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Retrovirus Infection: Effect of Time and Target Cell Number

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Using a model amphotropic recombinant retrovirus encoding the *Escherichia coli lacZ* gene and quantitative assays to measure virus infection, we have determined the effects of time and target cell number on infectivity. Infection of various numbers of NIH 3T3 fibroblasts showed that the extent of *lacZ* virus infection was dependent on virus concentration and independent of target cell number. These results demonstrate that multiplicity of infection is not an accurate predictor of the efficiency of retroviral infection. Varying the time of viral infection revealed that maximal infection occurred after greater than 24 h of exposure of the cells to the *lacZ* virus. Half-maximal infection occurred after 5 h of exposure. After 2 h of adsorption at 37°C, the majority of infectious virus was not adsorbed to cells but was unbound and able to infect other cells. These results are discussed in terms of both their relevance to the fundamental biology of retrovirus infection and the use of recombinant retroviruses for retrovirus-mediated gene transfer with purposes of gene therapy.

Retrovirus infection is a multistep process which begins with adsorption of the virus particle to the cell surface via a specific cellular receptor (45). For murine leukemia retroviruses (MuLV), this interaction is mediated by the gp70 envelope protein found on the surface of viral particles. Purified gp70 binds cellular receptors with specificity and high affinity (9, 10, 12, 13, 16, 18, 21). Viruses of different host ranges (ecotropic, xenotropic, amphotropic, and polytropic) recognize distinct cellular receptors, and this specificity is determined by the amino acid sequence of gp70 (8, 17, 34, 38). The critical domains of the gp70 molecule which specify host range have been located (8, 17, 34, 38). In addition, the genes encoding the cellular receptors for several retroviruses have been cloned, including the receptor for the ecotropic virus (3, 23, 46), amphotropic virus (31, 44), gibbon ape leukemia virus (22, 37), and the human immunodeficiency virus (HIV) (11, 24), and the critical domains of some of these receptors have been located (2, 50).

While much is known about the individual viral envelope proteins and cellular receptors from studies of purified proteins and isolated genes, little is understood about the detailed mechanism by which the viral particle binds to and enters the target cell. For example, since the surface of the viral particle is composed of numerous gp70 molecules embedded in the lipid bilayer of the particle, it is difficult to define the contribution of the individual gp70 proteins to the effective particle affinity. Information on these interactions and the properties of retroviral virions is important to the understanding of retrovirus-cell interactions and is potentially applicable to the use of recombinant retroviruses for the purposes of gene transfer and human gene therapy (7, 36).

In the current study, we have used a recombinant retrovirus encoding the *Escherichia coli lacZ* gene as a model retrovirus along with a combination of assays to investigate the parameters which govern retrovirus infection. The results presented indicate that retrovirus infection is dependent on virus concentration and independent of target cell number over a greater than 10-fold range in cell number and virus concentration. Moreover, maximal infection requires prolonged exposure of the cells to virus, and after 2 h of adsorption, the majority of infectious virus is not bound to the cells but is free in solution.

MATERIALS AND METHODS

Chemicals. Nonidet P-40, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), glutaraldehyde (ultrapure), rhodamine B, 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT), and 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene) were purchased from Sigma Chemical Co., St. Louis, Mo. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was purchased from Boehringer Mannheim GmbH.

Cell culture. NIH 3T3 cells and *lacZ* virus-producing cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, Md.) with 10% bovine calf serum (HyClone Labs Inc., Logan, Utah) containing 100 U of penicillin and 100 μg of streptomycin (Gibco BRL) per ml. The amphotropic packaging cell line ψ^- CRIP, producing the α-SGC-*lacZ* virus, was kindly provided by L. K. Cohen of Somatix Therapy Corporation, Alameda, Calif. (15). *lacZ* virus-containing medium was harvested from confluent cultures of the virus-producing cell line (overnight incubation of 10 ml of culture medium in a 10-cm tissue culture dish), frozen on pulverized dry ice in 1-ml aliquots, and stored at −85°C.

Diluted titer assay. Tenfold serial dilutions of the *lacZ* virus stock were made in DMEM with 10% calf serum and Polybrene (8 μg/ml). Three milliliters were used to infect 3×10^5 3T3 cells plated onto a 60-mm dish the previous day. The medium was changed after an overnight incubation, and 2 days after the start of infection, the cells were fixed and stained for β-galactosidase activity with X-Gal (40). Colonies of $lacZ^+$ cells, which were typically clusters of two, four, or eight blue cells, were counted with the aid of a dissecting microscope. At appropriate dilutions of the virus stock, the clusters of blue cells were sufficiently spread over the 60-mm dish that each cluster arose from a single infectious event. From triplicate plates, the number of $lacZ^+$ CFU per milliliter was $4 \times 10^5 \pm 0.8 \times 10^5$.

MOI. The multiplicity of infection (MOI) is the ratio of infectious virus to target cells. To determine the percentage of cells expected to be infected with at least one active virus at a given MOI, we used the expression $100 \times (1 - e^{-[MOI]})$ (29). This expression assumes that virus particles distribute into individual cells randomly and that their distribution will follow the Poisson equation $p(r) = s^r e^{-s}/r!$, where $p(r)$ is the probability of having exactly r virions in a given cell, s is the average number of virions per cell (MOI), and r is the actual number of virions in a given cell. The percentage of cells which have been infected is equal to $100 \times [1 - p(0)] = 100 \times (1 - e^{-s}] = 100 \times (1 - e^{-(MOI)}]$, where $p(0)$ is the fraction of cells which have not been infected.

β-Galactosidase assay. We have developed a microplate assay to measure virus infectivity (33). The day before infection with the *lacZ* virus, a 10-cm dish of confluent 3T3 cells was treated with trypsin, and the cells were counted with a Coulter counter model ZM (Coulter Electronics, Hialeah, Fla.). Five thousand cells in 100 μl of medium were plated per well in a 96-well flat-bottomed tissue culture dish with a low-evaporation lid (Costar Corp., Cambridge, Mass.). The next day (19 to 25 h later), the medium was removed, and dilutions of *lacZ* virus in culture medium with Polybrene (8 μg/ml) were added to each well (final

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volume, 100 μl per well unless otherwise specified). Two days after infection, the culture medium was removed, and the cells were washed once with 100 μl of phosphate-buffered saline (PBS)–1 mM $MgCl_2$. After removal of the wash solution, 50 μl of lysis buffer was added (PBS with 1 mM $MgCl_2$ and 0.5% Nonidet P-40) to each well, and the plate was incubated at 37°C. After 30 min, 50 μl of lysis buffer with 6 mM ONPG warmed to 37°C (28) was added to each well, and the plate was incubated at 37°C for another 15 min. The reactions were halted by the addition of 20 μl of stop buffer (1 M Na_2CO_3) (41). The plate was brought to room temperature, and the optical density at 420 nm (OD_{420}) was read, nonspecific background at 650 nm was subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the averages for at least triplicate wells.

Percent infection assay. Confluent wells of the 96-well dish infected with the *lacZ* virus were washed once with PBS–5 mM EDTA and treated with 100 μl of trypsin solution as described above. Cells were dispersed by repeated pipetting, and 20 μl of the trypsinized cells was diluted into 1 ml of medium. Ten microliters of this dilution was plated into a 10-cm dish. After 10 days, when between 100 and 200 macroscopic colonies had grown, the plates were washed once with PBS and fixed in PBS containing 0.5% glutaraldehyde for 5 min at room temperature. Plates were washed with PBS–1 mM $MgCl_2$ and stained for *lacZ* activity by incubation in a solution containing PBS, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 3.3 mM $K_3Fe(CN)_6$, and 1 mg of X-Gal per ml for 3 to 4 h at 37°C. Reaction mixes were removed, and the colonies were counterstained with a solution of 1% rhodamine B, rinsed with water, and air dried. Values for the percentage of $lacZ^+$ colonies at each point (blue colonies/total colonies × 100) are the averages of triplicate wells of the 96-well dish.

MTT assay for cell number. The density of viable cells was measured by a modified MTT assay (35). The tetrazolium salt of MTT is cleaved by the mitochondrial dehydrogenase of viable cells only and forms a colored precipitate, the amount of which is proportional to viable-cell number. After removal of the *lacZ* virus-containing medium, 10 μl of a MTT stock (10 mg of MTT per ml in PBS, filter sterilized) was added to each well. After incubation of the plate at 37°C for 4 h, the medium was gently removed, and the cells containing dark blue formazan precipitate were solubilized by addition of 150 μl of 10% sodium dodecyl sulfate (SDS) and incubation overnight at 37°C. The OD_{570} was read, and nonspecific background at 650 nm was subtracted.

RESULTS

Model retrovirus. To quantitatively measure the parameters which control retrovirus infection, we used a model retrovirus, α-SGC-*lacZ*. The α-SGC-*lacZ* recombinant retrovirus encodes the *E. coli lacZ* gene for β-galactosidase, which is expressed from an internal promoter and enhancer (alpha-globin promoter and cytomegalovirus enhancer) (15). The α-SGC-*lacZ* retrovirus is a replication-defective recombinant retrovirus with an amphotropic host range harvested from the ψ CRIP packaging cell line (12).

The number of infectious virions per milliliter of our frozen stock of α-SGC-*lacZ* virus was determined by serial dilution of the stock. Aliquots were subjected to 10-fold serial dilution and used to infect NIH 3T3 cells in the presence of Polybrene (8 μg/ml). Two days after infection, the cells were fixed and stained for β-galactosidase with X-Gal. Clusters of $lacZ^+$ cells stained blue and were counted on plates infected with virus diluted greater than 1,000-fold. The α-SGC-*lacZ* stock contained $4 \times 10^5 \pm 0.8 \times 10^5$ CFU/ml.

Dose-response of *lacZ* virus. To measure the parameters which control the infectivity of more concentrated stocks of the α-SGC-*lacZ* virus, we infected cells in a microplate assay with neat and diluted stocks of recombinant *lacZ* virus (33). NIH 3T3 cells (5,000 per well) were plated in a 96-well dish, and the next day, the medium was replaced with 100 μl of various dilutions of *lacZ* virus (from undiluted to 6 μl of viral stock) containing Polybrene (8 μg/ml). Two days after infection, the cells were washed and lysed, and the extracts were measured for β-galactosidase activity with the substrate ONPG by determining the OD_{420} of the reactions.

As shown in Fig. 1A, the dose-response curve of *lacZ* virus has two components, a linear portion (6 to 50 μl per well, $r^2 = 0.99$), in which β-galactosidase activity in the infected 3T3 cells is directly proportional to the volume of *lacZ* virus added, and a plateau region (above 50 μl), at which infectivity is maximal.

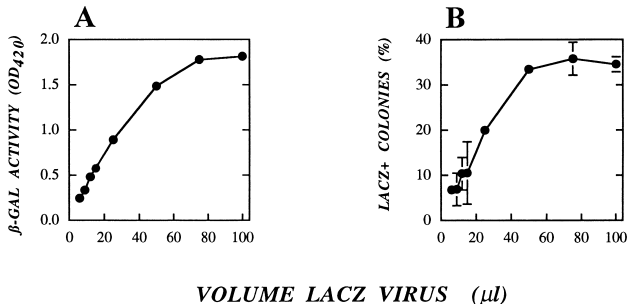

FIG. 1. Dose-response curve for α-SGC-*lacZ* recombinant retrovirus. (A) NIH 3T3 cells were plated in a 96-well dish (5,000 per well) and, the next day, infected with 100 μl of medium containing various amounts of *lacZ* virus with Polybrene (8 μg/ml). Two days later, the cells were assayed for β-galactosidase activity. The infected 3T3 cells were washed with 100 μl of PBS–1 mM $MgCl_2$, lysed with 50 μl of lysis buffer for 30 min at 37°C, and incubated with an additional 50 μl of lysis buffer containing 6 mM ONPG at 37°C. After 15 min, the reactions were stopped by the addition of 20 μl of 1 M Na_2CO_3. The OD_{420} was read and the OD_{650} was subtracted, with uninfected 3T3 cells used as a plate blank. Each point shows the mean and standard deviation for three replicates. (B) Parallel wells of cells infected with the same virus dilutions were trypsinized, diluted, and plated onto a 10-cm dish. After 10 days of growth, macroscopic colonies were fixed, stained for *lacZ* activity with the X-Gal substrate, and counterstained with rhodamine B. Values for the percentage of $lacZ^+$ colonies at each point (blue colonies/total colonies × 100) are the average ± standard deviation of triplicate wells of the 96-well dish.

As an independent measurement of the dose-response of *lacZ* virus, we determined the percentage of $lacZ^+$ cells after infection. Two days after infection, parallel wells of cells infected with the same *lacZ* virus dilutions were treated with trypsin, diluted, and plated onto 10-cm dishes. After 10 days, when the colonies had grown to macroscopic size, the cells were fixed and stained with X-Gal. Cells were counter stained with rhodamine B to reveal colonies of uninfected cells, and the percentage of $lacZ^+$ colonies for each virus dose was determined. As shown in Fig. 1B, a dose-response curve similar to the previous one was seen for the percentage of $lacZ^+$ colonies versus virus dose. The curve has a linear portion (6 to 50 μl per well, $r^2 = 0.98$), in which the percentage of infected cells is directly proportional to the volume of *lacZ* virus added. The maximal percentage of infected cells at the highest dose (undiluted) was 34.5% ± 1.7%.

One explanation for the shape of the dose-response curves could be that viral infectivity becomes saturated at the highest doses of virus. We ruled out this possibility by showing that conditioned medium contained an inhibitory activity. Each of the virus dilutions used in the dose-response experiments contains not only different virus concentrations but also different concentrations of conditioned medium from the packaging cell line mixed with different amounts of fresh medium. Experiments were performed to determine the effects of these components on the dose-response curve. The *lacZ* virus was removed from the viral stock by centrifugation, and various amounts of this virus-free conditioned medium were mixed with a constant amount of *lacZ* virus and adjusted to 100 μl with fresh medium. As the fraction of virus-free conditioned medium was increased, infectivity was depressed in a dose-dependent manner. Inhibitory activity was also found in virus-free conditioned medium from an ecotropic virus stock as well as normal NIH 3T3 cells (data not shown). These results suggest that the plateau at the highest virus doses is not due to a saturation phenomenon related to the virus or cells but is due to inhibitory effects of high concentrations of conditioned medium.

Another unexpected result of the dose-response experiments was the relatively low percentage of cells infected at the

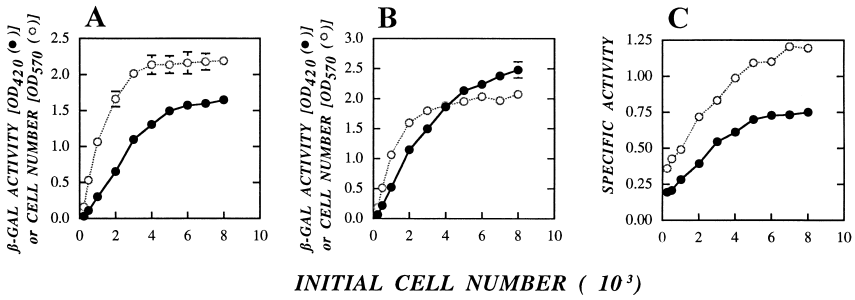

FIG. 2. Varying target cell number. (A) Various numbers of 3T3 cells (250 to 8,000 cells per well) were plated in duplicate 96-well plates. The next day, the medium was replaced with medium containing α-SGC-*lacZ* virus (100 μl) with Polybrene. Two days after infection, β-galactosidase activity was measured in one plate (OD_{420}), and cell number was determined by the MTT assay in the other plate (OD_{570}). Six microliters of a 10-mg/ml MTT solution was added to each well, and the plate was incubated at 37°C for 4 h. The cells were solubilized with SDS, and the OD_{570} minus the OD_{650} was determined. Each point shows the mean ± standard deviation of three replicates. (B) Various numbers of a *lacZ*$^+$ 3T3 cell line (250 to 8,000 cells per well) were plated in duplicate 96-well plates. Three days after plating, β-galactosidase activity was measured in one plate (OD_{420}), and cell number was determined by the MTT assay in the other plate (OD_{570}). (C) Specific activities of the infected cells (solid circles) and the *lacZ*$^+$ cell line (open circles) were calculated by normalizing the β-galactosidase activity of the cells to cell number (OD_{420}/OD_{570}) for each of the cell densities tested.

high viral doses. At the highest viral doses, the MOI was between 4 and 8 active viruses per cell, yet only 34.5% ± 1.7% of the cells were infected. At MOIs of between 4 and 8, greater than 98% of the cells would be expected to be infected with at least one active virus. Although this discrepancy might be explained by inhibition by conditioned medium at high viral doses, we wanted to determine if MOI was an accurate predictor of the efficiency of retroviral infection.

Infection efficiency is constant over a 10-fold range of cell number. To further examine the relationship between the efficiency of infection and MOI, various numbers of NIH 3T3 cells (250 to 8,000 cells per well) were plated in duplicate 96-well dishes, and the next day, the medium was replaced with medium containing α-SGC-*lacZ* virus (100 μl) with Polybrene. Two days after infection, β-galactosidase activity was measured by the ONPG assay in one plate, and cell number was determined in the duplicate plate, in order to normalize infectivity to cell number.

Cell number was measured by the MTT assay, and the OD_{570} minus the OD_{650} was determined. As expected, 2 days after infection, the number of cells varied according to the original number plated (Fig. 2A). The wells in which 4,000 to 8,000 cells had been plated reached confluence after 2 days and showed similar levels of absorbance at 570 nm, whereas the wells in which 250 to 3,000 cells had been plated did not reach confluence and had proportionally lower absorbance at 570 nm. When the β-galactosidase activity of infected cells was measured (OD_{420}), a similar trend was found (Fig. 2A). The wells in which 4,000 to 8,000 cells had been plated contained comparable levels of β-galactosidase activity, whereas the wells in which 250 to 3,000 cells had been plated contained proportionately less β-galactosidase activity. To normalize the infection to cell number, we calculated the specific activity of the infected cells (β-galactosidase activity per cell, OD_{420}/OD_{570}) and plotted this value for each of the cell numbers plated (Fig. 2C). Surprisingly, the specific activity declined with fewer cells plated at the start of the infection, even though the ratio of virus to cell number (MOI) increased.

To control for effects due to cell density in the assay, we prepared an NIH 3T3 cell population transduced with the α-SGC-*lacZ* virus which contained 37% ± 5.2% *lacZ*$^+$ cells. Various numbers of this *lacZ*$^+$ cell line (250 to 8,000 cells per well) were plated in a 96-well dish. Three days after plating (a time equivalent to that in the infection assay), the cells were assayed for β-galactosidase activity and cell number (Fig. 2B). Curves similar to those in the infection assay for β-galactosidase activity and the MTT assay were seen with the stable *lacZ*$^+$ cell line. Moreover, the specific activity of the cells also declined with decreasing cell number plated at the start of the assay (Fig. 2C). Since this cell line had been infected prior to plating in the assay, the decline in specific activity appears to be unrelated to any of the steps of viral infection (adsorption, uncoating, reverse transcription, or integration) and is probably attributable to a decrease in *lacZ* reporter gene expression at low cell densities. When corrected for the decline in *lacZ* gene expression due to low cell densities, the specific activity of viral infection appears to remain relatively constant over the cell densities tested.

The relationship between the efficiency of infection and MOI was also tested by infecting either a low or high cell number and measuring β-galactosidase activity when both had reached similar cell densities (confluence). NIH 3T3 cells (500 or 5,000 cells per well) were plated, and the next day, the medium was replaced with various dilutions of α-SGC-*lacZ* virus with Polybrene. Two days after infection, the wells initiated with 5,000 cells were confluent and were assayed for β-galactosidase activity. Four days after infection, the wells initiated with 500 cells were confluent and were assayed for β-galactosidase activity. As shown in Fig. 3A, the efficiency of infection, as measured by β-galactosidase activity, at both cell

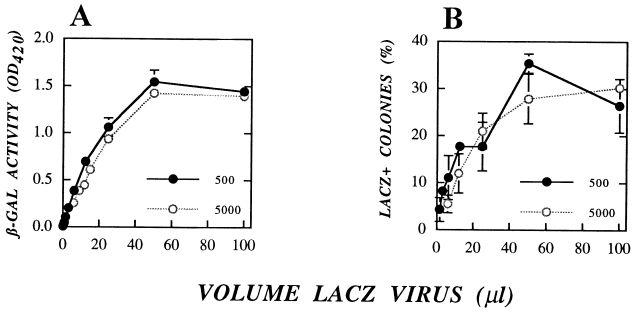

FIG. 3. Effect of target cell number. (A) NIH 3T3 cells (500 or 5,000 cells per well) were plated in a 96-well dish, and the next day, the medium was replaced with medium containing various dilutions of α-SGC-*lacZ* virus with Polybrene. The wells initiated with 5,000 and 500 cells were assayed for β-galactosidase activity at 2 and 4 days after infection, respectively. (B) The percentage of *lacZ*$^+$ colonies was determined for each of the virus doses.

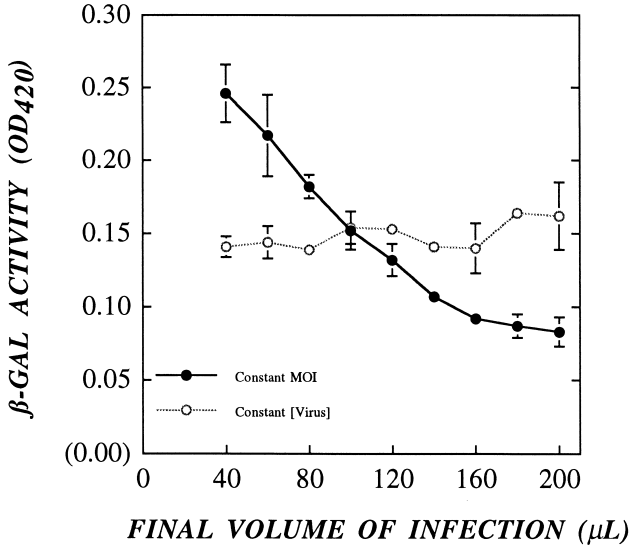

FIG. 4. Virus concentration, not total number of virions, determines infectivity. (A) NIH 3T3 cells (5,000 per well) were plated and, the next day, infected with various final volumes (40 to 200 μl) of two α-SGC-*lacZ* virus stocks. In one series of infections, the total number of virus particles in the well was constant (constant virus-to-cell ratio, constant MOI), and thus the virus concentration (virions per milliliter) decreased as the volume of infection was increased. In the other series of infections, the total number of virus particles in the well increased (increasing virus-to-cell ratios, increasing MOI), and thus the virus concentration was constant. To avoid any problems associated with the effects of small volumes on cell viability, the infections were performed for 2 h and the viral stock was removed and replaced with 100 μl of normal medium. Two days after infection, the β-galactosidase activity of infected cells was measured.

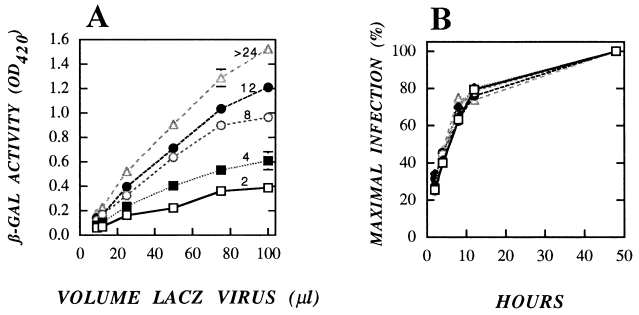

FIG. 5. Rate of virus infection. (A) NIH 3T3 cells (5,000 per well) were plated in a 96-well dish, and the next day, the medium was replaced with various dilutions of medium containing *lacZ* virus with Polybrene (8 μg/ml). At hourly intervals after the start of infection, the virus was removed from a series of dilutions by aspiration and replaced with normal medium, and incubation was continued at 37°C. Two days after the start of infection, *lacZ* activity was measured as described above. Each point shows the mean ± standard deviation of three replicates. (B) Plot of time versus percent maximal infection for each of the virus doses.

densities was comparable. Likewise, when the percentage of infected cells was determined for these infections, a similar dose-response curve was found for both 500 and 5,000 cells (Fig. 3B), even though the starting target cell number differed by 10-fold. The percentage of infected cells at the maximum virus dose (undiluted) for 500 and 5,000 cells was 26.4% ± 5.7% and 30.2% ± 1.5%, respectively.

Finally, the relationship between the efficiency of infection and MOI was further examined by changing the virus-to-cell ratio, using a constant cell number and varying the final volume of the infection. NIH 3T3 cells (5,000 per well) were plated and, the next day, infected with various final volumes (40 to 200 μl) of two α-SGC-*lacZ* virus stocks. In one series of infections, the total number of virus particles in the well was constant (constant virus-to-cell ratio, constant MOI), so that the virus concentration (virions per milliliter) decreased as the volume of infection increased. In the other series of infections, the total number of virus particles in the well increased (increasing virus-to-cell ratios, increasing MOI), and thus the virus concentration was constant. To avoid any problems associated with the effects of small volumes on cell viability, the infections were performed for 2 h and the viral stock was removed and replaced with 100 μl of normal medium. Two days after infection, the β-galactosidase activity of infected cells was measured.

As shown in Fig. 4, the efficiency of infection was dependent on the concentration of virus and independent of MOI. When the concentration of virus declined as the volume of infection was increased, the β-galactosidase activity of the infected cells likewise decreased, even though the MOI was constant, whereas when the virus concentration remained constant as the volume of infection was increased, the β-galactosidase activity of infected cells remained relatively constant over the volumes tested, even though the MOI increased fivefold.

Maximal infection requires prolonged exposure to virus. In the next set of experiments, we investigated the kinetics of retrovirus infection. NIH 3T3 cells (5,000 per well) were plated in a 96-well dish, and the next day, the medium was replaced with various dilutions of α-SGC-*lacZ* virus with Polybrene. At intervals after the start of infection, the virus was removed from a set of dilutions and replaced with normal medium, and incubation was continued at 37°C. Two days after the start of infection, β-galactosidase activity was measured as described above. As shown in Fig. 5A, infection for all virus dilutions tested increased with time and reached a maximum after greater than 24 h of exposure to virus. A plot of time versus percent maximal infection for each virus dose tested is shown in Fig. 5B. For all virus doses, half-maximal infection occurred after 5 h of exposure to virus.

Most infectious virus is not adsorbed. To determine if virus adsorption might be a rate-limiting step in the retrovirus life cycle, we estimated the proportions of adsorbed infectious virus and free unbound infectious virus after a 2-h adsorption period at 37°C. NIH 3T3 cells (5,000 per well) were plated in a 96-well dish, and the next day, various dilutions of *lacZ* virus containing Polybrene were adsorbed to the cells for 2 h at 37°C, removed, and used to infect new 3T3 cells for another 2 h at 37°C. To control for nonspecific adsorption and decay of the virus at 37°C, *lacZ* virus was incubated under similar conditions in wells without cells, removed after 2 h, and used to infect new 3T3 cells for 2 h. Two days later, the β-galactosidase activity of infected cells was measured. As shown in Fig. 6, significant amounts of *lacZ* virus were present in the medium after a 2-h incubation with cells. Compared with the result in the control experiment without cells, very little of the virus was actually removed from the viral stock by adsorption to the cells. The drop in viral activity was not dependent on the presence of cells and is probably a result of viral decay and/or nonspecific adsorption. Thus, after 2 h of adsorption, most of the infectious virus is not bound to the cell but is free in solution.

DISCUSSION

We have studied the effects of time and target cell number on retroviral infection and have found that maximal infection efficiency requires prolonged exposure of the cells to the retrovirus, that a small fraction of the infectious virus is adsorbed to cells, and that MOI is not an accurate predictor of the

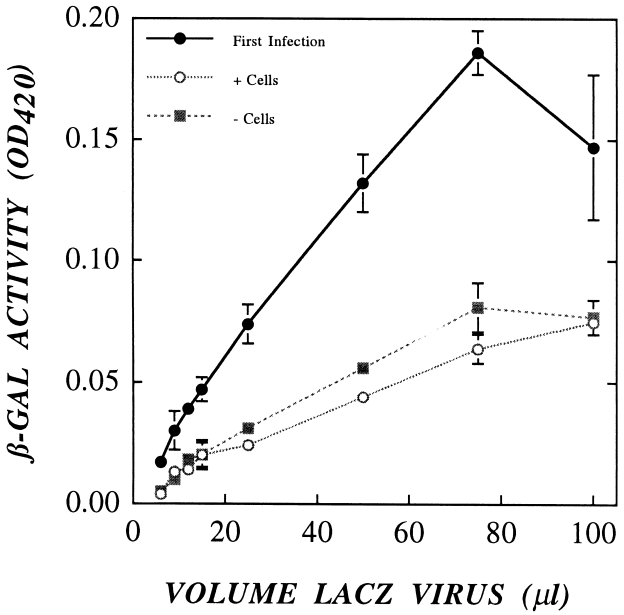

FIG. 6. Adsorption of infectious virus. NIH 3T3 cells (5,000 per well) were plated in a 96-well dish, and the next day, various dilutions of *lacZ* virus-containing Polybrene were adsorbed to the cells or empty wells for 2 h at 37°C, removed, and used to infect new 3T3 cells (5,000 per well) for another 2 h at 37°C. Two days later, β-galactosidase activity was measured for replicate wells.

efficiency of infection. We used a model amphotropic recombinant retrovirus encoding the *E. coli lacZ* gene and quantitative assays to measure β-galactosidase activity. By measuring *lacZ* gene expression, only successful infectious events are assayed. Unlike some assays which measure reverse transcriptase activity or radiolabeled virus particles, the expression of an integrated *lacZ* gene is a bona fide infectious event, whereas noninfectious virus particles can be radiolabeled and can contain active reverse transcriptase. This virus is replication defective, and thus it is possible to examine a single step in the infectious cycle without the complications of continued viral replication.

The first steps of retrovirus infection are the transport of a virus particle to the cell surface, adsorption of the particle to the cell surface, and binding of a specific cellular receptor by the gp70 proteins of the virus particle, followed by an ill-defined process which results in the internalization of the viral genetic material. While each of these events could be a rate-limiting step in the process of retrovirus infection, little is known about the relative importance of each step.

Purified gp70 protein has been used to estimate the number of cellular receptors. DeLarco and Todaro detected approximately 5.3×10^5 ecotropic receptors per cell (13), and Bishayee et al. reported 4×10^5 ecotropic receptors per 3T3 cell (9). Moldow et al. used gp70 purified from amphotropic virus and estimated between 1×10^5 and 5×10^5 amphotropic receptors per cell on CCL64 cells (32). Although it is not clear how many cellular receptors are required to bind a retrovirus for a single infectious event to occur, it would appear that during typical infections, the number of receptors is in excess of the virus particles as well as gp70 proteins. Consistent with this conclusion is the observation that infection frequencies were similar in cell lines expressing different levels of a transfected clone of the ecotropic receptor (47).

Maximal binding of purified gp70 protein from ecotropic virus occurs in less than 2 h at 37°C (13, 16, 18, 21). In one report, maximal binding of ecotropic virus, as measured by particle-associated reverse transcriptase activity, was attained within 20 min at 37°C (1). Others have shown that radiolabeled ecotropic virions and infectious virus accumulated on the cells with similar kinetics and reached half-maximal levels between 1.5 and 2 h at 37°C (6). However, these investigators measured total radioactivity inside the cells, and therefore the measurement reflects both virus binding and virus internalization. In addition, their measurements of infectious virus were complicated by the use of a replication-competent virus. More recently, investigators used a fluorescently labeled monoclonal antibody to gp70 to monitor the kinetics of binding of an amphotropic virus (19). Cells were exposed to a viral stock and then incubated with the labeled antibody to determine cell surface binding of gp70. In this assay, maximal binding occurred after 10 min of exposure to the viral stock. However, the assay is complicated by the fact that binding of the labeled antibody could be due to the cell surface binding of both gp70 associated with virus particles and gp70 free in solution.

Our data show that over a greater than 10-fold range of virus concentrations, maximal infection required prolonged exposure of the cells to virus. Maximal infection occurred after greater than 24 h of exposure, and half-maximal levels were reached after 5 h. Moreover, it has been reported that retrovirus loses activity at 37°C, with reported half-lives of 8 to 9 h (27), 6.5 h (39), and 7.2 ± 1.3 h (26). Maximal infection, which is a function of the concentration of active virus and the rate of infection, is also quite likely limited by the half-life of the virus and the associated loss of infectivity with time.

One parameter often used to predict the efficiency of infection for many types of mammalian viruses (DNA and RNA viruses) is the MOI (29). MOI is a measure of the ratio of infectious virus to cell number and is typically used to determine the probability and distribution of infectious events in a population of cells. It is also used to determine the likelihood that all cells in a population are infected with at least one virus and widely used to describe the in vitro conditions of most MuLV and HIV infections.

Surprisingly, MOI was not an accurate predictor of the efficiency of infection in our studies with the *lacZ* retrovirus. When cells were infected at an MOI of between 4 and 8, only 34.5% ± 1.7% of the cells were infected. An MOI in this range would have predicted to infect >98% of the cells. When the MOI was varied greater than 10-fold by changing the target cell number, there was little effect on the extent of infection. Likewise, our experiments in which the MOI was changed fivefold by increasing the volume of infection showed little effect on infectivity. Kahn et al. also reported that increasing MOI by altering target cell number did not increase the efficiency of infection of a recombinant retrovirus (20). Thus, the percentage of infected cells appears to be governed predominantly by virus concentration and is independent of cell density over at least a 10-fold range. Provided that the cells are able to divide (a strict requirement for successful MuLV infection) (30), retrovirus infection is first order with respect to virus concentration, as would be the case for a typical receptor-ligand interaction.

Typical receptor-ligand interactions for a simple system and a small molecule are controlled predominantly by the concentration of the ligand and its affinity for the receptor. At equilibrium, ligand binding is also independent of cell number, because receptors are in excess, and so the critical parameter is the affinity constant (K_a). These may be true for small molecules, such as gp70, for which values for K_a are in the range of 3.5×10^8 to 8.3×10^9 M^{-1} for ecotropic gp70 (9, 10, 21) and 2.5×10^8 M^{-1} for gp120 of HIV (25). But binding of retroviral

particles may be far more complex and governed by additional parameters. For example, retroviral virions, like most viruses, contain multiple gp70 molecules and are thought to be multivalent. The expression N_tK_a, where N_t is the total number of envelope proteins per particle and K_a is the affinity constant of a single envelope protein, has been used to describe the enhancement in affinity of virus particles as a result of this multivalency (48). For a retroviral particle, this enhancement of affinity could be on the order of 100-fold. Yet we found that the majority of infectious virus was not bound after 2 h at 37°C, a result consistent with the observations of others, who showed that after 2 h at 37°C, approximately 5% of an infectious ecotropic virus encoding human growth hormone was adsorbed (47). Although these studies cannot be used to accurately measure the affinity of retrovirus particles, they do suggest that the binding of infectious retrovirus does not occur in the range of affinities expected of a high-affinity multivalent particle in a typical receptor-ligand interaction or that the time to reach equilibrium is much greater than 2 h.

One complication might be that the assumption that receptors are in vast excess is not correct. Although receptor numbers have been verified by several groups, each of these studies defined the receptor by its ability to bind purified gp70 protein (9, 10, 13, 14, 16, 18, 21). It is possible that only a fraction of the gp70-binding receptors are competent to mediate an infectious event. Receptor competency could be controlled by any of a number things, including clustering of receptors, accessory factors, and correct membrane trafficking (47, 49). Nevertheless, if binding of gp70 is necessary but not sufficient to mediate an infectious event, the number of competent receptors might be overestimated.

Another complication might be that the affinity of retroviral particles is not as high as expected. The receptor-binding domains of gp70 proteins that are assembled as trimers in the lipid bilayer of the virus particle may not be as accessible to receptor binding as the purified protein, and thus the K_a determined with purified gp70 protein is higher than the affinity of gp70 in viral particles. Conversely, the cellular receptor for the virus may not be as accessible to gp70 proteins incorporated into viral particles as they would be to purified gp70. Likewise, other steric factors such as protein conformation and/or the charge density of the cell surface could influence the binding of virus particles. Another indication that retroviral virions may not have high affinities is the common use of polycations such as Polybrene. These polycations are commonly used to enhance retrovirus infectivity as much as 10-fold and are thought to aid in overcoming electrostatic repulsion of virus binding (42). Our experiments were all done in the presence of Polybrene, so electrostatic interactions are not the explanation for these observations.

A third complication might be that multivalent retroviral particles do have high affinities but that virus attachment to cell surface receptors is diffusion limited (4, 5, 43). The diffusion constant of a 100-nm-diameter retrovirus particle, as estimated by the Stokes-Einstein equation ($D = kT/6\pi\eta a$) is 2×10^{-8} cm^2/s, which is more than 20-fold lower than the estimated diffusion constant of soluble gp70 protein ($D = 7 \times 10^{-7}$ cm^2/s). Kinetics studies of gp70 binding, which reached equilibrium binding within 15 to 90 min, are not relevant to the binding of slowly diffusing virus particles. Given the large disparity between their diffusivities, it is possible that virus binding is diffusion limited and soluble gp70 binding is reaction limited. Reactions which are diffusion limited are typically first order with respect to the diffusing reactant (in this case, viruses) regardless of the intrinsic kinetics. Indeed, if virus binding is diffusion limited, it may be possible to enhance the rate of binding and infection by improving the mass transfer of virions to the cell surface. Issues of diffusivity may also explain differences in the kinetics of attachment to suspension and attached cell lines. The attachment of viruses to cells in suspension is less likely to be diffusion limited because both the particles and cells are in motion, which minimizes the distances through which virions must diffuse.

Even after a virus particle has diffused to near the surface of a cell, other mass transport limitations may be operable. Virions must overcome potentially repulsive colloidal forces once near the cell surface. The particle may be subjected to unfavorable electrostatic forces with the negatively charged cells as well as steric repulsion from the thick polymeric glycocalyx of the cell. Such constraints are probably minimal for purified gp70 protein. In summary, the intrinsic high affinity of multivalent retroviral particles may be masked by the combination of inefficient mass transport and repulsive surface forces between the particles and cells which combine to limit the rate of attachment of retroviruses to their receptors.

Additional studies are required to determine which of these steps are limiting for retrovirus infection. Future studies should include measurements of the rate of deposition of retroviral particles on the cell surface, the affinity of viral particles, and the contribution of individual gp70 molecules to particle affinity.

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