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Quantitation of Human Immunodeficiency Virus Type 1 Infection Kinetics

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Tissue culture infections of CD4-positive human T cells by human immunodeficiency virus type 1 (HIV-1) proceed in three stages: (i) a period following the initiation of an infection during which no detectable virus is produced; (ii) a phase in which a sharp increase followed by a peak of released progeny virions can be measured; and (iii) a final period when virus production declines. In this study, we have derived equations describing the kinetics of HIV-1 accumulation in cell culture supernatants during multiple rounds of infection. Our analyses indicated that the critical parameter affecting the kinetics of HIV-1 infection is the infection rate constant $k = \ln n/t_i$, where n is the number of infectious virions produced by one cell (about 10^2) and t_i is the time required for one complete cycle of virus infection (typically 3 to 4 days). Of particular note was our finding that the infectivity of HIV-1 during cell-to-cell transmission is 10^2 to 10^3 times greater than the infectivity of cell-free virus stocks, the inocula commonly used to initiate tissue culture infections. We also demonstrated that the slow infection kinetics of an HIV-1 *tat* mutant is not due to a longer replication time but reflects the small number of infectious particles produced per cycle.

In general, laboratory strains of human immunodeficiency virus type 1 (HIV-1) have markedly different biological properties than virus recently isolated from an infected individual. The prototype isolate used in tissue culture experiments, HIV-1_{Lai} (also known as HIV-1_{IIIB}), rapidly replicates to high titers in peripheral blood lymphocytes (PBLs) and CD4-positive T leukemia cell lines but not in monocyte-derived macrophages, readily induces large syncytia in H9 and SupT1 cells, and causes cell death. The host range of typical "fresh" HIV-1 isolates is usually limited to PBLs and monocyte-derived macrophages and does not include CD4-positive T-cell lines. Recent HIV-1 isolates commonly exhibit slow replication kinetics and low to negligible cytopathicity and frequently fail to induce syncytia, particularly when recovered from seropositive, asymptomatic individuals (8, 30).

Although very little is known quantitatively about the multiplicity of factors that determine the rate and levels of HIV-1 spread in tissue culture systems, even less is understood about the critical parameters affecting virus replication in an infected person. By tradition, virologists commonly monitor and characterize virus infections by using cell-free virus stocks. This approach facilitates and "standardizes" quantitative comparisons of different virus isolates and mutants because the number of extracellular "infectious units" can frequently be monitored in plaque assays on cultured cell monolayers after limiting dilutions of the initial virus stock have been made. While cell-free HIV-1 is readily detectable in tissue culture infections, significant plasma viremia has been difficult to demonstrate in infected individuals until relatively recently. In most instances, no cell-free virus is detected in asymptomatic seropositive individuals and is only demonstrable later in the clinical course, at a time when severe immune dysfunction becomes evident (4, 5, 11, 13). During the clinically latent period, the virus isolated has frequently been given such qualitative labels as rapid/high and slow/low (8, 9), neither of which permits a precise quantitation of two critical determinants of virus load in an infected person: (i) the time it takes for an HIV-1 isolate to complete a single cycle of replication and (ii) the number of infectious virions produced by an individual cell during one cycle of infection.

In this study, we have used data from tissue culture experiments in which a variety of critical parameters were examined to derive the equation $k = \ln n/t_i$, where k is the infection rate constant, n is the number of infectious progeny virions produced by a single cell, and t_i is the time required for one cycle of virus replication. When this equation was used to analyze HIV-1 infections in tissue culture systems, several interesting conclusions were reached, including: (i) the "infectivity" of virus-producing cells is 10^2 to 10^3 times greater than that of a cell-free virus inoculum derived from the same cells in initiating a de novo infection, and (ii) the marked delay in replication of a Tat^- mutant of HIV-1 compared with wild-type virus (31 versus 10 days to peak reverse transcriptase [RT] production) simply reflects a reduction in the number of infectious progeny virions produced per cell per cycle (12 versus 180, respectively) and not a prolongation of the virus replicative cycle.

MATERIALS AND METHODS

Virus and cells. HeLa cells, maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were trypsinized, diluted, and transferred to 25-ml flasks (Costar) 20 to 24 h prior to DNA transfection. Individual cultures (3×10^6 cells in 4 ml of medium), 80 to 90% confluent, were transfected with 40 μg of the full-length, uncleaved HIV-1 proviral DNA clone pNL4-3 (1) by the calcium phosphate method. The culture supernatant, containing the NL4-3 cell-free virus inoculum used in subsequent studies, was collected 48 h after transfection, filtered

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through 0.45-μm-pore-size filters, and frozen at −70°C until use.

The HIV-1 Tat$^-$ mutants were generated by polymerase chain reaction site-specific mutagenesis, which changed amino acid residue 9 (glutamic acid) to an amber stop codon (TAG) and amino acid residue 10 (proline) to alanine. To generate the *dl*.Sp1/CMV Tat$^-$ mutant [designated HIV-$1_{TAT(B)}$], nucleotide sequences containing the three Sp1 binding sites (located between positions −136 and −176 [the transcriptional initiation site is at +1]) of the HIV-1 long terminal repeat (LTR) were replaced with a 243-bp cytomegalovirus enhancer fragment (derived from pCATdl23 [14]) that had been amplified by polymerase chain reaction. Details of the construction of the Tat$^-$ mutants are described in detail elsewhere (2a).

The number of physical particles present in the inoculum was estimated to be approximately 10^9 per ml of virus stock by counting virions labeled with the fluorescent dye R18 by image-enhanced videomicroscopy (20). This assay was calibrated with virus stock (obtained from ABT, Cambridge, Mass.) containing a known number of virions as measured by electron microscopy. One count per minute (cpm) of RT activity corresponded to approximately 50 physical virus particles. The titer (50% infectious dose [ID_{50}] per ml of inoculum) of the NL4-3 virus stock, as determined by endpoint dilution (21, 22, 28), was 10^6/ml or 10^5/ml in assays in which virus was adsorbed to 12D7 cells (10^6 cells) in 0.02 or 0.2 ml of medium, respectively (7). One ID_{50} of the NL4-3 virus stock was therefore equivalent to about 10^3 to 10^4 physical particles and 20 to 200 cpm of RT activity. The limit of detection by the RT assay was 50 to 100 cpm.

The 12D7 subclone (27a) of the CD4$^+$ lymphocytic leukemia cell line A3.01 (10) was maintained in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum at a concentration of 10^6 cells per ml. The infection kinetics of Tat$^-$ HIV-1 mutants was studied in the AA2 (2) lymphoblastoid cell line. The AA2 cells were diluted threefold every 3 to 4 days. H9 cells chronically infected with HIV-1_{IIIB} were propagated (5×10^5 cells per ml) in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum for more than 1 month (29). These chronically infected HIV-1_{IIIB} cells produced 0.4×10^6 to 2×10^6 cpm (^{32}P) of RT activity per ml during a 48-h period when seeded in fresh medium at a density of 5×10^5 cells per ml. The supernatant collected from these cells by centrifugation and filtration through a 0.22-μm filter contained 0.7×10^6 cpm (^{32}P) of RT activity per ml, and the virus titer was 10^4 ID_{50} per ml. Infected H9 cells and companion supernatants were used for comparisons of cell-to-cell and cell-free virus infections, respectively.

RT assay. Virion-associated RT activity was measured as described previously (32). Briefly, 10-μl samples of culture supernatants were mixed with 50 μl of an RT reaction buffer, which contained a template primer [poly(A) (5 μg/ml) and oligo(dT)$_{12-18}$ (1.57 μg/ml)] in 50 mM Tris (pH 7.8)–7.5 mM KCl–2 mM dithiothreitol–5 mM $MgCl_2$–0.05% Nonidet P-40–0.5 μCi of [^{32}P]dTTP (400 Ci/mmol). Following a 90-min incubation at 37°C, 10 μl (for the pNL4-3/12D7 system) or 6 μl (for the H9 system) of the reaction mixture was spotted onto DEAE ion-exchange paper, dried, washed three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to remove unincorporated [^{32}P]dTTP, rinsed twice with ethanol, and dried at 70°C, and radioactivity was counted in a scintillation counter.

Infection kinetics. Cell-free HIV-1_{NL4-3} (100 μl) was mixed with 10^6 12D7 cells in 100 μl of medium and incubated for 1 h at 37°C. The unbound virus was then removed by washing with fresh medium, and the cells were cultured in 25-ml flasks (Costar). In a parallel experiment, the infected cells were maintained in 24-well plates. No difference in infection kinetics was observed between cells cultured in 10 ml of medium (flasks) or 1 ml of medium (24-well plates). Cells were diluted threefold on day 4 and every 2 days thereafter to maintain an average cell concentration of approximately 10^6 cells per ml. Prior to each dilution, cell viability was evaluated by trypan blue dye exclusion. Morphological changes (e.g., cell aggregation) were monitored by bright-field and phase contrast microscopy. The size of cell aggregates was determined by measuring their average diameters in at least three different microscopic fields. This average diameter was then divided by the maximal average diameter observed during the course of infection. In some experiments, undiluted virus was preincubated with 40 μg of soluble CD4 (sCD4) (obtained from ABT) per ml at 4°C for 1 h, mixed with 12D7 cells for 1 h at 37°C, and then washed and cultured.

H9 cells (10^6 cells in 0.1 ml of medium) were incubated with 0.1 ml of undiluted or diluted cell-free HIV-1_{IIIB} for 1 h at 37°C, washed, and then cultured in 2 ml of medium in 24-well plates. On day 2, the cells were transferred to 25-ml flasks containing 10 ml of medium. Parallel cultures were also maintained in the 24-well plates. Supernatant samples were collected each day to assay for RT activity, and 50% of the medium was changed. Cell-to-cell infections were established by mixing equal numbers (5×10^5) or different ratios of chronically infected and uninfected H9 cells in 2 ml of medium. Virus production was monitored as described for the cell-free infection. Formation of syncytia (defined as cells with a diameter larger than four uninfected-cell diameters) was also monitored in the infected H9 cultures.

RESULTS

Tenfold dilution of cell-free HIV-1 inoculum results in a 2-day delay in the peaks of cell aggregation and cell death. The kinetics of HIV-1 infections initiated by cell-free virus preparations was monitored by three parameters: (i) the formation of stable cell aggregates; (ii) the increase in the number of dead cells divided by the decrease in the total number of cells in the culture; and (iii) progeny virion production, measured as the virion-associated RT activity released into the medium. The appearance of stable cell aggregates is the first in a series of very early events that are associated with a productive HIV-1 infection. In H9 cells, for example, aggregated cells can be identified 30 to 60 min following infection and gradually progress to the formation of visible syncytia over the next several hours (data not shown). Cell aggregation is also induced by cell-free preparations of HIV-1 in 12D7 cells, a clonal derivative of the CEM T-cell line but, in contrast to infected H9 cells, does not progress to detectable syncytium formation. As shown in Fig. 1A, the aggregation of infected 12D7 cells proceeded rapidly after a variable lag time, reaching a peak within 2 to 4 days, at which point most of the cells were present in aggregates, the largest of which consisted of 100 or more cells. Cell aggregation decreased markedly over the ensuing 2 to 4 days. Both the lag times and the times required to reach peak aggregation, t_p, were longer when the HIV-1_{NL4-3} stock was diluted. Each 10-fold dilution of the cell-free virus stock resulted in a 2-day delay in the lag time and the time to peak cell aggregation (Fig. 1A). The kinetics of cell aggregation did not change when the cell concentration was decreased

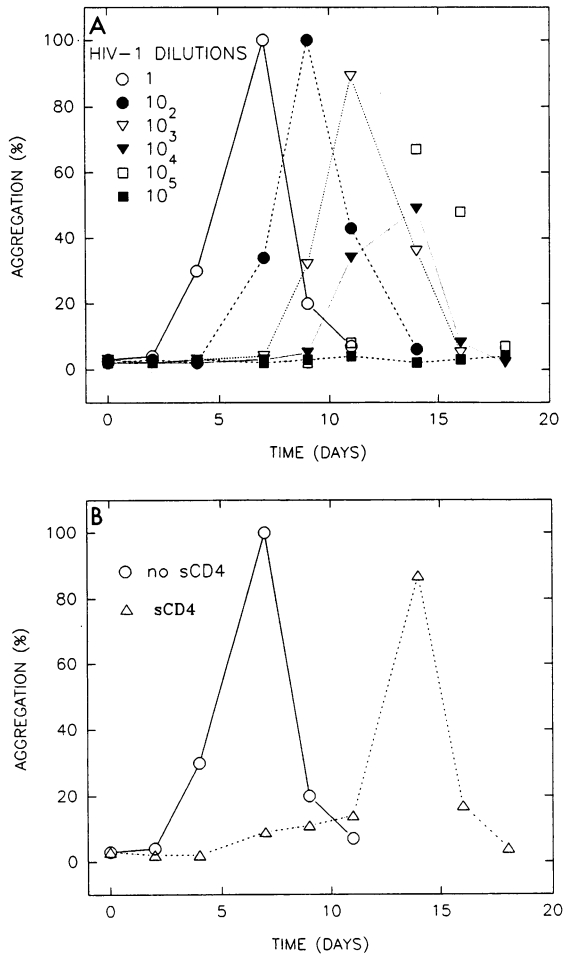

FIG. 1. Kinetics of HIV-1-induced cell aggregation. (A) 12D7 cells were infected with the indicated dilutions of HIV-1_{NL4-3}. Cell aggregates were measured as described in Materials and Methods and are presented as a percentage of their maximal size. (B) Undiluted virus was untreated or incubated with sCD4 (40 μg/ml, 4°C, 1 h).

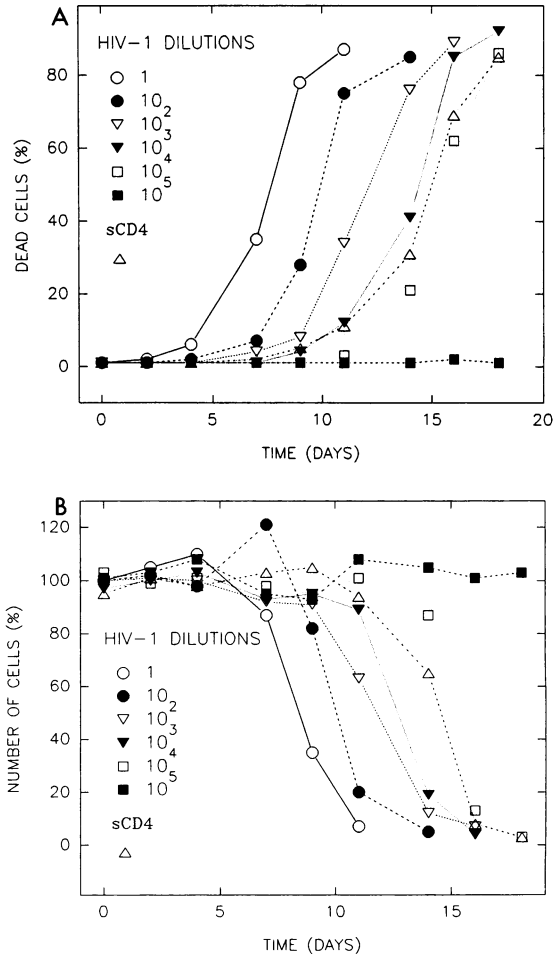

FIG. 2. Kinetics of HIV-1-induced cytopathicity. (A) 12D7 cells were infected with various dilutions of HIV-1_{NL4-3}, and cell viability was monitored by trypan blue dye exclusion. The number of cells that did not exclude trypan blue dye is presented as a percentage of the total number of cells in the culture at the various time points after infection. (B) 12D7 cells were infected with different dilutions of HIV-1_{NL4-3}, and the number of cells present at the indicated time points is shown as a percentage of the number of cells present in a parallel noninfected culture. △, incubation with sCD4 (40 μg/ml, 4°C, 1 h).

from 10^6 to 10^5 cells per ml (data not shown); at lower cell concentrations, however (10^4 and 10^3 cells per ml), the times of peak aggregation were delayed by an additional 1 to 2 days (data not shown). As shown in Fig. 1B, preincubation of the undiluted virus stock with sCD4 markedly delayed the peak of 12D7 cell aggregation.

The second parameter of virus infection, HIV-1-induced cell death, paralleled the time course of 12D7 cell aggregation. At the times of peak cell aggregation, 20 to 40% of the cells were no longer viable (Fig. 2A) and the total number of cells in the culture had decreased by 10 to 30% (Fig. 2B). Large aggregates were not detected when more than 80% of the starting 12D7 cells were dead and the total number of cells had decreased by more than 80 to 90%.

Kinetics of virus production parallels cell aggregation and cell death and can be described by an exponential function. As shown in Fig. 3, the third parameter used to monitor HIV-1 infection kinetics, virus accumulation in the culture supernatants as monitored by RT activity, paralleled the time course of virus-induced cell aggregation and cell killing. As was observed previously in the experiments measuring cell aggregation and cell death, the number of progeny virions increased sharply (after a variable lag period, the duration of which depended on the dilution of the virus inoculum) and reached a peak over the next 3 to 4 days, after which RT production decreased to background levels during a 2- to 6-day period (data not shown). The lag and peak times for RT production were virtually identical to those observed for cell aggregation (compare Fig. 1A and 3). As noted previously in the experiments in which cell aggregation and cell viability were monitored, RT activity was markedly delayed when the undiluted HIV-1_{NL4-3} inoculum was pretreated with sCD4. This sCD4 effect did not alter the kinetics of RT production after the extended lag period.

In an independent experiment (Table 1), the effect of the initial virus and cell concentrations on HIV-1 infection kinetics was evaluated by incubating a constant amount of cells and virus (i.e., a fixed multiplicity of infection [MOI]) in different volumes of medium. A 10-fold decrease in the

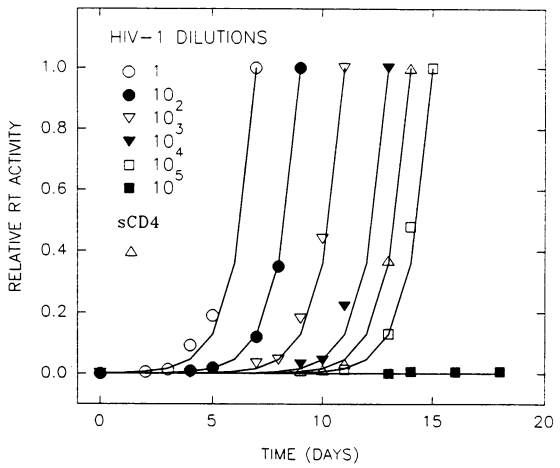

FIG. 3. Kinetics of HIV-1 virion production. 12D7 cells were infected with different dilutions of the HIV-1_{NL4-3} virus stock; culture supernatants were collected at the indicated times and assayed for the presence of virion-associated RT activity as described in Materials and Methods. RT activity is presented relative to its peak value (25,300 ± 2,800 cpm/μl). Symbols represent the experimental data, and lines show the least-square fit of the data with equation 1. △, incubation with sCD4 (40 μg/ml, 4°C, 1 h).

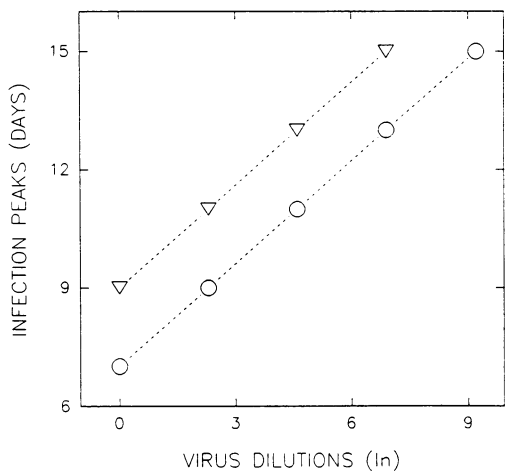

FIG. 4. Peak times of virus production at different virus dilutions and adsorption volumes. Two independent stocks of HIV-1_{NL4-3} [pNL4-3(A) (○) and pNL4-3(B) (▽) were serially diluted 10-fold and used to infect 10^6 12D7 cells in 0.2 ml of medium. The peaks of infection were determined by measuring the RT activity.

incubation volume reduced the time for peak cell aggregation and RT production by 2 days and also resulted in an apparent 10-fold increase in virus infectivity, as measured by the endpoint dilution assay. As shown in Table 1, this 2-day delay in peak virion production was observed over a 10^5-fold range of virus dilutions.

The shapes of the curves describing RT activity as a function of time in Fig. 3 are similar for all virus dilutions examined and appeared to be exponential. This would imply that a relationship exists between RT production and the time from the beginning of the infection and can be described by the equation

$$RT = e^{k(t - t_p)} \quad (1)$$

where RT is the measured relative RT activity (normalized to its maximal value of 1 at $t = t_p$), k is a rate constant independent of time t, and t_p is the time at which peak RT production occurs. When theoretical curves based on equation 1 are generated, there is an excellent fit with the actual datum points, as shown in Fig. 3. In this experiment, the HIV-1 infection rate constant k was found to be 1.1 day^{-1}, determined by fitting the experimental data in Fig. 3 with equation 1, using the computer program SigmaPlot 4.0.

TABLE 1. Times to peak virus production[a]

Virus dilution	Time to peak virus production (days) at incubation volume:	
	0.02 ml	0.2 ml
1	7	9
10^1	9	11
10^2	11	13
10^3	13	15
10^4	15	NVP[b]
10^5	NVP	NVP

[a] HIV-1 (10 μl) was incubated with 10^6 12D7 cells in 0.02 or 0.2 ml of medium for 1 h at 37°C. The unbound virus was removed by washing, and the cells were cultured for 21 days.

[b] NVP, no virus production.

Time required to reach peak virus production can be used to determine the MOI. The relationship between the delay to peak RT activity and the virus dilution was next examined in an experiment in which 10^6 12D7 cells were incubated with various concentrations of two independent stocks of HIV-1_{NL4-3}. As shown in Fig. 4, the times of peak RT production (t_p) are proportional to the logarithm of the virus dilution (D) and can be described by the equation

$$at_p = \ln D + b, \quad (2)$$

where a and b are independent of the dilution. The value determined for constant a (1.1 day^{-1}) in equation 2 was the same as the rate constant k determined from the data presented in Fig. 4. k could therefore be substituted for a in equation 2. When equation 2 is rewritten to describe two different virus dilutions, D_1 and D_2 (which correspond to two different MOIs, MOI_1 and MOI_2), which are subtracted to eliminate constant b, one obtains the equation

$$k(t_{p1} - t_{p2}) = \ln(D_1/D_2) = \ln(MOI_2/MOI_1) \quad (3)$$

where MOI is defined as the ratio of the number of infectious units to the total number of uninfected cells.

The extent to which the infectivity of a particular virus isolate or stock is affected by exposure to antiviral agents can be determined from equation 3 if the peak time of virus production and the infection rate constant can be measured. Thus, the "effective infectivity" of the undiluted HIV-1 stock after incubation with sCD4 can be determined from the data shown in Fig. 3. In that experiment, the t_{p1} for the undiluted, non-sCD4-treated virus is 7 days, k = 1.1 day^{-1}, and the MOI is 10^{-2} (10^4 50% tissue culture-infective doses of HIV-1 and 10^6 12D7 cells). After incubation with sCD4, the peak of RT production shifts to 14 days (t_{p2} = 14), which corresponds to an effective MOI of 3.0×10^{-6} as calculated from equation 3 and represents a 3.3×10^3-fold ($10^{-2}/3 \times 10^{-6}$) reduction in virus infectivity.

Rate of HIV-1 infection is proportional to the logarithm of the number of infectious virions produced by one cell and inversely proportional to virus replication time. In the previ-

ous sections, we presented the experimental data used to formulate empirical equations 1 and 2, which describe HIV-1 infection kinetics. These equations enabled us to determine the values of the infection rate constant k and to define the relationship between virus dilution and the peak times of virus infection. Empirical equations 1 and 2 cannot be used to calculate the number of infectious virions produced by a single cell or the time of one virus replicative cycle. However, if one assumes that the duration of each cycle of virus infection, t_i, is constant and that each HIV-1-infected cell produces a constant number of infectious units, n, per cycle, then the infection rate constant can be calculated as function of n and t_i:

$$k = \ln n/t_i \quad (4)$$

(see Appendix for its derivation). If it is also assumed that the peak of the infection occurs when most of the cells are infected and the production of progeny virions (determined by RT activity in the medium) is proportional to the number of infected cells, then the replication time, t_i, and the rate constant k (and therefore n) can be determined from the equation

$$t_p = t_i - (1/k)\ln(\text{MOI}),\ \text{MOI} = N_0/N_t \quad (5)$$

(see Appendix for its derivation), where t_p is the time needed to reach the peak of infection, N_0 is the number of initially infected cells, and N_t is the total number of cells in the tissue culture.

When equations 4 and 5 are applied to the experiment depicted in Fig. 3, one can calculate that a single infection cycle of HIV-1_{NL4-3} in 12D7 cells takes 3 days (t_i = 3 days) and 26 infectious particles (n = 26) are produced per cell per cycle (k = 1.1 day^{-1}).

HIV-1 infection rate constants must be corrected for the progeny virus-infected cells discarded during the maintenance of infected tissue cultures. HIV-1-infected human T cells are frequently maintained in culture for several weeks, during which time a significant portion (two-thirds to three-fourths) of the cells and medium are periodically discarded and replenished with fresh medium to maintain appropriate cell densities. This removal of cells and medium could markedly retard or even stop a spreading HIV-1 infection if the elimination of infected cells and virions exceeds the rate of progeny virus production, particularly when small numbers of infectious units are produced per cell.

As shown in the Appendix, the infection rate constant k must be adjusted for the dilutional effects attending the maintenance of infected tissue cultures. An effective rate constant k can be represented as

$$k = k_i - k_d \quad (6)$$

where $k_i = \ln n/t_i$ and

$$k_d = \ln m/t_d \quad (7)$$

where m is the fold dilution and t_d is the time between tissue culture changes or dilutions. The dilution rate constant, k_d, must be added to the measured rate constant k to obtain k_i for the calculation of the number of infectious particles produced per cell, n. In the experiment presented in Fig. 3, k = 1.1 day^{-1}, k_d = 0.55 day^{-1} (calculated from equation 7 with m = 3 and t_d = 2 days), t_i = 3 days, and the corrected value for n is 140 rather than the 26 determined previously without taking the dilutional effects of the medium changes (discarding two-thirds of the cells and medium every 2 days) into account.

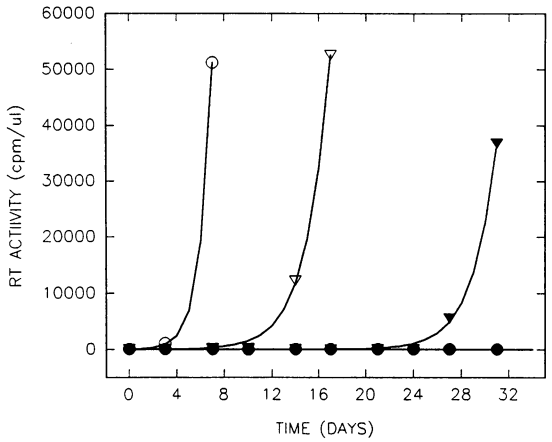

FIG. 5. "Slow" kinetics of an HIV-1 Tat mutant infection. AA2 cells were infected with two concentrations of the HIV-$1_{TAT(B)}$ mutant, undiluted (▼) and 25-fold concentrated (▽), generated by transfecting HeLa cells. A stock of the HIV-$1_{TAT(A)}$ mutant was also obtained from a transfection supernatant and used undiluted to infect AA2 cells (●). Wild-type HIV-1 (○) was used at an MOI of 0.05 (25-fold concentrated). The RT activity was monitored by assaying culture supernatants at intervals of 3 or 4 days for up to 55 days.

An HIV-1 Tat mutant replicates rapidly but produces only a few infectious virions per cycle. As an example of a practical use of the equations described in the previous section, the number of infectious particles released per cycle and the length of each cycle of infection were determined for an HIV-1 Tat-deficient mutant which produced progeny virions following a consistent 4- to 5-week delay compared with its wild-type parent. Two different HIV-1 Tat mutant viruses (each containing a termination codon at residue 9 and a proline-to-alanine substitution at residue 10 of the Tat protein) were examined. One [HIV-$1_{TAT(A)}$] contained the usual wild-type LTR, and in the other [HIV-$1_{TAT(B)}$], the two NFκB motifs located in the U3 region of the LTR were replaced by a human cytomegalovirus immediate-early enhancer element that increases basal levels of LTR-directed gene expression (2a). Stocks of wild-type HIV-1 and the two Tat-deficient viruses were prepared by transfecting HeLa cells with the appropriate cloned proviral DNAs. When the input virus stocks used for a subsequent infection of AA2 cells were adjusted for equivalent amounts of RT activity (MOI of approximately 0.002, as determined for the wild-type HIV-1_{NL4-3}), the peak production of wild-type HIV-1 was observed on day 10 (data not shown), whereas no HIV-$1_{TAT(A)}$ mutant progeny were ever detected, even after 55 days of observation. In contrast, the HIV-$1_{TAT(B)}$ mutant reproducibly directed the synthesis of significant amounts of progeny virus, but peak production was consistently delayed to 30 to 40 days after infection (day 31 in the experiment presented in Fig. 5), in both primary and "second-cycle" reinfections of AA2 cells. Nucleotide sequence analysis of the HIV-$1_{TAT(B)}$ progeny virions indicated that the original *tat* mutation was still present (data not shown).

Since it is possible to determine the rate constant, k, for HIV-$1_{TAT(B)}$ infections from the times of peak virus production at different MOIs with equation 3, AA2 cells were infected with wild-type HIV-1 (HIV-1_{WT}) and HIV-$1_{TAT(B)}$ at a 25-fold-higher MOI (Fig. 5). In this case, peak HIV-$1_{TAT(B)}$ production was observed on day 17, compared with

day 7 for wild-type HIV-1. By using equations 4 to 7 to calculate t_i and n for HIV-1_{WT} and HIV-$1_{TAT(B)}$ infections corrected for medium changes, the time required for a single infectious cycle was indistinguishable for the two viruses (4 days), but HIV-$1_{TAT(B)}$ produced fewer (n = 12) infectious particles per cycle than HIV-1_{WT} (n = 180).

The HIV-$1_{TAT(A)}$ mutant failed to produce detectable RT activity in the experiment shown in Fig. 5, indicating that either the infected cells did not produce any virus or the amount of viral progeny was below the limits of detection. As discussed in the previous section, the dilution of infected cell cultures associated with their maintenance may preclude the detection of progeny virion production. Equations 6 and 7 allow the calculation of the minimal number of infectious particles produced by a single cell per infectious cycle, n_m, needed to sustain a spreading HIV-1 infection. The number of infected cells increases with time if $k = (k_i - k_d) > 0$. At $k = 0$, the number of infected cells remains constant. n_m can therefore be calculated from the relationship $k = 0$, which leads to $\ln(n_m) = (t_i/t_d)\ln m$. For example, if the cultured infected cells are diluted threefold every 3 days and the replication time, t_i, is 3 days, the minimal number of infectious virions produced per cell (n_m) required to merely maintain the status quo would be 3. No peaks in the progeny virus production would be detected in such a system if each infected cell produced three or fewer infectious virions. As shown in Fig. 5, we were unable to ascertain whether any HIV-$1_{TAT(A)}$ mutant particles were produced. However, assuming that the HIV-$1_{TAT(A)}$ MOI and replication times are the same as for HIV-$1_{TAT(B)}$, it is possible to calculate that the production of four or fewer infectious particles by HIV-$1_{TAT(A)}$ could result in a time to peak progeny virion synthesis of more than 55 days.

HIV-1-infected cells are 10^2- to 10^3-fold more infectious than cell-free virus inocula. The ability to calculate the number of infectious particles synthesized in virus-producing cells permits an evaluation of the infectivity of cell-free and cell-associated HIV-1 inocula. It has recently been reported that cell-to-cell transmission of HIV-1 is significantly more rapid than are infections initiated with cell-free virus preparations (29). At a donor/recipient cell ratio of 1:4, virus-induced cell-cell fusion was observed within 15 min of cocultivation, and substantial amounts of full-length linear viral DNA were detectable by 4 h. We therefore decided to directly compare the kinetics of HIV-1 infections initiated by virus-producing cells with those of infections initiated by a cell-free virus inoculum generated contemporaneously by the same infected cells. Infection kinetics was examined by cocultivating infected $H9_{IIIB}$ (donor) with uninfected H9 (recipient) cells at different ratios or by inoculating uninfected H9 cells with different concentrations of cell-free virus released from known numbers of the same donor $H9_{IIIB}$ cells. As shown in Fig. 6, the cell-free $H9_{IIIB}$ virus exhibited infection kinetics similar to that observed previously with cell-free preparations of HIV-1_{NL4-3} (see Fig. 3). However, the kinetics of the cell-free $H9_{IIIB}$ infection was markedly delayed compared with those of the cell-to-cell virus transmission observed after cocultivation. For example, RT production in the infection initiated with undiluted cell-free $H9_{IIIB}$ (obtained by filtering the medium from 0.5×10^5 cells) coincided with that observed in the infection in which only 10^2 virus-producing $H9_{IIIB}$ cells were used as the inoculum. Thus, the infectivity of the filtered medium from 0.5×10^5 infected H9 cells was equivalent to that observed when the inoculum consisted of 500-fold fewer infected HIV-1-producing cells; a similar disparity in infectivities was observed when the supernatant was diluted 100-fold (compare open and solid triangles in Fig. 6). However, it should be noted that the numbers of infectious particles produced per cell and the replication times for the cocultivation (n = 300, t_i = 3 days) and the cell-free virus infection (n = 330, t_i = 4 days) experiments were similar. This result indicates that once an HIV-1 infection is initiated (by either type of inoculum), subsequent infectious cycles are kinetically indistinguishable, presumably because they are driven by cell-associated inocula.

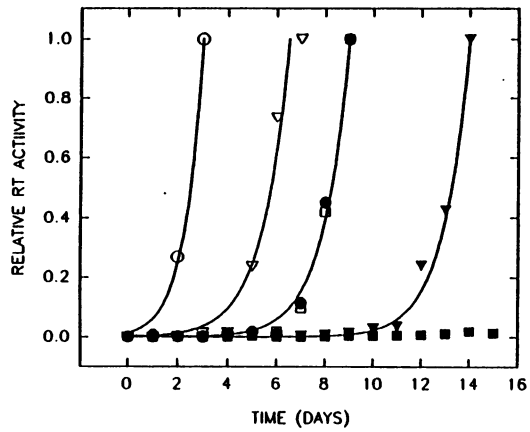

FIG. 6. Kinetics of HIV-1 virion production in cells cocultured with chronically HIV-1-producing cells or infected with cell-free virions. H9 cells chronically infected with HIV-1_{IIIB} were cocultured with uninfected H9 cells at $H9_{IIIB}$/H9 cell ratios of 1:1 (○), $1:10^2$ (▽), and $1:10^4$ (□). The total number of cells was 10^6. In a separate experiment, H9 cells were infected with 1:1 (●), $1:10^2$ (▼), or $1:10^4$ (■) dilutions of cell-free HIV-1_{IIIB} virus stock (produced by 0.5×10^5 chronically infected H9 cells). The RT activity was normalized to its peak value. The peak value for different dilutions did not vary by more than 10% and was 20,600 cpm/μl at an H9IIIB/H9 ratio of 1:1. The lines represent the best fits as determined by equation 1 (see text).

DISCUSSION

Two approaches are commonly used to measure HIV-1 infectivity: endpoint dilution of a virus stock, which is reported as the amount of inoculum having a 50% probability of initiating an infection during a defined observation period (such endpoint-diluted virus stock is assumed to contain 1 ID_{50}) (21), and the quantitation of syncytium formation at a defined time during virus infection, an assay which assumes that each infected cell will generate a single syncytium. HIV-1 infectivity measured this way is reported as syncytium-forming units (18, 19, 24). The first approach requires serial dilutions of each virus preparation and, for large numbers of samples of unknown infectivity, may be impractical. Syncytium assays are obviously limited to virus-cell systems which readily form large and stable syncytia. For example, HIV-1_{NL4-3} does not induce syncytia visible by light microscopy during infections of 12D7 (CEM) cells. The empirical equations that we have derived, relating the peak of virus production to the MOI, offer a means to rapidly quantitate the effect of antiviral agents, such as sCD4 (7, 23), on HIV-1 infectivity. This approach may be particularly relevant for measuring the kinetics of virus inactivation, when endpoint dilution of a virus stock may be deleterious to a potential inhibitor of HIV-1 being assayed. In addition to

TABLE 2. Infection rate constants (k), replication times (t_i), and number of infectious particles produced by one cell (n) for infection by cell-free and cell-associated HIV-1

Virus	Cells	k (day^{-1})	t_i (days)	n
HIV-1_{NL4-3}	CEM	1.1	3	140
HIV-$1_{TAT(B)}$	AA2	0.25	4	12
HIV-1_{NL4-3}	AA2	1.1	4	180
HIV-1_{IIIB}	H9	1.1	4	330
H9IIIB	H9	1.5	3	300
HIV-1_{SF2}	PBMCs	1.2	4	120
HIV-1_{SF13}	PBMCs	1.8	3	220

quantitating HIV-1 infectivity, the equations presented may also be used to estimate the time required for the completion of a single cycle of infection, t_i, and the number of infectious particles, n, produced by a single cell. For the HIV-1_{NL4-3}-12D7 system, one cycle of infection takes 3 days, a value which is in agreement with other published reports for HIV-1-infected CEM cells (26).

The equations that we have derived are also in agreement with previously reported HIV-1 infection kinetics in diverse experimental systems. For example, the infection rate constant k, calculated from the data for human PBLs infected with HIV-1_{LAV} (21), is equal to 1.4 day^{-1}. This is similar to the rate constant reported in our study (1.1 day^{-1}), in which the closely related virus strain HIV-1_{NL4-3} was used. The slightly higher rate constant (1.4 versus 1.1 day^{-1}) may be due to differences in the cell cultures (PBLs versus the 12D7 leukemic cell line) or may reflect the contribution of the HIV-1_{NY5} *gag*, *pol*, and *vif* genes in HIV-1_{NL4-3} (1) rather than those derived from the HIV-1_{LAI} isolate. In another study (3), peripheral blood mononuclear cells (PBMCs) and several cell lines were infected with the primary isolates HIV-1_{SF2} and HIV-1_{SF13}. HIV-1_{SF13} was isolated 5 months after HIV-1_{SF2} from the same individual (3). The calculated infection rate constants, k, and number of infectious virions, n, for PBMCs were higher for the later virus isolate HIV-1_{SF13} (k = 1.8 day^{-1}, n = 220) than for HIV-1_{SF2} (k = 1.2 day^{-1}, n = 120). The replication time for HIV-1_{SF13} (t_i = 3 days) was somewhat shorter than that for HIV-1_{SF2} (t_i = 4 days), suggesting that HIV-1_{SF13} produces nearly twice as many infectious progeny and replicates faster than HIV-1_{SF2} (Table 2).

The number of physical particles released from the HIV-1-producing cells was estimated to be about 10^9 virions per 10^6 cells (in 1 ml of supernatant), based on image-enhanced videomicroscopy of fluorescent dye-labeled virus and RT activity. This corresponds to about 10^3 physical particles released per infected cell, a value which is in reasonable agreement with published data for HIV-1-infected human PBLs (21) and CEM cells (31). Since the culture supernatant contains 10^9 virus particles per ml and 10^5 to 10^6 infectious units per ml, the infectivity of HIV-1, defined as the ratio of infectious units to virus particles, is on the order of 10^{-4} to 10^{-3} infectious units per virus particle (a similar number was obtained previously [17, 21]). Our calculations indicate, however, that during virus transmission in tissue cultures, each infected cell produces about 100 infectious virions, and therefore its infectivity is on the order of 10^{-1} infectious unit per virus particle. This value is about 10^2 to 10^3 times higher than the infectivity of cell-free virus present in the supernatants of infected cells. The infectivity of chronically infected H9 cells was also much higher (about 10^3 times) than that of cell-free HIV-1 derived from the same cells (Fig. 6) (29). Even though the infectivity of HIV-1 during transmission in cell cultures is quite high (1 infectious unit per 10 virus particles), it is actually low compared with that of other viruses (e.g., the particle-to-infectious unit ratio for Semliki Forest virus has been reported to be 2 [12]).

Several factors are probably responsible for the greater infectivity of HIV-1 than of cell-free virions during multicycle infections. Sato et al. (29) proposed that cell contact favored virus spread by maximizing the effects of cell-cell fusion. Intimate contacts between cells in tissue culture either allow freshly budded (and possibly more stable) virions to infect adjacent cells or promote the process of cell-cell fusion more efficiently than a typical cell-free preparation of HIV-1 (6, 7, 29). Syncytium formation plays a minor role in virus transmission, as indicated by the similarity in the rate constants (k), number of infectious virions per cell, and time required for one round of infection observed for the 12D7-HIV-1_{NL4-3} (which do not form syncytia) and the H9-HIV-1_{IIIB} (which do form syncytia) systems.

The relatively low infectivity of cell-free virus inocula may also reflect interference by noninfectious virus particles and/or HIV-1 components, such as gp120, that are not associated with virions. As noted above, the ratio of noninfectious to infectious particles is 10^2- to 10^3-fold greater in the cell-free virus preparations than in tissue cultures. Consequently, the effect of viral interference on the infection kinetics observed during cell-to-cell spread is relatively minor. In infected individuals, viral interference may not play a significant role in HIV-1 transmission in lymphoid tissues because the high density and proximity of virus-producing lymphocytes would favor cell-to-cell transmission. The converse is true for susceptible CD4-positive cells in the general circulation, where defective virus particles and/or free gp120 may block infection. The effects of viral interference, HIV-1 genomic variability, and other factors that may affect HIV-1 infection in vivo could be quantitatively analyzed by a generalization of our model, currently under development.

The equations described here have also proved useful for evaluations of viral isolates such as the HIV-1 Tat-deficient derivative HIV-$1_{TAT(B)}$, which consistently exhibited markedly delayed infection kinetics. An unexpected conclusion of the HIV-$1_{TAT(B)}$ analysis is that a rather modest reduction (about 15-fold) in the number of infectious progeny virions produced per cell results in a profound prolongation (4 weeks) in time to peak virus production compared with wild-type HIV-1. In the case of HIV-$1_{TAT(B)}$, the effect of generating only 12 infectious units per cell per cycle rather than 180 leads to an infection that is so delayed (4 to 6 weeks) that it very likely would not be detected by conventional protocols. Under the same conditions, the HIV-$1_{TAT(A)}$ mutant, which contains a wild-type LTR, failed to produce detectable progeny during a 55-day experiment. Our calculations would predict that HIV-$1_{TAT(A)}$ generates four or fewer infectious progeny particles per cell per cycle. Taken together, these results suggest that the "window" of HIV-1 infectivity (measured as the number of infectious units released per cell per cycle) is quite narrow, as monitored in typical tissue culture infections, and may, in fact, be limited to a 10- to 20-fold range. If such a restriction is also applicable to HIV-1 infections in vivo, then it may be possible to develop permanently enfeebled strains of virus,

similar to those described for simian immunodeficiency virus (15), which are unable to replicate to high titers and, as a consequence, do not cause disease.

The quantitation of HIV-1 infection kinetics that we have described may assist in the interpretation of experiments in which the host range and replicative and cytopathic properties of different virus isolates are compared (3, 16). This approach may also prove useful when considering the kinetics of HIV-1 infection in infected individuals or when developing mathematical models of AIDS progression (25) and in vivo HIV-1 infections (27) by providing the necessary parameters and relationships for such analyses. However, it must be pointed out that our approach does not take into account possible virus interference and heterogeneity, which could significantly affect the HIV-1 infection kinetics.

APPENDIX

Model of HIV-1 infection kinetics. Assuming that each infected cell produces n infectious progeny virions that are transmitted as cell-free particles and/or by HIV-1 induced cell-cell fusion and lead to a multicycle infection which spreads throughout the culture, then the number of infected cells, N, after r rounds of infection will be

$$N = N_0 n^{r-1} \quad \text{(A1)}$$

where N_0 is the initial number of infected cells after the first cycle of infection is completed. It is also assumed that the number of rounds of infection, r, is equal to the time after the start of infection, t, divided by the time required to complete one cycle of replication, t_i. The substitution of $r = t/t_i$ in equation A1 leads to

$$N = N_0 \exp\{[(t/t_i) - 1]\ln n\} = (N_0/n)\exp[(t/t_i)\ln n] \quad \text{(A2)}$$

Assuming that the number of RT-active virions in the culture supernatant is proportional to the number of infected cells (i.e., RT is proportional to N), equation A2 leads to the same functional dependence on time t as equation 1 and allows the determination of the rate constant k:

$$k = \ln n/t_i \quad \text{(A3)}$$

Assuming that the RT activity is proportional to the number of infected cells, N, equation A2 can be represented as

$$RT = N/N_t = (N_0/nN_t)\mathrm{e}[(\ln n/t_i)t] \quad \text{(A4)}$$

where RT is relative RT activity normalized to its value at $N = N_t$ and N_t is the total number of cells. Assuming that all of the cells are infected at the peak of RT activity ($N = N_t$ at $t = t_p$), equation A4 leads to $N_0/nN_t = \exp(-kt_p)$, which can be represented as

$$t_p = t_i - (1/k)\ln(\mathrm{MOI}), \ \mathrm{MOI} = N_0/N_t \quad \text{(A5)}$$

where the relationship $k = \ln n/t_i$ was used. Equation A5 is identical to empirical equation 2. Equations A3 and A5 allow the calculation of the time for one cycle of infection, t_i, and the number of infectious virions produced by one cell per cycle, n, from the experimental data.

HIV-1 infection rate constants for cell cultures which are periodically diluted. When a cell culture is periodically diluted m-fold to maintain an optimal number of cells, the number of infected cells and progeny virions in the medium are reduced m-fold. To calculate the number of infected cells which are left after t/t_d dilutions, where t_d is the time period between two dilutions, one must divide the number of the infected cells, N, by a factor of $m^{t/td}$. This leads to exactly the same expression as that given by A2 but with

$$k = k_i - k_d \quad \text{(A6)}$$

where k_i is given by A3 and

$$k_d = \ln m/t_d$$

Equation A5 can then be represented as

$$t_p = t_i - [1/(k_i - k_d)]\ln(\mathrm{MOI}) \quad \text{(A7)}$$

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