Replication of patient isolates of human immunodeficiency virus type 1 in T cells: A spectrum of rates and efficiencies of entry
(slow/low patient isolate/rapid/high patient isolate/single cycle infections/leu3a entry block/dideoxycytidine reverse transcription block)

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ABSTRACT Isolates of human immunodeficiency virus type 1 (HIV-1) undergo many different rates of replication, with the time course of replication being determined by the host cell and the virus. Recently, we demonstrated that the permissiveness of four CD4* T-cell lines for the laboratory strain NL4-3 correlated with the rate and efficiency of virus entry. In this study, we have analyzed the replication of a “slow/low” isolate from the pre-AIDS period of infection and two “rapid/high” isolates from the AIDS period of infection to determine which steps in the virus life cycle determine differences in the patient characteristics of isolates. Differences in the growth characteristics of patient isolates correlate with differences in entry but not postentry steps of the virus life cycle. The two rapid/high patient isolates (SF33 and SF216) underwent 50% entry in ≤0.5 hr in C8166 cells, in ≤1 hr in mitogen-stimulated peripheral blood mononuclear cells, and in ≥2.5 hr in H9 cells. In contrast, a class 3 slow/low patient isolate required 1 hr for 50% entry into C8166 cells, 3 hr for 50% entry into peripheral blood mononuclear cells, and 5–6 hr for 50% entry into H9 cells. Entry efficiency correlated with entry rate, with the rapid/high virus having a 2-fold higher titer and the slow/low virus having a 5-fold higher titer on C8166 than H9 cells. The laboratory strain NL4-3 displayed intermediate rates and efficiencies of entry. These data demonstrate that entry characteristics are major determinants of the pathogenic potential of patient isolates.

Human immunodeficiency virus type 1 (HIV-1) isolated from patients during the long seropositive pre-AIDS period of disease has been reported to be slowly replicating, low-yielding viruses (slow/low viruses) that do not cause syncytia and do not grow well on cell lines. In contrast, isolates from patients with fully developed AIDS tend to be rapidly replicating, high-yielding viruses (rapid/high viruses) that cause syncytia and readily establish productive infections in T-cell as well as monocyte/macrophage cell lines (1–5). Laboratory strains of HIV-1 also exhibit virus- and cell-line-specific differences in growth potential and syncytium forming activity.

Recently we demonstrated that differences in the ability of four cell lines to support the replication of a laboratory strain of HIV-1 (NL4-3) correlated with the rate and efficiency of virus entry (6). Virus entry was measured as the time required for 50% of the infectious units to escape being blocked by the anti-receptor antibody leu3a (7). Fifty percent entry occurred within 30 min in infections of the highly permissive C8166 cells. In contrast, 50% entry required an average of 4 hr in infections of three less permissive cell lines (H9, Jurkat, and A3.01). The rate of entry correlated with the efficiency of entry. Stocks of NL4-3 had three times higher titers on C8166 cells than on the less permissive cells. Interestingly, although entry depended on the presence of CD4, differences in entry did not correlate with surface levels of CD4 (6).

The current study was undertaken to determine whether virus as well as T-cell factors could affect HIV-1 entry. To optimize our chance of detecting viral factors that might affect entry, the life cycles of three patient isolates were compared with that of NL4-3. Two of the patient isolates, SF94 and SF216, represent serial isolates from a single patient (2). SF94, from the pre-AIDS period of disease, has a limited ability to replicate on H9 cells (slow/low, class 3 phenotype) (3). In contrast, SF216, from the AIDS period of disease, establishes productive infections on various cell lines (rapid/high phenotype). The third patient isolate, SF33, is a rapid/high virus with an unusually broad tissue tropism that includes an osteosarcoma cell line (8).

MATERIALS AND METHODS

Cells. C8166, a human T-lymphotrophic virus type 1 immortalized but nonexpressing line of cord blood lymphocytes (9), and H9 cells, a subline of HUT78 cells (10), were obtained from the AIDS Repository, Rockville, MD. Peripheral blood mononuclear (PBM) cells were cultured from seronegative donors according to the consensus protocol used by the National Institutes of Health-sponsored AIDS Clinical Trials Units. Cells were cultured at densities of 1–2 × 10^6 per ml in RPMI 1640 medium supplemented with 10–15% fetal bovine serum.

Viruses. NL4-3, a laboratory strain that has been constructed to encode all known HIV-1 gene products (11), was recovered from pNL4-3 DNA by DEAE-dextran transfection of H9 cells. Three patient isolates, SF33, SF94, and SF216, were obtained from C. Cheng-Mayer and J. Levy (University of California, San Francisco, CA) (2, 8). Stocks of the SF viruses were produced during a single passage on H9 cells. Serial passage of NL4-3 or SF94 on H9 cells resulted in stocks with altered growth potentials (R.F.-L. and H.L.R., unpublished observations). Viruses were titered for infectious units on C8166 cells (12).

Single Cycle Infections. Infections were carried out at 37°C in the presence of 2 μg of Polybrene per ml at multiplicities of 0.2–0.8 C8166 infectious unit per cell (6). At the time of virus addition, cultures were aliquoted in prewarmed 24-well plates. The time course for entry was followed by timed additions of leu3a, an anti-receptor monoclonal antibody (Becton Dickinson) (7). The time course for reverse transcription was followed using timed additions of dideoxycytidine (ddC), a reverse transcriptase inhibitor (Sigma) (13). Addition of leu3a (250 ng/ml for NL4-3, SF94, and SF216; 750 ng/ml for SF33) or ddC (2 μM) 10 min prior to the addition of virus completely blocked each of the infections.

Abbreviations: HIV-1, human immunodeficiency virus type 1; PBM, peripheral blood mononuclear; ddC, dideoxycytidine; IFA*, immunofluorescent antibody positive.

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Escape from the leu3a and ddC blocks was scored by monitoring cultures for plateau levels of virus-expressing cells using indirect immunofluorescence (6, 12). The time course for virus expression was determined by washing an untreated aliquot at 12–15 hr after infection and growing the washed cells in the presence of leu3a and ddC to prevent the spread of progeny virus. Aliquots of these cultures were analyzed at sequential times for virus-expressing cells using indirect immunofluorescence (6, 12) and for levels of viral proteins using an antigen capture ELISA. The ELISA used 99% pure immunoglobulins obtained from pooled patient plasma for the capture and for detecting antibodies. These immunoglobulins recognized several HIV-1 proteins, including p24, gp41, and gp120 (14).

RESULTS

To identify steps in the HIV-1 life cycle that determine differences in the replication of patient isolates, single cycle analyses were conducted for two rapid/high patient isolates (SF33 and SF216), one slow/low patient isolate (SF94), and a laboratory strain (NL4-3). Growth studies were done on the highly permissive C8166 cells, the less permissive H9 cells, and mitogen-stimulated PBM cells. In these analyses, timed additions of an anti-receptor antibody (leu3a) and of an inhibitor of reverse transcriptase (ddC) were used to define the times required for entry, reverse transcription, and expression.

An example of such a single cycle analysis is given for the replication of the slow/low virus (SF94) in PBM cells (Fig. 1). The time course for escape from the leu3a block (○), the time course for escape from the dideoxycytidine block (●), and the time course for virus expression (□) are indicated in Fig. 1. These three time courses allowed estimation of the times required for 50% of the infectious units to undergo entry (escape the leu3a block), undergo reverse transcription (pass from the leu3a block past the ddC block), and undergo expression (pass from the ddC block to virus-expressing cells).

Data from 12 such single cycle infections are summarized in Fig. 2. These 12 infections followed the life cycles of the two rapid/high viruses, the slow/low virus, and NL4-3 in C8166 cells, H9 cells, and mitogen-stimulated PBM cells. The data reveal striking differences in the times required for virus entry. In sharp contrast, little or no differences were observed in the times required for reverse transcription and expression.

Entry rate depended on the virus and the T cell (Fig. 2). For each of the viruses, the most rapid entry occurred in C8166 cells, the intermediate rates of entry occurred in mitogen-stimulated PBM cells, and the slowest rates of entry took place in H9 cells. Entry rates within a cell type were consistently 2- to 3-fold faster for the rapid/high viruses than the slow/low virus. The laboratory strain NL4-3 had an intermediate entry profile. To test whether virus concentration would affect entry rates, two dilutions of a SF216 stock were analyzed for entry. The 5-fold dilution in these samples did not affect the "rapid/high" entry characteristics of SF216 (R.F.-L. and H.L.R., unpublished observations). Entry rates did show some variation from experiment to experiment (6). However, within each experiment, the relative differences for the different HIV-1 isolates were always the same.

The efficiency of the entry of each virus correlated with its rate of entry (Fig. 3). When titers of virus were determined from data obtained in independent single cycle infections, the titers in H9 infections were consistently lower than those in the highly permissive C8166 infections. To directly test for the relative titers on C8166 and H9 cells, virus stocks were titered on C8166 cells and then diluted to initiate H9 infections with 0.5 C8166 infectious unit per cell (a multiplicity of infection that gives 50% infected C8166 cells). The leu3a entry block was added to cultures at various times after

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Fig. 1. Single cycle infection of SF94 in PBM cells. Entry, reverse transcription, and expression times are defined as the hours required for 50% of the infectious units to escape the leu3a block (entry), the hours for 50% of the infectious units to traverse from the leu3a block past the ddC block (reverse transcription), and the hours for 50% of the infectious units to pass from the ddC block to scoring for viral antigens in an indirect immunofluorescence antibody assay (expression). IFA+*, immunofluorescent antibody positive. ○, Time of leu3a addition; ●, time of ddC addition; □, time for expression of viral antigens in 15-hr leu3a plus ddC point. The vertical dotted lines indicate how the times for entry, reverse transcription, and expression were determined.

Fig. 2. Hours required for 50% of the infectious units to undergo entry, reverse transcription, and expression. Data for each of the T-cell types were collected from parallel, simultaneous single cycle infections with the four tested viruses. For an example of a single cycle infection see Fig. 1. PBM, PBM cells. (Top) Entry. Values represent the hours required for 50% of the infectious units to escape the leu3a block. (Middle) Reverse transcription. Values represent the hours required for 50% of the infectious units to pass from the leu3a block past the ddC block. (Bottom) Expression. Values represent the hours required for 50% of the infectious units to pass from the ddC block to the expression of viral antigens.
infection and the cultures were scored for the percent infected cells at 60 hr after infection. In each of the infections, the plateau level of virus-expressing cells that occurred when all of the infectious units had passed the leu3a block was <50%. Only 10% of the H9 cells became infected by the slow/low virus. Approximately 25% of the H9 cells became infected by the two rapid/high viruses. These results indicate that the slow/low virus underwent a 5-fold reduction of its C8166 titer on H9 cells, whereas the two rapid/high viruses underwent 2-fold reductions in their C8166 titers on H9 cells. Consistent with its intermediate entry rate, NL4-3 exhibited an intermediate (3-fold) reduction of its C8166 titer on H9 cells (6).

The levels of virus expression per infected cell were cell type specific and not a general predictor of whether an isolate had slow/low or rapid/high growth characteristics (Fig. 4).

Under our conditions of single cycle infections, between 10% and 50% of the cells are infected. This means that the majority of virus-expressing cells are infected by a single infectious unit. Assuming a Poisson distribution, two active proviruses would be expected in 10% of the cells in which 50% of a culture is infected and in <1% of the cells in which 10% of the culture is infected. Thus, normalization of the level of virus proteins per infected cell provides an estimate of the relative levels of expression per infectious unit. When this was done for H9 infections, the two rapid/high viruses and NL4-3 were found to have similar levels of expression, which were ~3-fold higher than that of the slow/low virus (Fig. 4). In contrast, on PBM cells, the slow/low virus was expressed at levels similar to one of the rapid high viruses (SF33) and NL4-3. This level of expression was about 2-fold lower than that of the other rapid/high virus (SF216) (Fig. 4). These results indicate that differences in the levels of virus expression per infected cell do not necessarily correlate with the slow/low and rapid/high phenotype.

DISCUSSION

Spectrum of Rates and Efficiencies of HIV-1 Entry. Our results clearly demonstrate that HIV-1 isolates have a whole spectrum of rates and efficiencies of entry. In our tests, 50% entry required from <20 min to >5 hr, depending on the virus isolate and the host T cell (Fig. 2). Entry rates also affected entry efficiencies (Fig. 3). For our slowest entry virus (SF94), the titer of infectious units measured in the most permissive cell was five times higher than the titer of infectious units determined in the least permissive cell.

Steps that take place during HIV-1 entry require the viral envelope glycoproteins gp120 and gp41 and the host cell receptor CD4. The first step in entry is the binding of virus to cells. This step is facilitated by gp120 binding to CD4 (for a recent article, see ref. 15). gp120–gp41 oligomers are then thought to undergo post-binding conformational changes that expose the fusion domain of gp41 (16, 17). These conformational changes are pH independent (18), may involve shedding of gp120 (19–21), and may require proteolytic cleavage of the third variable region of gp120 (V3 loop) (22, 23). Once the conformational changes have taken place, virus enters the cytoplasm by fusion through the cytoplasmic or an endosomal membrane (24, 25). Our results indicate that virus and host cell factors affect the rate and efficiency of these entry phenomena (Fig. 2).

In terms of viral factors, we think it likely that differences in the envelope glycoproteins of patient isolates will determine differences in entry characteristics. During HIV-1 infections, envelope sequences undergo selection for neutralization-resistant variants by the host’s immune response (26, 27). Concomitant with this selection and the establishment of the long seropositive period of infection, viruses with slow/low growth characteristics emerge as the predominant isolates (1–5, 28, 29). This would suggest that selection of envelope sequences for antibody resistance may also select for slow entry. Such would be consistent with the coselection of neutralization resistance and poor replication in a series of molecularly characterized envelope genes from an experimentally infected chimpanzee (27).

In terms of host cell factors that might affect entry rates and efficiencies, differences in the permissiveness of the test cells did not correlate with differences in the surface densities of the CD4 receptor (6). However, differences in susceptibility to HIV-1-induced syncytium formation did correlate with rate and efficiency of virus entry. The entry-permissive C8166 cells are much more susceptible to syncytium formation than either mitogen-stimulated PBM cells or H9 cells.

The entry times given in Fig. 2 are distinctly longer than those that have been previously reported (24, 25). Our study
differs from prior studies on entry in that (i) we did not prebind virus and (ii) we followed the entry of infectious units (rather than physical particles) of virus.

**Entry Characteristics and Pathogenic Potential.** This study represents our first comparative study on the life cycles of slow/low and rapid/high patient isolates. Unexpectedly, the data demonstrate that a class 3 slow/low virus differs from two rapid/high viruses in entry characteristics (Fig. 2). The data reveal no differences in the time courses for reverse transcription and expression (Fig. 2). Data on the levels of expression per infected cell do reveal differences among the viruses. Moreover, these were cell type dependent and not a general correlate with the slow/low or rapid/high phenotype (Fig. 4).

The discovery of regulatory genes in HIV-1 raises high interest in the possibility that such would determine differences in the growth potentials and tissue tropism of patient isolates. Interestingly, our analyses on the growth of patient isolates in T cells and the work of others on tissue tropism have demonstrated viral-encoded entry, rather than viral-encoded regulatory phenomena, determining differences in pathogenic potential (Fig. 2; refs. 30–34). However, our studies, as well as those on tropism, have involved only a few patient isolates, with the isolates chosen for study representing those that could be grown in mitogen-stimulated PBM cells or monocyte/macrophages. Thus, these studies do not preclude a role for regulatory genes and proviral latency in pathogenic potential. Rather, they highlight the importance of the entry step of the HIV-1 life cycle in the determination of pathogenic potential.

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