bilinear dependence of number of transduced cells on both target cell and retrovirus densities was shown experimentally. Either the initial virus density (C10) or target cell density (C70) was kept constant while varying the other (Fig. 2). Experimentally, these relationships were found to be linear, as shown in Fig. 2, A and B, thus confirming the expectation that infectivity is proportional to the likelihood of a collision between the retrovirus and the target cell, as described by Equation 2.

The number of transduced cells did not depend on the total number of viruses present. Experiments were performed where the number of transduced cells was measured over time for different amounts of virus supernatant overlaying the target cell bed. Different numbers of virus were obtained by varying the depths of retrovirus solution in seeded tissue culture wells. Retroviral concentrations, CV-1 target cell densities, and transduction time were held constant. Figure 2C shows that no significant difference in the number of cells transduced was observed as the depth of the solution layer exceeded the mean displacement distance λ0.5 for all time points. Even though there was a higher number of retroviruses in the infection medium, they did not lead to an increase in the number of cells transduced, confirming the diffusion limitation predicted by the analysis presented above.

Vertical flow-through could substantially increase the number of cells transduced (Fig. 3). Two target cell lines, NIH-3T3 and CV-1, were used to examine the flow-through method of retrovirus delivery. Static transductions (no fluid flow) were carried out on collagen membranes with infection fluid overlaying the target culture. For static transductions, the target cells were exposed to 2 ml of virus medium for the entire 10-hr period, which should yield a maximal number of transduced cells (Fig. 2C plateau). Using flow-through, the degree of transduction increased linearly with the volume of retrovirus solution flowed. The number of flow-transduced cells far exceeded the number of static-transduced cells (Fig. 3B,C). Thus, the number of transduced cells was proportional to the number of retroviruses contacting the target cell bed. Visually, the increase in the number of transduced target cells was quite dramatic, as shown in Fig. 4. This experimental result is again consistent with the theoretical predictions made above.

Polybrene, a polycation, has been essential to obtaining high transduction efficiencies in RNA virus (static) infection systems (Manning et al., 1971; Cornetta and Anderson, 1989). Polybrene is believed to increase contact between the virus and target cell by overcoming (repulsive) electrostatic forces between the like-charged virus and cell (Coelen et al., 1983). On the basis of the analysis presented above, one would expect that the convective force applied in the flow-through procedure will alleviate the need for Polybrene. The data in Fig. 3C confirm

FIG. 2. Gene transfer (as enumerated by CFU) after static transduction is shown as a function of initial retroviral concentration (relative units) with an 8-hr transduction time (A), (B) target cell density (units in thousands of CV-1 cells per/well) with an 8-hr transduction time, and time, with increasing depths of infection solution (C): 520 μm (500 μl = □), 832 μm (800 μl = Δ), and 1,559 μm (1500 μl = ●). All measurements were performed in triplicate. Individual data points are shown.
FIG. 3. Transduction in flowing medium. A schematic of the retrovirus solution flowing through the target cell bed is shown in A. Transduction enhancements using virus solution flow (relative to a no-flow, or static transduction) are shown for CV-1 cells in B and NIH-3T3 cells in C. No Polybrene was used in infecting the NIH-3T3 cells.

this expectation: More than 35% of 3T3 target cells were transfected without the use of Polybrene. Using the static method, less than 1% of the cells could be transduced.

Several control experiments were performed to verify the above results. Mock infections using both the static and flow-through methods of exposure resulted in no cells being transduced, as determined by both X-gal and flow cytometry. Thus, the flow-through procedure did not result in a greater background of (false) gene expression. Also, various methods of mixing the infection cultures (continuous shaking and periodic tilting) did not result in any additional cells being transduced (Chuck, 1995) over those obtained by static virus exposures. The enhancements from flow-through were not due to the collagen substrate; similar enhancements as reported above were observed with polyester membranes with Polybrene present (Chuck, 1995). In addition, the numbers of cells recovered (~3 days post infection, at the time of analysis) and the percentages of cells transduced were the same for static transductions whether the target cells grew on membrane substrate or tissue culture plastic. Finally, no difference in the number of cells recovered was observed for flow-through versus static transductions, or for the concentration of retrovirus used in either of these transduction methods (Chuck, 1995).

DISCUSSION

In widely used methods of static transductions using conditioned medium from packaging cell lines, the transport of retrovirions to target cells is, in all probability, limited by the slow delivery by Brownian motion relative to the half-life of the retrovirus. Thus, the virus adsorption rate is dictated by the virus mass transfer rate to the target cell bed. It has been demonstrated here that most of the volume of virus solution overlaying a target cell bed does not contribute toward increasing the transduction rate. Under these diffusion limitations, the number of virions reaching the cell bed can be increased by increasing the number of retroviruses per unit volume in the infection medium, but high retroviral titers have proven difficult to obtain (Belmont et al., 1988; Bodine et al., 1990; Lynch and Miller, 1991). Retrovirus-mediated gene transfer has been shown to be enhanced through co-culture of the target cells on the virus-producing cell line (Hock and Miller, 1986; Bodine et al., 1991). The reasons for this enhancement can be explained by the current results: The higher rates are due to the proximity of target cells to viral source.

The limitations imposed by Brownian motion can be managed using directed convective flow. By inducing liquid (and thus retroviral) motion in the desired direction only, we do not rely on Brownian motion to deliver the virus to the target cells. Fluid flow through a porous cell growth surface allows for very effective contact between the gene carrier and the target cell. The net rate of transport of retrovirus to the target cell bed, and thus the net rate of adsorption, can be significantly increased. Flow-through should be an attractive method of delivery of other gene transfer vehicles (e.g., adenoviruses, adeno-associated viruses, and liposomes), which also have very low diffusivities.

Selective motion of viruses can be obtained by means other than fluid flow. Elevated gravity will increase the settling rate of the virus. Indeed, it has been shown that centrifugation does enhance retrovirus-mediated gene transfer into peripheral blood lymphocytes from humans and nonhuman primates (Bunnel et
FIG. 4. Photographs of transduced CV-1 target cells. Two milliliters of virus medium in static transduction (A) is compared to increasing volumes in flow-through transduction: 1 ml (B), 3.5 ml (C), and 5 ml (D).

al., 1995). The mechanism leading to this increase in gene transfer rate obtained by centrifugation is outlined in this manuscript. This study thus shows that eliminating the limitation of Brownian motion will also lead to increased gene transfer into primary human cells.

The virus can be adhered to the membrane without having the target cells present at the time of fluid flow. The target cells can be introduced subsequently and allowed to grow on a virus-containing surface (Chuck and Palsson, 1996b). This approach allows for the separation of the process of localization of the virus and the exposure to the target cell. This separation offers a number of advantages for gene therapy because the medium containing the gene carrier and the cell culture medium can be separated. Retroviral supernatants are known to contain products that alter the growth of the target cells (Xu et al., 1994). Using the flow-through approach, one can "load" the gene carriers onto the growth surface and then remove the infection medium prior to introducing the target cells in their own growth medium. The same procedure can be used for a gene carrier that needs to be present in serum-free medium, as is the case with liposomes, and target cells that have to be in serum-containing medium. Other advantages of the flow-through approach for gene therapy include insensitivity to titer (Chuck and Palsson, 1996a) and shortened virus exposure times (Chuck and Palsson, 1996b).

In summary, we have shown that retrovirus-mediated gene transfer is limited by random Brownian motion. This limitation can be overcome by directing the motion of the retrovirus toward the target cells. The flow-through approach may significantly help the development of gene therapy (Chuck and Palsson, 1996a), which is currently hampered by low gene transfer rates (Buchshacher, 1993; Cassel et al., 1993).

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