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Binding Kinetics of Ecotropic (Moloney) Murine Leukemia Retrovirus with NIH 3T3 Cells

HONG YU, NAYWEI SOONG, AND W. FRENCH ANDERSON*

Gene Therapy Laboratories, Norris Cancer Center, University of Southern California School of Medicine, Los Angeles, California 90033

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A quantitative analysis of the binding kinetics of intact Moloney murine leukemia retrovirus (MoMuLV) particles with NIH 3T3 cells was performed with an immunofluorescence flow cytometry assay. The virus-cell binding equilibrium dissociation constant (K_D), expressed in terms of virus particle concentration, was measured to be 8.5 (±6.4) × 10^{-12} M at 4°C and was three- to sixfold lower at temperatures above 15°C. The K_D of virus binding is about 1,000-fold lower than the K_D of purified MoMuLV envelope. The association rate constant was determined to be 2.5 (±0.9) × 10^9 M^{-1} min^{-1} at 4°C and was 5- to 10-fold higher at temperatures above 15°C. The apparent dissociation rate constant at 4°C was 1.1 (±0.4) × 10^{-3} min^{-1} and was doubled for every 10°C increase in temperature over the range tested (15 to 37°C).

The envelope protein (Env) of ecotropic murine leukemia virus (MuLV) binds specifically to a receptor on the surface of mouse cells. This multimembrane-spanning receptor protein has been identified as a cationic amino acid transporter (20, 34). Genetic and chimeric analyses have localized the receptor binding domain of ecotropic and amphotropic MuLV to the N-terminal 200 amino acids of the envelope protein surface subunit (SU), or gp70 (15, 16, 27, 29, 31). Similarly, studies have shown that the third extracellular domain of the receptor is essential for interaction with the envelope (1, 37). The precise nature of the envelope-receptor interaction remains a subject of investigation.

Kinetic analyses have been used to define the binding parameters of different viruses to their cognate receptors (3, 5, 7, 8, 11, 13, 19, 21, 24, 25, 35) and can thus provide quantitative information on the nature of the binding interaction. In studies on the interaction of human immunodeficiency virus (HIV) envelope gp120 with its receptor CD4, the equilibrium dissociation constant (K_D) has been used to identify the receptor residues critical for the binding of virus (3, 24) and the virus envelope region critical for interaction with the receptor (21). So far, kinetic studies on the interaction of ecotropic MuLVs with their receptors have focused on the binding of purified envelope proteins with target cells or cell membranes (8, 13, 19). These studies have demonstrated that the binding of MuLV gp70 to target cells is a noncooperative and saturable process and is dependent on the concentrations of both receptors (cells) and gp70, as well as on temperature and pH. The K_Ds measured in these studies ranged from 10^{-8} to 10^{-9} M.

It has been estimated that there are about 5×10^5 ecotropic MuLV receptors on the surface of an NIH 3T3 fibroblast (9, 13) and about 10^4 receptors on a thymus cell (8). Therefore, the association of virus with cells is essentially a multivalent interaction whereby viral particles, each bearing multiple copies of oligomeric gp70, bind to target cells, each bearing multiple copies of the receptor. To more closely approximate the nature of virus-cell interaction, we performed a quantitative kinetic analysis of the binding of NIH 3T3 cells with intact viral particles which were derived from the Moloney MuLV (MoMuLV) producer line GP+E86/LNCX. Unlike earlier MuLV binding studies (8, 13, 19), which involved the measurement of bound radiolabeled gp70, our study used a convenient indirect immunofluorescence assay with flow cytometry analysis (18) to quantitate the amount of bound virus. Similar assays have been used to study the binding kinetics of HIV gp120 to its receptor CD4 (6, 11, 24). In this study, we measured the K_D, the association rate constant (k_a), and the dissociation rate constant (k_d) for the binding of MoMuLV to NIH 3T3 cells.

Virus supernatant. Replication-incompetent MoMuLV virus particles were produced from the ecotropic viral vector producer cell line GP+E86/LNCX, which was derived from a stable transfection of GP+E86 (22) with vector LNCX (23). The GP+E86/LNCX cell line was maintained in D10 medium, which is Dulbecco's modified Eagle's medium (Gibco/BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum (Gemini Bioproducts, Inc., Calabasas, Calif.) and 2 mM glutamine. GP+E86/LNCX cells were cultured to about 80% confluency, and fresh D10 medium was added. After 48 h of incubation at 32°C, the supernatant was collected and filtered through a 0.45-μm-pore-size filter to remove cell debris. The virus particle concentration of the supernatant was estimated by comparing its reverse transcriptase (RT) activity with that of a standard MoMuLV stock with known particle concentration. The RT assay was performed as previously described (36).

To prepare the standard virus stock, viral supernatant from producer cell line GP+E86/LNCX was centrifuged at 9,500 rpm in a Beckman JA-14 rotor for 12 h. The pellet was resuspended in TNE buffer (0.01 M Tris-HCl [pH 7.2], 0.1 M NaCl, 0.001 M EDTA) and centrifuged through a 10 to 60% linear sucrose gradient in a Beckman SW40 rotor at 30,000 rpm for 2 h at 20°C. The virus band was recovered by using a syringe, suspended in TNE buffer, and pelleted by centrifugation at 17,000 rpm in a Beckman JA-17 rotor for 2 h. The virus stock was mixed with latex particles of known concentration (Nanosphere size standard; Duke Scientific Corp., Palo Alto, Calif.). This mixture of latex and viral particles was counted by electron microscopy. The virus particle concentration of this stock was then determined by measuring the ratio of viral particle number to latex particle number (16a). The virus concentration in virus particles per liter was divided by Avogadro's number to obtain the molar concentration.

* Corresponding author. Mailing address: Norris Cancer Center, Room 614, University of Southern California School of Medicine 1441 Eastlake Ave., Los Angeles, CA 90033. Phone: (213) 764-0612. Fax: (213) 764-0097.

Binding assay. NIH 3T3 cells were maintained in D10 medium and were detached with a solution of trypsin (0.05%)–EDTA (0.53 mM). Rat monoclonal antibody (MAb) 83A25, specific for the C-terminal region of MuLV gp70, was obtained as a stock of hybridoma supernatant from L. Evans (12). Mouse MAb 273, specific for the N-terminal region of MuLV gp70, was obtained from the AIDS Reagent Repository (Rockville, Md.). Anti-rat immunoglobulin G (IgG) and anti-mouse IgG, both conjugated with fluorescein isothiocyanate (FITC), were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.). MoMuLV gp70, kindly provided by Larry O. Arthur, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, Md.), was from virus supernatant that was subjected to repeated freezing and thawing. The E_{229} protein is a truncated form of gp70 which consists of the N-terminal 229 amino acids of MoMuLV gp70. It lacks the epitope for the MAb 83A25. The E_{229} protein was expressed in a baculovirus system and purified by Mono Q Fast protein liquid chromatography. Both protein preparations were determined to be over 90% pure by analysis of a silver-stained sodium dodecyl sulfate-polyacrylamide gel. The concentrations of the proteins were determined by using a detergent-compatible protein assay kit from Bio-Rad (Hercules, Calif.).

The virus bound on the cell surface was quantitated by indirect immunofluorescence using fluorescence-activated cell sorting (FACS) analysis described previously (18), with some modifications. One milliliter of virus was mixed with 2×10^5 NIH 3T3 cells at the specified temperature for 2 h or as indicated. The cells were then centrifuged at 16,000 × *g* for 15 s to remove the supernatant and washed once with 1 ml of ice-cold wash buffer (phosphate-buffered saline [PBS] containing 10% goat serum). The cells were resuspended in 250 μl of MAb 83A25 and incubated at 4°C for 1 h. They were then washed again with 1 ml of cold wash buffer and resuspended in 100 μl of wash buffer containing 0.5 μg of FITC-labeled goat anti-rat IgG for 30 min at 4°C. After a final wash, they were fixed in 330 μl of 4% paraformaldehyde in PBS. The fluorescence intensity of the cell samples was analyzed on a FACStar Plus flow cytometer (Becton Dickinson). The measured mean channel number of the samples was converted to fluorescence intensity according to the formula $\log(f) = a \cdot \mathrm{MN} + b$, in which f represents fluorescence intensity, MN is the mean channel number as measured by FACS, and a, and b are the constants derived from a linear regression of a standard curve generated by using FITC-labeled RCP-70-5 microbeads with known fluorescence intensities (Spherotech, Inc., Libertyville, Ill.). Background fluorescence was measured from cells mixed with D10 medium and the antibodies and was subtracted from the experimental values after the fluorescence conversion.

Env-cell binding assays were performed both with purified envelope protein (gp70) and with truncated envelope protein (E_{229}). The assay was similar to the virus-cell binding assay, with the following modifications. The cells were resuspended in 1 ml of wash buffer containing envelope protein at the specified concentration. After binding and washing, the cells were labeled with 100 μl of MAb 273 at 4°C for 1 h. Following the washing step, 100 μl of washing buffer containing 0.5 μg of FITC-conjugated anti-mouse IgG was added, and the mixture was incubated at 4°C for 30 min. The cells were then fixed and analyzed by FACS.

Model for virus-cell binding. Virus binds to a cell to form a virus-cell complex which can either be internalized or be dissociated to release the free virus and the cell. The events can be modeled by the kinetic scheme

$$V + C \underset{k_d}{\overset{k_a}{\rightleftharpoons}} VC \xrightarrow{k_i} C_V$$

Scheme 1

where V represents virus, C represents cells, VC represents the virus-cell complex, and C_v represents the cells with internalized virus, and k_i is the internalization rate constant. The kinetic parameters being measured are macroscopic descriptions of the system. To simplify our analysis, we assume that internalization is negligible. This assumption is valid at low temperatures at which the membrane fluidity is low (17). At high temperatures (25 and 37°C), we assume that k_i is small relative to k_a and k_d and will not make a significant contribution. The equilibrium dissociation constant can then be expressed as

$$K_D = k_d/k_a = [V] \cdot [C]/[VC] \quad (1)$$

where $[V]$, $[C]$, and $[VC]$ represent the equilibrium concentrations of free virus, free cell, and virus-cell complex, respectively. At temperatures above 15°C, internalization does occur to some degree. We address this internalization component during our measurements of k_d by using fixed cells (see below). The relative amount of viral particles bound on the cell surface, $[VC]$, can be obtained by measuring the cell surface fluorescence in the indirect immunofluorescence FACS assay (described above). Under conditions such that virus is in excess, the K_D can be calculated by using the hyperbolic equation

$$f = f_{\max} \cdot [\mathrm{V}]/(K_D + [V]) \quad (2)$$

where f represents the fluorescence measured at virus concentration $[V]$ and $f_{\max}$ represents the fluorescence when $[V]$ reaches infinity.

If the virus concentration is in sufficient excess, the original virus concentration will be essentially unchanged after binding and can be used to approximate the free virus concentration at equilibrium. To test if this condition was satisfied, we examined the binding capacity and particle concentration of the virus supernatant after successive rounds of binding to fresh batches of cells. After incubation of 1 ml of virus supernatant (6×10^9/ml) with 10^5 NIH 3T3 cells for 30 min at 15°C, the cells were centrifuged, and the virus supernatant was recovered and reincubated with a second batch of cells. After four rounds of successive incubations with fresh cells and the same virus supernatant, the RT activity in the virus supernatant (as an indicator of virus concentration) and the cell surface fluorescence (as a measurement of virus binding capacity) remained essentially unchanged (data not shown), which suggests that the virus concentration was not significantly depleted after binding with 10^5 cells. This result was not due to the unbound virus particles being defective for binding, since the virus could be depleted when a larger number of cells was used (data not shown). This observation suggests that under the conditions (10^5 cells) that we used for measuring K_D, the virus is in sufficient excess, and the original virus concentration can be used to approximate the free virus concentration at equilibrium to calculate K_D by using equation 2.

***K_D*.** To measure K_D, a series of virus dilutions (3×10^7 to 6×10^9 virus particles per ml) were mixed with 10^5 NIH 3T3 cells. The incubations were performed for 1 h (25 and 37°C) or 2 h (4 and 15°C), which was sufficient for equilibrium to be achieved. The fluorescence on the cell surface was measured and plotted against virus concentration. K_D was determined by a nonlinear least-squares fit to equation 2 or by a linear least-

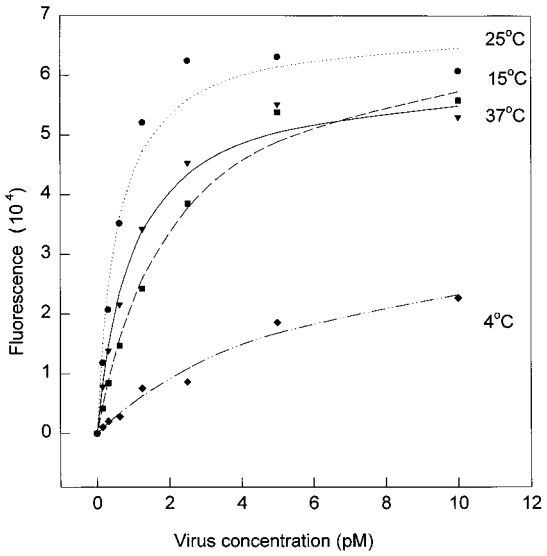

FIG. 1. Measurement of K_D. Serial dilutions of virus supernatant were mixed with 2×10^5 NIH 3T3 cells at 4°C (◆), 15°C (■), 25°C (●), or 37°C (▼) for 1 (25 and 37°C) or 2 (4 and 15°C) h to reach equilibrium. The virus-bound cells were labeled by MAb 83A25 and assayed by FACS. The original virus concentration of the supernatant obtained from the ecotropic MuLV producer cell line GP+E86/LNCX was 6×10^9 particles per ml, determined by comparing its RT activity with that of a standard viral stock of known concentration. The K_D was obtained by a nonlinear least-squares fit of the fluorescence versus virus concentration to equation 2. The symbols represent the experimental data, and the lines represent the computer fit to the data.

squares fit to a double-reciprocal equation (Lineweaver-Burk), using SigmaPlot software (Jandel Scientific, San Rafael, Calif.).

Virus binding, as quantitated by cell surface fluorescence, is dependent on virus concentration and is saturable. The data fit equation 2 very well, consistent with a simple, bimolecular, noncooperative model for virus-cell interaction (Fig. 1). The K_D values were determined to be 5.1×10^9, 2.2×10^9, 0.78×10^9, and 0.9×10^9 virus particles per ml at 4, 15, 25, and 37°C, respectively. The corresponding K_D values expressed in molar units are 8.5×10^{-12}, 3.6×10^{-12}, 1.3×10^{-12}, and 1.5×10^{-12} M, respectively (Table 1). Maximal binding at 4°C is about twofold lower than that at 15°C or higher. K_D measured under these conditions is independent of cell concentration within the range of 5×10^4 to 5×10^5 cells per ml (data not shown). The standard free energy change for association was calculated from the K_D, using the equation $\Delta G^0 = -2.3RT \cdot \log(1/K_D)$, where R is the gas constant (1.98×10^{-3} kcal [1 cal = 4.184 J] mol^{-1} degree^{-1}) and T is the absolute temperature on the Kelvin scale. The values are −13.7, −15.0, −16.1, and −16.7 kcal/mol at 4, 15, 25, and 37°C, respectively, indicating that the associated state of the virus-cell complex is highly favored at equilibrium.

Free gp70 can be shed from virus particles (2, 33) or secreted directly from producer cells as in the case of simian immunodeficiency virus (32). Significant amounts of free gp70 in the virus supernatant could confound our analysis of virus-cell interaction. Free gp70 can interfere with fluorescence measurements by occupying binding sites and blocking virus particles from binding, thus decreasing the observed fluorescence. To examine the degree of free gp70 interference, several experiments were performed. Virus supernatant was passed through Centriprep-100 concentrators (100-kDa cutoff; Amicon, Inc., Beverly, Mass.) to separate the free gp70 from the virus supernatant. The fluorescence intensity obtained by performing a binding assay using the flowthrough fraction (containing free gp70) was about 10-fold lower than that of the retentate, which generated fluorescence similar to that generated by the original viral preparation (data not shown). This observation suggests that free gp70 in the virus supernatant has little effect on the actual K_D measurement. Furthermore, the K_D of a virus stock, purified by sucrose gradient to remove free gp70, was measured and found to be similar to that obtained for an unpurified virus supernatant (data not shown). These results suggest that the contribution of free gp70 in the unpurified virus supernatant is not significant. Therefore, the K_D estimates obtained in our studies using the unpurified virus supernatant can be considered to represent the actual K_D of virus-cell binding.

The K_D of virus binding to cells is about 1,000-fold lower than the K_D of free gp70 as measured by other methods (8, 13, 19). To exclude the possibility that the quantitative differences in the K_D values observed between intact virus and gp70 were due to the difference in assay systems, we also performed the binding assays with purified MoMuLV envelope protein. Two versions of the envelope protein, the entire gp70 molecule and the E_{229} truncated protein (see above), were used in these experiments. Both proteins can bind to NIH 3T3 cells and be immunolabeled with the gp70 N-terminus-specific MAb 273, but neither can be immunolabeled with the gp70 C-terminus-specific MAb 83A25. Using the FACS assay, we determined the K_Ds at 4°C of gp70 and E_{229} to be 26 and 3.3 nM, respectively. The possible alternative explanations for the K_D difference between gp70 and E_{229} are (i) the gp70 protein might be partially damaged as a result of the purification procedures and (ii) the C-terminal region of gp70 (which is absent in E_{229}) might have a negative effect on its binding. Previous estimates of K_D at 24 or 37°C for the purified Rauscher MuLV gp70 binding to NIH 3T3 cells or cellular membrane fractions were in the order of 2 to 8 nM (13, 19). Our data for virus-cell binding suggest that the K_D is reduced by about 1.5- to 2-fold for every 10°C increase in temperature. Thus, the K_D estimates

TABLE 1. Kinetic parameters of virus-cell interaction

Binding temp (°C)	K_D[a] (M)	k_a (min^{-1} M^{-1})	k_d (min^{-1})	k_d/k_a (M)
4	$8.5 (\pm 6.4) \times 10^{-12}$ (18)[b]	$2.5 (\pm 0.9) \times 10^9$ (15)	$1.1 (\pm 0.4) \times 10^{-3}$ (5)	4.4×10^{-13}
15	$3.6 (\pm 3.4) \times 10^{-12}$ (5)	$1.5 (\pm 0.9) \times 10^{10}$ (8)	$2.9 (\pm 0.4) \times 10^{-3}$ (2)	1.9×10^{-13}
25	$1.3 (\pm 0.7) \times 10^{-12}$ (5)	$2.6 (\pm 0.2) \times 10^{10}$ (3)	$5.6 (\pm 0.1) \times 10^{-3}$ (2)	2.2×10^{-13}
37	$1.5 (\pm 0.6) \times 10^{-12}$ (6)	$3.0 (\pm 1.8) \times 10^{10}$ (6)	$14.1 (\pm 0.2) \times 10^{-3}$ (2)	4.7×10^{-13}

[a] The virus particle concentration of the supernatant was estimated by comparing the RT activity of the supernatant with that of a sucrose gradient-purified virus stock whose particle concentration was determined by counting via electron microscopy. The particle number per liter was divided by Avogadro's number to obtain the molar concentration.

[b] Each number in parentheses represents the number of experiments performed. The ± values represent 1 standard deviation of the mean.

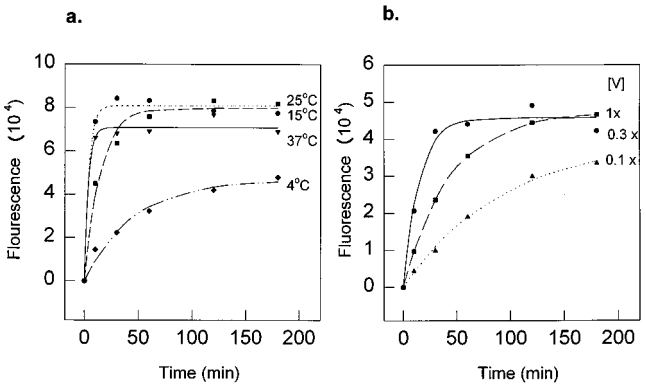

FIG. 2. Effect of temperature and viral concentration on k_a. NIH 3T3 cells (2×10^5) were suspended with 1 ml of virus supernatant for various periods of time in which the incubation temperature was at 4°C (◆), 15°C (■), 25°C (●), or 37°C (▼) and the virus supernatant concentration was 6×10^9 virus particles per ml (a) or the virus concentration, was 6×10^9 (●), 2×10^9 (■), or 0.6×10^9 (▲) virus particles per ml and the incubation temperature was 15°C (b). Cell surface fluorescence was measured as described in the text, and the k_a was analyzed by nonlinear least-squares fit to the equation $f = f_{max} \cdot [1 - \exp(-k \cdot t)]$, where $k = k_a \cdot [V]$.

for gp70 determined by our laboratory and those in the literature are similar when adjustment for the different temperatures is made.

The difference in K_D values between gp70 and intact virus might reflect the multivalent interactions of virus with cell, as opposed to the monovalent interaction of individual gp70 molecules with cellular receptors. It is possible that the differences in K_D observed between intact viruses and purified envelope proteins can be accounted for quantitatively by the number of envelope proteins on the virus. Assuming that there are 300 envelope protein molecules on each MoMuLV particle, based on a comparison with the number of envelope molecules on HIV (14), the theoretical K_D for the binding of each envelope in a virus particle would be 2.6×10^{-9} M ($300 \times 8.5 \times 10^{-12}$) at 4°C. This value is close to the measured K_D of the E_{229} protein (3.3×10^{-9} M). The agreement between these values suggests that there may be no cooperative or synergistic effect in the binding of multiple envelope proteins in a single virus.

***k_a*.** To measure k_a, time course experiments were performed with different virus concentrations at different temperatures. NIH 3T3 cells (2×10^5) were suspended with 1 ml of virus supernatant (6×10^9 virus particles per ml or as indicated) for various periods of time at the indicated temperature. Binding was terminated by centrifuging the cells at 16,000 $\times$ *g* for 15 s and washing them. Cell surface fluorescence was measured as described above, and the data were plotted as cell surface fluorescence versus virus-cell mixing time. The k_a was analyzed by a nonlinear least-squares fit of the data to the equation $f = f_{max} \cdot [1 - \exp(-k \cdot t)]$, where $k = k_a \cdot [V]$, f represents the fluorescence at time t, and f_{max} represents fluorescence when t is infinity (Fig. 2).

k_a was determined to be 2.5 ($\pm$0.9) $\times 10^9$ M^{-1} min^{-1} at 4°C and was about 10-fold higher at 15°C and above (Table 1). The amount of virus bound is also temperature dependent. The maximum level of virus binding to the cell surface above 15°C is about twofold higher than at 4°C (Fig. 2a). The maximal binding at 37°C is lower than that at 25 and 15°C (Fig. 1 and 2), although the binding affinity at 37°C is equal to or higher than that at 25 and 15°C. This observation may be explained by the rapid internalization of occupied receptors at 37°C, resulting in a decrease in the number of binding sites on a cell. Since our calculations do not account for internalization, the interpretation of our data at 37°C is limited by this caveat.

Lower virus concentrations required longer periods of time to reach equilibrium, as shown in Fig. 2b. The overall binding rate (k) was dependent on the virus concentration. A threefold decrease in virus concentration resulted in a corresponding threefold decrease in the overall binding rate (Fig. 2b). However, k_a, calculated by the equation $k_a = k/[V]$, was independent of virus concentration within the tested range of 10^{-11} to 10^{-12} M (6×10^8 to 6×10^9 virus particles per ml) and had a value of 2×10^{10} M^{-1} min^{-1}. These results are consistent with a bimolecular, noncooperative model when virus is in excess.

The binding of MoMuLV to cells is a rapid event (Fig. 2a). With a virus concentration of 6×10^9/ml and a cell concentration of 2×10^5/ml, half-maximal binding occurs in about 3 min. At temperatures above 15°C, maximal binding was attained after 30 min. At 4°C, half-maximal binding occurs at approximately 30 min, and maximal binding was attained after 2 h. Comparing the k_a of 3×10^{10} M^{-1} min^{-1} at 37°C for MoMuLV binding, the k_a of soluble CD4 binding to membrane-bound gp120-gp41 of HIV type 1 is more than 1,000-fold lower (about 10^7 M^{-1} min^{-1}) (11). Considering that there are 100 to 300 Env molecules per MoMuLV particle, the k_a for each Env molecule in a virus can be estimated to be about 10^8 M^{-1} min^{-1}. This value is about 10-fold higher than the k_a for soluble CD4 binding.

The binding of some viruses to target cells, e.g., the binding of *Autographa californica* nuclear polyhedrosis virus to insect cells (35), is thought to be diffusion limited. Theoretically, the diffusion-limited association rate, k_{diff}, is the maximum possible association rate; i.e., every encounter of a virus with a cellular receptor results in a productive binding event. k_{diff} can be calculated from the diffusion coefficient, D_v, of the virus. Assuming that MoMuLV is a perfect sphere with a radius of 50 nm (28), its theoretical D_v can be calculated to be 4.3×10^{-8} cm^2/s at 22°C, using the Stokes-Einstein equation (4). The k_{diff} is then calculated to be 3.2×10^{-8} cm^3/min or 1.9×10^{13} M^{-1} min^{-1} from the equation $k_{diff} = 4a\pi D_v$ (30), where a is the radius of the cell. The k_a that we measured at 25°C is about 640-fold smaller than the k_{diff}. This result suggests that the binding of MoMuLV to the cell is not diffusion limited. Apparently, the binding reaction step, secondary to the encounter step, is the rate-limiting step.

***k_d*.** k_d was determined by measuring the loss of fluorescent signal from virus-bound cells over time. NIH 3T3 cells (2×10^6) were mixed with 10 ml of virus supernatant (6×10^9 particles per ml) and incubated at 15°C for 30 min to allow virus to bind to the cells. The cells were centrifuged at 16,000 $\times$ *g* for 15 s to remove the unbound virus and resuspended in fresh D10 medium containing 10 nM E_{229} protein. E_{229} acts as a binding competitor to prevent the dissociated virus from rebinding to the cells. Because it lacks the epitope for MAb 83A25, the E_{229}-cell complex will not be detected by the indirect immunofluorescence FACS assay. The cell suspension was incubated at different temperatures as indicated to allow dissociation to occur. After the specified dissociation time, a 1-ml aliquot was labeled with MAb 83A25 followed by fluorescent secondary antibody. FACS analysis was then performed. The fluorescence representing the virus remaining on the cell surface was used to plot f/f_0 versus dissociation time (Fig. 3).

The cell surface fluorescence disappeared slowly at 4°C, with only about 30% of the signal lost over a 5-h incubation period (Fig. 3). In contrast, cell surface fluorescence disappeared rapidly at 37°C. After a 2-h incubation at 37°C, about 90% cell surface fluorescence was lost. These results are consistent with those reported by Kalyanaraman et al. (19). In that study, it

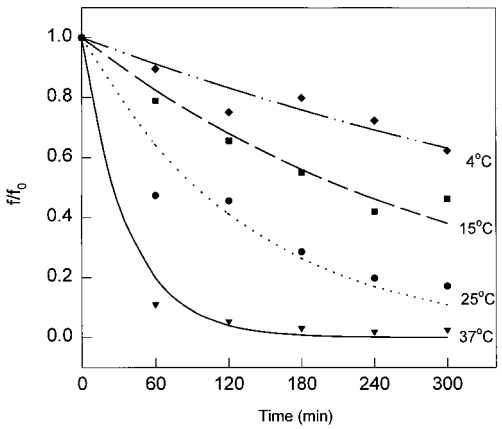

FIG. 3. Measurement of k_d. After a 30-min incubation with virus supernatant at 15°C, NIH 3T3 cells were resuspended in fresh medium containing E_{229} protein as a binding competitor to prevent rebinding of the dissociated virus. The cell suspension was incubated at different temperatures as indicated to allow dissociation to occur for a specified dissociation time, and then the fluorescence on the cell surface was measured by the FACS assay. The k_d was obtained by a linear regression of a plot of $\ln(f/f_0)$ versus dissociation time, t, where f_0 and f are fluorescence at times zero and t, respectively. The symbols ◆ (4°C), ■ (15°C), ● (25°C), and ▼ (37°C) represent the experimental data, and the lines represent the computer fit to the data.

was observed that 95% of Rauscher MuLV gp70 remained bound to membrane fractions after 3 h at 4°C but was essentially completely dissociated after 3 h at 37°C. The data fit well to an exponential decrease equation, $f/f_0 = \exp(-k_d \cdot t)$, where f_0 and f are fluorescence at times zero and t, respectively. The apparent k_d was obtained by a linear regression of a plot of $\ln(f/f_0)$ versus dissociation time, t. The apparent k_d values were calculated to be about 1.1×10^{-3}, 2.9×10^{-3}, 5.6×10^{-3}, and 14×10^{-3} min^{-1} at 4, 15, 25, and 37°C, respectively (Table 1). The value of k_d is approximately doubled for every 10°C increase in temperature within the range tested (4 to 37°C). The half-lives of the virus-cell complex are 630, 239, 123, and 50 min at 4, 15, 25, and 37°C, respectively. The k_d measured here is also similar to that determined for CD4-gp120 dissociation in the HIV type 1 binding study of Dimitrov et al. (10).

At higher temperatures (25 and 37°C), loss of fluorescence on the cell surface could be due to a combination of virus-receptor internalization and the true dissociation of the virus from cellular receptors. To eliminate the contribution of virus-receptor internalization, the NIH 3T3 cells were fixed with 3.7% formaldehyde in PBS prior to binding. Internalization is blocked in fixed cells (17), and the loss of fluorescence on the fixed cell surface is mostly due to virus dissociation. The k_d values with the fixed cells were measured to be 6.5 ($\pm$0.2) $\times$ 10^{-4} and 6.0 ($\pm$0.3) $\times$ 10^{-3} min^{-1} at 4 and 37°C, respectively. These values are about twofold lower than those obtained with unfixed cells, which may reflect the contribution of internalization with the live cells.

In studies of the interaction of HIV type 1 envelope with its receptor CD4, it has been shown that soluble CD4 binding can cause envelope dissociation from the membrane (10, 11, 26). This effect of envelope binding may also occur with MoMuLV. The gp70 molecule on MoMuLV may dissociate from the viral membrane after it binds to the receptor. Thus, the virus is shed from the cell, leaving the gp70 still bound to the receptor. This event may contribute to the disappearance of the fluorescence signal. Therefore, the k_d that we measured here may have two components: (i) the true dissociation of the gp70 (with virus) from the receptor and (ii) the shedding of virus from a gp70-receptor complex.

The equilibrium dissociation constants can also be calculated from the ratio of the dissociation and association rate constants, $K_D = k_d/k_a$ (equation 1). The K_D values directly measured by equilibrium binding are within 3-fold (at 37°C) to 20-fold (at 4°C) of the K_D values calculated from the ratio k_d/k_a (Table 1). These discrepancies could represent the deviations in our experimental systems from the assumptions made in the theoretical model.

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