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Relationships between Infectious Titer, Capsid Protein Levels, and Reverse Transcriptase Activities of Diverse Human Immunodeficiency Virus Type 1 Isolates

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Most studies on human immunodeficiency virus type 1 (HIV-1) replication kinetics or fitness must rely on a particular assay to initially standardize inocula from virus stocks. The most accurate measure of infectious HIV-1 titers involves a limiting dilution-infection assay and a calculation of the dose required for 50% infectivity of susceptible cells in tissue culture ($TCID_{50}$). Surrogate assays are now commonly used to measure the amount of p24 capsid, the endogenous reverse transcriptase (RT) activity, or the amount of viral genomic RNA in virus particles. However, a direct comparison of these surrogate assays and actual infectious HIV-1 titers from $TCID_{50}$ assays has not been performed with even the most conserved laboratory strains, let alone the highly divergent primary HIV-1 isolates of different subtypes. This study indicates that endogenous RT activity, not p24 content or viral RNA load, is the best surrogate measure of infectious HIV-1 titer in both cell-free supernatants and viruses purified on sucrose cushions. Sequence variation between HIV-1 subtypes did not appear to affect the function or activity of the RT enzyme in this endogenous assay but did affect the detection of p24 capsid by both enzyme immunoassays and Western blots. Clear groupings of non-syncytium-inducing (NSI), CCR5-tropic (R5), and SI/CXCR4-tropic (X4) HIV-1 isolates were observed when we compared the slopes derived from correlations of RT activity with infectious titers. Finally, the replication efficiency or fitness of both the NSI/R5 and SI/X4 HIV-1 isolates was not linked to the titers of the virus stocks.

Human immunodeficiency virus type 1 (HIV-1) is a complex lentivirus comprised of an envelope studded with approximately 400 transmembrane glycoproteins (gp120 and gp41) forming trimers surrounding a nucleoprotein core (53). During virus assembly, approximately 2,000 Gag molecules along with 50 to 100 Gag-Pol precursor proteins ($p55^{gag}$ and $p160^{gag\text{-}pol}$) form homodimers and then capture and encapsidate two copies of the viral RNA genome and about 20 copies of host $tRNA^{Lys}$ isoacceptors ($tRNA^{Lys}_{1,2}$ or $tRNA^{Lys}_{3}$) (5, 6, 11, 15, 19, 37, 53). This nucleoprotein complex associates with the inner plasma membrane prior to viral budding through a succession of interdependent steps (9, 45, 46, 48).

The disruption or inhibition of almost any step during replication can block or reduce the successful production of infectious virus particles (or virions). The high frequency of mutations in the HIV-1 genome introduced during reverse transcription can set the replication cycle astray and contribute to the production of defective or dead virus particles. It is estimated that approximately 1% of virus particles in an HIV-1-infected individual are actually infectious (13). Of this 1%, most HIV-1 clones in the infectious virus population will vary in fitness due to variations in the coding sequence (1 to 10 nucleotide [nt] substitutions per genome [36]) or an inefficient assembly process. Both host and viral factors will affect the HIV-1 mutation frequency and the assembly process and will lead to varying proportions of infectious virions to total virus particles (4, 28, 51).

Comparisons of biological properties from different HIV-1 isolates or mutants typically measure replication kinetics or fitness during infection of a susceptible cell type (38). An accurate standardization of virus inocula is required to detect a difference in replication efficiencies between mutant and wild-type strains. It is widely accepted that an actual measure of infectious titer is the best method to determine the quantity of HIV-1 (11). Unfortunately, the turnover rate and the lytic potential of HIV-1 are not sufficient to allow the measurement of titers via PFU counting on a monolayer of susceptible cells (16, 23). Instead, many studies (3, 8, 10, 22, 29, 32–34, 39, 40, 43, 55) employ a technique developed by Reed and Muench in 1938 (41). Susceptible cells are exposed to a serially diluted virus for several days. The dose required for 50% infectivity ($TCID_{50}$) is determined by the last virus dilution that is still capable of infecting the cells. This assay is typically cumbersome, time-consuming, and somewhat variable depending on the cell type employed and the assay used to detect virus production. More rapid techniques have been adopted to equalize virus inocula for phenotypic studies, namely p24 antigen capture assays-enzyme immunoassays (EIAs) (52) and reverse transcriptase (RT) assays (1, 12, 50). EIAs measure the amount of HIV-1 p24 capsid (CA) or $p55^{gag}$ precursor proteins released into the supernatant. In contrast, the RT assay is an indirect measure of virus titer that involves the use of the endogenous viral RT enzyme to extend an exogenous, ho-

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TABLE 1. Comparison of infectious titers of HIV-1 stocks with endogenous RT activities and p24 concn of antigen concentrations

Virus sample	Subtype	$TCID_{50}$ (log_{10} IU/ml)	Relative $TCID_{50}$ value[a]	Mean RT activity (10^3 cpm/ml)	Relative RT/ relative $TCID_{50}$[b]	Mean of HIV-1 p24 level (ng/ml)	Relative p24/relative $TCID_{50}$[b]
NSI/R5 Isolates							
B-BEK33	B	1.75	1	2,000 ± 79.8	1.00	666 ± 18.0	1
B-BEU0	B	12.5	5.62	22,600 ± 2,650	2.01	471 ± 592	1.26
B-BEU11	B	2.5	5.62	10,400 ± 1,870	0.926	5,260 ± 460	1.40
B-BEU24	B	2.25	3.162	5,770 ± 951	0.912	2,920 ± 141	1.39
B-BEU49	B	2.5	5.62	7,840 ± 502	0.698	2,510 ± 1,030	0.670
B-BEP48	B	1.75	1	2,420 ± 390	1.21	292 ± 50.3	0.438
B-BEP72	B	1.75	1	1,720 ± 515	0.858	117 ± 0.822	0.176
B-BEQ0	B	3.75	100	23,400 ± 1,800	0.117	7,290 ± 1,600	0.109
B-BET0	B	2.5	5.62	9,220 ± 1,880	0.820	7,260 ± 5.910	1.94
B-BET21	B	2.5	5.62	3,460 ± 36.9	0.307	4,090 ± 40.60	1.09
B-BET38	B	2	1.78	2,740 ± 171	0.770	25,600 ± 32,600	21.6
B-BET46	B	2.5	5.62	7,860 ± 569	0.699	4,820 ± 1,250	1.29
B-BET49	B	2.25	3.16	2,390 ± 252	0.377	1,910 ± 733	0.905
B2-92BR017	B	3.5	56.2	14,500 ± 1,140	0.129	4,010 ± 944	0.107
A1-92RW009	A	3.5	56.2	ND[c]	ND	15,300 ± 11,600	1.29
C3-97ZA012	C	4.5	562	490,000 ± 78,200	0.435	1,820 ± 131	0.00486
C5-97ZA003	C	4.5	562	549,000 ± 12,000	0.488	1,050 ± 108	0.00280
C8-96USNG58	C	4.5	562	924,000 ± 177,000	0.823	4,710 ± 329	0.0126
Mean					0.704 ± 0.462		1.93 ± 4.95
SI/X4 isolates							
E6-CMU06	E	2.75	1	2,490 ± 325	1.00	3,460 ± 477	1
D1-92UG021	D	2.75	1	2,470 ± 45	0.993	172 ± 13.9	0.0498
C20-ZMTCDD08	C	4.35	40	5,170 ± 108	0.0519	3,450 ± 164	0.0250
D4-93UG067	D	2.5	0.56	3,180 ± 135	2.28	12,000 ± 4,980	6.18
E7-CMU02	E	2.7	0.892	3,550 ± 318	1.60	2,000 ± 452	0.649
A8-92UG029	A	2.75	1	1,440 ± 175	0.580	19,880 ± 1,640	5.75
Mean					1.08 ± 0.779		2.28 ± 2.88

[a] Relative $TCID_{50}$ (set to 1) to that of the first isolate listed for a biotype.
[b] Relative RT or p24 level (set to 1) to that of the first isolate listed for the biotype divided by the relative $TCID_{50}$, value.
[c] ND, not determined.

mopolymeric DNA primer on an RNA template. In recent literature, approximately 50% of studies comparing the replication efficiencies of different HIV-1 isolates or mutants measured virus titers by $TCID_{50}$ assays (10, 22, 32, 33, 55), 45% used p24 antigen capture assays (14, 21, 26, 44, 49), and <5% used RT assays (17, 27, 54). As outlined below, most reports measuring relative in vitro HIV-1 fitness levels by using sensitive competitive replication assays have employed the $TCID_{50}$ assay to obtain an accurate measure of virus titers to equalize inocula (3, 8, 29, 34, 39, 40, 43).

Adding to the complexity of measuring accurate virus titers is the increasing use of genetically diverse HIV-1 isolates for in vitro studies. It is quite likely that surrogates for the infectious titer may be influenced by the 15 to 30% diversity in nucleotide sequence between HIV-1 isolates from different group M subtypes (A through J) and intersubtype recombinants (24, 35). In addition, differences in biological phenotype (non-syncytium-inducing [NSI] versus SI) or coreceptor usage (CCR5-tropic versus CXCR4-tropic) may further affect virus titers measured on different primary cells and tumor cell lines (2, 16, 23).

This study was designed to compare different surrogate assays (measuring RT activity, p24 content, and viral RNA load) as a measure of infectious titers of highly divergent HIV-1 isolates. We also determined if the stock titers of these isolates were related to their relative replication efficiencies or fitness. Infectious titers ($TCID_{50}$ values) were measured in peripheral blood mononuclear cell (PBMC) cultures for 44 primary HIV-1 isolates from different subtypes (A, B, C, D, CRF01, and group O) and biotypes, i.e., 24 NSI/R5, 17 SI/X4, and 3 dual-tropic isolates. RT activity, not p24 content or viral RNA load, appeared to be the best correlate of infectious titer. The poor relationship between the p24 content and infectious titer was observed in both p24 EIA and anti-p24 Western blot analyses. Interestingly, the ratios of relative RT activity to infectious titer appeared to group according to virus tropism (i.e., R5, X4, or R5-X4) but did not appear to be related to the overall diversity in the RT or *env* coding regions between the strains. Finally, the prolonged propagation of HIV-1 isolates revealed that even though RT activity can peak and then diminish over time, p24 levels will peak and then plateau.

MATERIALS AND METHODS

Cells and viruses. Healthy human PBMC were purified from HIV-1-seronegative donors by Ficoll-Paque gradient centrifugation. Purified PBMC were resuspended in RPMI 1640 medium (Mediatech, Inc.) supplemented with 10% fetal bovine serum (Mediatech, Inc.), 100 IU (100 μg) of penicillin-streptomycin/ml, 1 U of phytohemagglutinin/ml, and 1 ng of recombinant interleukin-2 (Invitrogen). The cells were suspended at 2×10^6 cells/ml and cultured for 3 days at 37°C and 5% CO_2 before infection. The viruses listed in Table 1 and in the figures were obtained from the NIH AIDS Research and Reference Program, Division of AIDS, NIAID, NIH, and from HIV-infected cohorts in Antwerp, Belgium (kindly provided by G. Vanham), in Kampala, Uganda, and in Harare, Zimbabwe (kindly provided by D. Katzenstein). The viruses were propagated on stimulated PBMC, harvested, and clarified at peak viremia as measured by a radioactive RT assay. $TCID_{50}$ values were determined by serially diluting supernatants of each stock of virus and performing quadruplicate infections of stimulated PBMC. The $TCID_{50}$ assay end point was determined on day 12 by a radioactive RT assay and was calculated by the method of Reed and Muench (41) as described previously (3, 39).

Reverse transcriptase assay. Reverse transcriptase activity was determined in cell-free supernatants by a radioactive reverse transcriptase assay, as described previously (12, 50). Briefly, clarified supernatant samples were diluted in triplicate (1:4 to 1:1,024 for the values shown in Fig. 2 and 1:4, 1:64, and 1:1,024 for

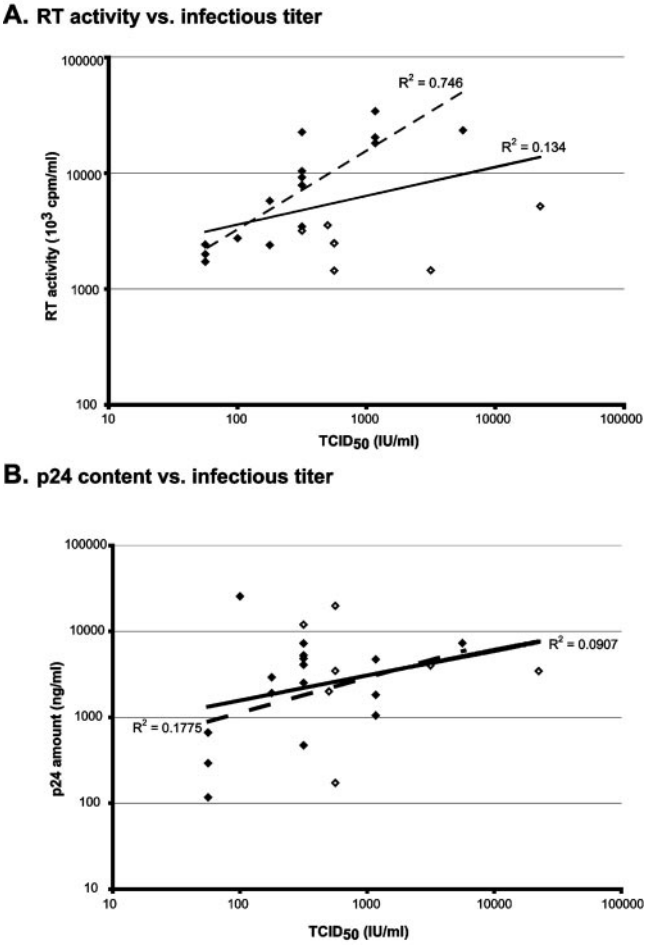


FIG. 1. Relationship of p24 capsid content and RT activity with infectious HIV-1 titer. The mean RT activities (cpm/ml) (A) and p24 capsid contents (ng/ml) (B) derived from dilutions of 24 primary HIV-1 virus stocks were plotted against the $TCID_{50}$ values (infectious units/ml). The counts per minute were adjusted for degradation of the ^{32}P isotope. One count per minute is equivalent to the incorporation of 0.000152 pmol of [α-^{32}P]TMP by RT. The open diamonds on each panel represent values for SI/X4 strains, whereas the closed diamonds represent values for NSI/R5 strains. The solid and dotted linear regression lines are for all isolates and for all isolates but with the SI/X4 isolates removed, respectively.

all other figures and tables), and 10 μl was added to 25 μl of RT master mix [50 mM Tris-HCl (pH 7.8), 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 5 μg of poly(rA)-poly(dT) per ml, 0.5% (vol/vol) NP-40, 1 μl of fresh 10-mCi/ml [α-^{32}P]TTP per ml]. The reactions were incubated at 37°C for 2 h, and then 10-μl samples were blotted onto a DEAE filter mat (Wallac). The filters were dried, washed five times with SSC (0.15 M NaCl, 0.015 M sodium citrate), rinsed two times with 80% ethanol, and dried. The radioactivity of the samples was quantified with a Packard Matrix 96 direct beta counter. It is important to note that RT assays were performed on large sets of diluted viruses with the same [α-^{32}P]TTP stock. In cases in which a different [α-^{32}P]TTP stock was employed, RT activity values were corrected for radioisotope degradation and were still plotted in counts per minute per milliliter. The actual concentration of the 3,000-Ci/mmol [α-^{32}P]TTP stock (10 mCi/ml) obtained from Perkin-Elmer was approximately 3.3 μM. One count per minute (or disintegration per minute) is equivalent to the incorporation of 0.000152 pmol of [α-^{32}P]TMP by RT.

Capsid p24 antigen capture assay-EIA. Capsid (CA) p24 antigen capture assays or enzyme immunoassays were performed according to the manufacturer's protocol (Beckman-Coulter). Briefly, clarified cell-free supernatant samples were diluted 1:5, 1:250, and 1:12,500 in duplicate, and 200 μl of each dilution was added to the assay plate. Dilutions of the p24 kinetic calibration standard (1,000, 500, 250, 125, 62.5, 31.25, and 15.63 pg/ml) were also added to the plates to quantify the amount of p24 in the virus stocks. Only optical densities at 450 and 570 nm in the linear range of 0.110 to 2.20 were used to calculate concentrations based on the kinetic calibration standard.

Western blot analyses. Twenty-five microliters of each virus stock was lysed in 225 μl of sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.04 M Tris, pH 6.8) and then heated to 95°C for 5 min. Samples and protein molecular weight standards (Fermentas) were separated in Tris-HCl–12.5% polyacrylamide precast gels (Bio-Rad) and then transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore) by electroblotting (Bio-Rad). The membranes were incubated with blocking reagent (5% milk–0.05% Tween in phosphate-buffered saline) for 1 h at room temperature and then hybridized with a mouse anti-p24 monoclonal antibody (diluted 1:1,000; Fitzgerald Industries International, Inc.) overnight at 4°C. After being washed, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse antiserum (diluted 1:40,000; Pierce) for 3 h. Immune complexes were visualized by use of the ECL system (Amersham) according to the manufacturer's instructions. Films from ECL immunoblots were analyzed with Kodak 1D software.

Viral load quantification assay. Cell-free supernatant samples (i.e., virus stocks) were diluted 1:100, 1:1,000 and 1:10,000 in RPMI medium. HIV-1 RNA loads were measured by use of a Roche Amplicor HIV-1 Monitor, version 1.5, kit. Briefly, the virus contained in the dilutions was lysed and the viral RNA was precipitated according to the manufacturer's protocol. The viral RNA was resuspended in a solution containing an RNA quantitation standard. This mixture was then subjected to reverse transcription and PCR amplification procedures. The products from these reactions were then serially diluted fivefold in microwell detection plates along with low and high positive controls. Undiluted standard and a 1:5 dilution of the quantitation standard for each sample were detected in separate wells of the plates. The concentrations of viral RNA in the virus stock samples were determined from the PCR products detected on this plate and through the use of algorithms provided by Roche.

Determination of relative HIV-1 fitness. The relative fitness or replication efficiencies of 18 primary HIV-1 isolates were measured via a competition with two reference HIV-1 isolates. NSI/R5 HIV-1 isolates were competed against NSI/R5 isolates A1-92RW009 and B2-92BR017, whereas the SI/X4 isolates were competed against isolates E6-CMU06 and A8-92UG029 (40). All dual infections were performed with PBMC from one donor in 24-well plates, using equal amounts of the control virus and the patient isolate (multiplicity of infection [MOI], 0.005, or 0.005 IU per cell). Virus mixtures were incubated with 10^6 PBMC for 2 h at 37°C in 5% CO_2. The cells were also washed three times with 1× phosphate-buffered saline and resuspended in complete medium (10^6/ml). The culture supernatants were removed every 5 days to monitor virus production by measuring the RT activity. Fresh phytohemagglutinin (PHA)- and interleukin-2 (IL-2)-treated PBMC from the same donor were added at 10 days to replenish the cells in the cultures. Supernatants and two aliquots of cells were harvested at day 15, resuspended in complete medium containing 10% dimethyl sulfoxide, and then stored at −80°C for subsequent analysis.

Proviral DNAs were extracted from lysed PBMC by use of a blood purification kit (Qiagen). As described previously (3, 40), the *env* C2-V3 region was PCR amplified and then employed in heteroduplex tracking assays with a radiolabeled probe of the same *env* region. Briefly, heteroduplex tracking assay reaction mixtures containing DNA annealing buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 2 mM EDTA), 10 μl of unlabeled PCR-amplified DNA from the competition culture, and approximately 0.1 pmol of radioactive probe DNA were denatured at 95°C for 3 min and then rapidly annealed on wet ice. After 30 min on ice, the DNA heteroduplexes were resolved in Tris-borate-EDTA–5% nondenaturing polyacrylamide gels (30:0.8 acrylamide-bisacrylamide) for 2.5 h at 200 V. The gels were dried, exposed to X-ray film (Eastman Kodak Co.), and analyzed with a Bio-Rad FX Pro MultiImager instrument and ImageQuant software (Bio-Rad).

The method for calculating relative fitness has been described previously (3, 40). The production of individual HIV-1 isolates in a dual infection (f_0) was divided by its initial proportion in the inoculum (i_0) to obtain a relative fitness value ($ws = f_0/i_0$), i.e., a fitness value for a particular isolate relative to a reference strain, not an absolute fitness value.

RESULTS

Estimating HIV-1 titer by various assays. Twenty-four primary HIV-1 isolates were propagated on PHA- and IL-2-

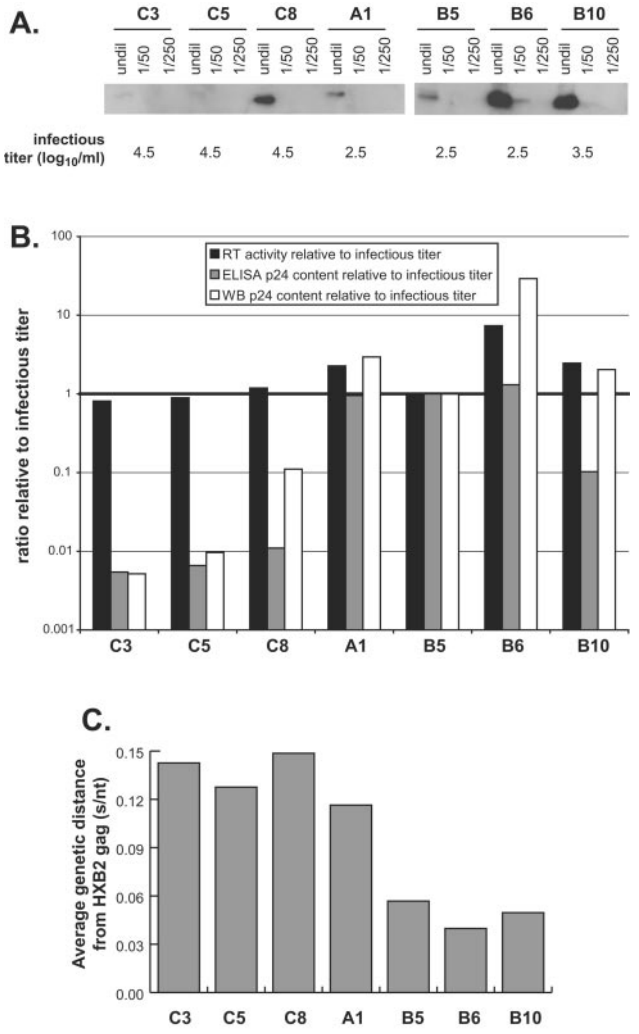


FIG. 2. Anti-p24 Western blot analyses of diverse HIV-1 stocks. Undiluted stocks and 1:50 and 1:250 dilutions of C3, C5, C8, A1, B5, B6, and B10 virus stocks were lysed and run in SDS-polyacrylamide gels, transferred to nylon membranes, and then probed with an anti-p24 mouse monoclonal antibody. Specific interactions with the p24 capsid were visualized and quantified after probing with a goat anti-mouse immunoglobulin G antibody and ECL development of the film (see Materials and Methods). Panel A shows the results of these blots, whereas panel B plots the amounts of undiluted bands on the Western blot, the RT activities, and p24 antigen levels from EIA, all relative to $TCID_{50}$ values. All ratios were plotted relative to the values obtained with the B5 HIV-1 stock (set to 1). Panel C compares the genetic distances between HXB2 and the HIV-1 isolates used in panels A and B. The p24 coding region of the *gag* gene was previously sequenced (3). The genetic distances (substitutions per nucleotide) were calculated by using the DNADIST program of the PHYLIP package.

treated PBMC (Table 1). Cell-free medium was harvested from these HIV-infected cultures at the peak of HIV-1 RT activity ($>10^5$ cpm/ml), typically 2 weeks after the initial infection. The supernatant was clarified, but the viruses were not purified for these initial comparisons. $TCID_{50}$ values ($\log_{10}$ IU/ml; Table 1) were calculated by using the standard Reed-Munch technique following the exposure of PBMC to 1/10 serially diluted HIV-1 isolates. Radioactive RT and p24 antigen capture assays were also performed on diluted virus-containing supernatant samples (see Materials and Methods). All p24 values (in nanograms per milliliter) were calculated based on a kinetic standard and then were adjusted for the dilution factor (Table 1). Duplicate p24 assays or triplicate RT assays were performed on separate dilution series. Each RT activity value presented in Table 1 and Fig. 1 represents the mean of values derived from at least two dilutions in the linear range (1:4, 1:64, and 1:1,024) (mean of six measurements), whereas p24 values were also calculated from the means of two dilutions (1:5, 1:250, or 1:12,500) (means of four measurements). Standard deviations were obtained for the replicates of each assay after adjustments for the dilution factor.

Upon first inspection, it appeared that the variation between RT assay values with the same virus samples was much less than the variation observed between p24 values (Table 1). The majority of the RT assay values (21 of 24) had standard deviations of <15% of the mean (percent coefficient of variance [% CV]). The standard deviations were larger for the p24 values (as high as 100% CV), and the p24 values of only 14 of 24 primary HIV-1 isolates had <15% CV (Table 1). Although p24 values were only determined in duplicate for at least two dilutions, the difference between the most disparate RT values was typically less than the difference between duplicate p24 values. A high variation (>15% CV) between replicate EIA assays performed with the HIV-1 p24 antigen has been reported by the manufacturer, Beckman-Coulter. Finally, the sensitivity range of the radioactive RT assay (5 to 3,000 cpm/μl; ~3 $\log_{10}$) in a single assay was significantly higher than that of the p24 antigen capture EIA (approximately 0.1 to 1.7 optical density values or 1.3 to 50 pg/μl; <1.6 $\log_{10}$). Repeat p24 EIAs performed on the same viral stocks sometimes resulted in lower or higher standard deviations from that reported in Table 1. However, the standard deviation of RT activity typically remained <15% CV.

Next, the p24 content and RT activity in various virus stocks were compared to the $TCID_{50}$ value. Ratios of the relative p24 content or relative RT activity to relative $TCID_{50}$ values should equal 1 if the infectious titer is considered the "gold standard" (Table 1). Several of these ratios were close to 1, but RT activity (mean RT/$TCID_{50}$ = 0.780 ± 0.456) appeared to provide a better approximation of virus titer than did the p24 content (mean p24/$TCID_{50}$ = 1.93 ± 4.95) (Table 1). The p24 values and RT activities in a virus stock were plotted against each other or against the $TCID_{50}$ values (Fig. 1). When all of the isolates shown in Table 1 were analyzed, there were no significant correlations between $TCID_{50}$ values and the mean RT activity or p24 level ($P > 0.05$, Pearson product moment correlation). A very weak coefficient for determination (R^2 = 0.1) was observed between p24 amounts and RT activities for these virus stocks (data not shown). This trend was not statistically significant but was consistent with an earlier report (31). The R^2 and slope derived from a plot of RT activity versus infectious titer increased when the SI/X4 isolates were removed from the analyses (increase in R^2 from 0.134 to 0.746). In addition, a significant correlation (r = 0.551; $P < 0.02$, Pearson product moment correlation) was now apparent between mean RT activities and $TCID_{50}$ values of the NSI/R5 virus stocks (Fig. 1A), whereas the p24 content still appeared unrelated to the $TCID_{50}$ values (R^2 = 0.091; $r = -0.0476$; P =

0.829, Pearson product moment correlation) (Fig. 1B), even when the SI/X4 isolates were removed. As described below, the correlation between RT activity and infectious titer for SI/X4 strains may still be significant, but it is different from the correlation observed for NSI/R5 HIV-1 isolates (i.e., lower slope values were derived from a regression analysis of RT activities and infectious SI/X4 HIV-1 titers).

Michael et al. (31) performed a similar analysis to that described for Fig. 1. Analogous to our initial findings and without a separation based on coreceptor usage or phenotype, they did not observe significant correlations when they compared infectious titers with RT activities, p24 contents, or viral RNA loads. To determine if differences in coreceptor usage or phenotype affected their analyses, we downloaded and analyzed 24 available envelope sequences of the 30 HIV-1 isolates used in their study. Based on a positive charged amino acid at position 306 or 322 (HXB2 sequence), we predicted that 6 of 24 sequences were SI/X4 HIV-1 isolates. NSI/R5 versus SI/X4 predictions based solely on the charge at position 306 or 322 are about 90% correct (20). Plots of the RT activities and infectious titers from only the predicted NSI/R5 isolates now revealed a strong correlation between these two parameters ($R^2 = 0.5429$; $r = 0.737$; $P < 0.0005$, Pearson product moment correlation). In contrast, analyses of the SI/X4 isolates did not result in a significant relationship between RT activities and infectious titers, but this lack of correlation could be attributable to the availability of fewer viruses for analysis, a similar problem encountered in our analyses. Finally, a comparison of the p24 contents and infectious titers of all of the isolates together or the NSI/R5 or SI/X4 isolates separately did not result in significant correlations. The best trend was between the p24 contents and infectious titers of NSI/R5 isolates, but it did not reach significance and was similar to our observations shown in Fig. 1 ($R^2 = 0.0638$; $r = 0.253$, $P = 0.312$, Pearson product moment correlation). These data and analyses will be provided upon request. Finally, it should be noted that even though Michael et al. performed [^{3}H]TTP-based RT assays, whereas we utilized a [^{32}P]TTP-based RT assay, the correlations with infectious titers were nearly identical and very convincing.

The poor correlation between p24 antigen content and infectious titer may be related to amino acid sequence variations between the isolates. For example, the nucleotide sequence diversity between the *gag* and RT genes of subtypes C and B is typically >10%. The ratio of p24 to infectious titer was <0.01 for three of the four subtype C isolates (C3, C5, and C8; Table 1), whereas the RT/TCID$_{50}$ ratio for these same isolates ranged from 0.4 to 0.8 (with the optimal value being 1) (Table 1). In general, the relationship between p24 and infectious titer appears to be weaker for non-subtype B isolates than for subtype B isolates (Table 1). In contrast, sequence variation in the RTs of different HIV-1 subtypes appeared to have minimal impact on relative enzyme activities. This was not surprising considering that previous studies have shown that even recombinant, purified HIV-2, HIV-1 group O, and group M, subtype B RTs had the same relative activity and shared identical functional properties, even though their primary amino acid sequences differed by 20 to 35% (18, 30, 42). Thus, it appears that the breadth of cross-clade binding by the anti-p24 antibody did not compare to the conserved cross-clade function of the RT enzyme.

The enzyme-linked immunosorbent assay-based p24 antigen capture assay quantifies the presence of the HIV-1 capsid protein through interactions with an anti-p24 monoclonal antibody coating each well of the plate. Serum and other cellular proteins could result in some nonspecific binding that may impact the accurate measurement of p24 content. A Western blot with anti-p24 antibodies should reduce any nonspecific binding and permit a simple identification and quantification of the correct protein. Undiluted stocks and 1/50 and 1/250 dilutions of seven NSI/R5 HIV-1 stocks were run in an SDS-polyacrylamide gel, transferred to nylon membranes, and probed with a primary mouse anti-p24 antibody and then with a goat anti-mouse antibody. In addition, HIV-1 p24 antigen capture assays and RT assays were performed on the same diluted stocks, and samples were added to SDS lysis buffer and utilized in the Western blot analyses. As shown in Fig. 2A, each virus isolate, regardless of its subtype, had an HIV-1 p24 capsid protein that migrated to roughly the same position on the gel. However, the quantitation of each p24 band on the Western blot revealed similar findings to those obtained with the p24 antigen capture assay, as shown in Table 1. Although the subtype C isolates had higher titers than the subtype B isolates, the amount of p24 capsid detected on the Western blot was orders of magnitude less, as shown in Fig. 2B. In contrast, the ratio of RT activity to infectious titer did not appear to differ among the subtype B or C isolates.

These findings suggest that the anti-p24 antibody binds more efficiently to the subtype B than the subtype C isolates. Unfortunately, the epitope in the HIV-1 p24 protein has not been mapped for this mouse monoclonal anti-p24 antibody (10-H30; clone M5101024). However, the p24 protein for mouse immunization (and its monoclonal antibody) was derived from the subtype B laboratory strain IIIB. Genetic distance analyses of the p24 coding region of the *gag* gene revealed that the subtype C isolates do not cluster with the subtype B isolates and only share about 85% nucleotide sequence identity with the HXB2 isolate (Fig. 2C). In contrast, the sequence identity between subtype B *gag* genes (or intrasubtype C) is over 95% (Fig. 2C). These analyses suggest that this inter- and possibly intrasubtype sequence diversity reduces the binding affinity of anti-p24 monoclonal antibodies derived from HXB2 p24 immunization of mice. In fact, there actually appears to be some correlation between the genetic distance of each isolate from the HXB2 sequence and the ability of the anti-p24 antibody to detect the Gag protein of infectious virus particles (compare Fig. 2B and C).

Finally, there was also a possibility that free p24 or Gag protein released from lysed cells or virus particles inflated the estimates of infectious titers based on the p24 antigen capture assay. The analyses for Table 1 and Fig. 1 were repeated with viruses pelleted from supernatants and then purified on a sucrose cushion (see Materials and Methods). The infectious titers of both unpurified viruses in cell-free supernatants and sucrose-cushion-purified viruses were compared to RT activities, viral RNA loads (obtained with the Roche Amplicor 1.5 assay), and p24 contents (Table 2). Sufficient material from the purified A1 and B2 viruses was not available for TCID$_{50}$ measurement, thereby limiting our interpretation of these analyses.

TABLE 2. Comparison of infectious titers of purified HIV-1 stocks with endogenous RT activities, p24 antigen contents, and viral RNA loads[a]

Virus	Subtype	Coreceptor usage/phenotype	$TCID_{50}$ value (log IU/ml)	RT activity (log cpm/ml)	$RT/TCID_{50}$	p24 content (log ng/ml)	$p24/TCID_{50}$	viral RNA load (log copies/ml)	$RNA/TCID_{50}$
Virus in cell-free supernatant									
A1	A	CCR5/NSI	3.5	6,160	1.95	5,500	1.74	7.38	7,590
B2	B	CCR5/NSI	3.5	8,710	2.75	398	0.126	9.69	1,550,000
A8	A	CXCR4/SI	3.25	4,170	2.34	5,130	2.88	8.81	363,000
E6	E	CXCR4/SI	3.5	7,410	2.34	7,240	2.29	9.22	525,000
Mean					2.35 ± 0.327		1.78 ± 1.18		$6.1 \times 10^5 \pm 6.6 \times 10^5$
Sucrose cushion-purified virus									
A1	A	CCR5/NSI	ND	10,200	ND	4,790	ND	9.48	ND
B2	B	CCR5/NSI	ND	692	ND	2,240	ND	6.66	ND
A3	A	CXCR4/SI	2.45	363	1.29	178	0.630	7.93	302,000
E6	E	CXCR4/SI	3.41	2,690	1.10	21,900	8.12	9.33	832,000
Mean					1.20 ± 0.134		4.38 ± 5.30		$5.7 \times 10^5 \pm 3.8 \times 10^5$

[a] ND, not done.

Regardless of whether the viruses were purified, RT activity was still a stronger surrogate of the infectious titer than the p24 content and viral RNA load (Table 2). It is important to note that all values in Tables 1 and 2 were obtained from separate assay runs.

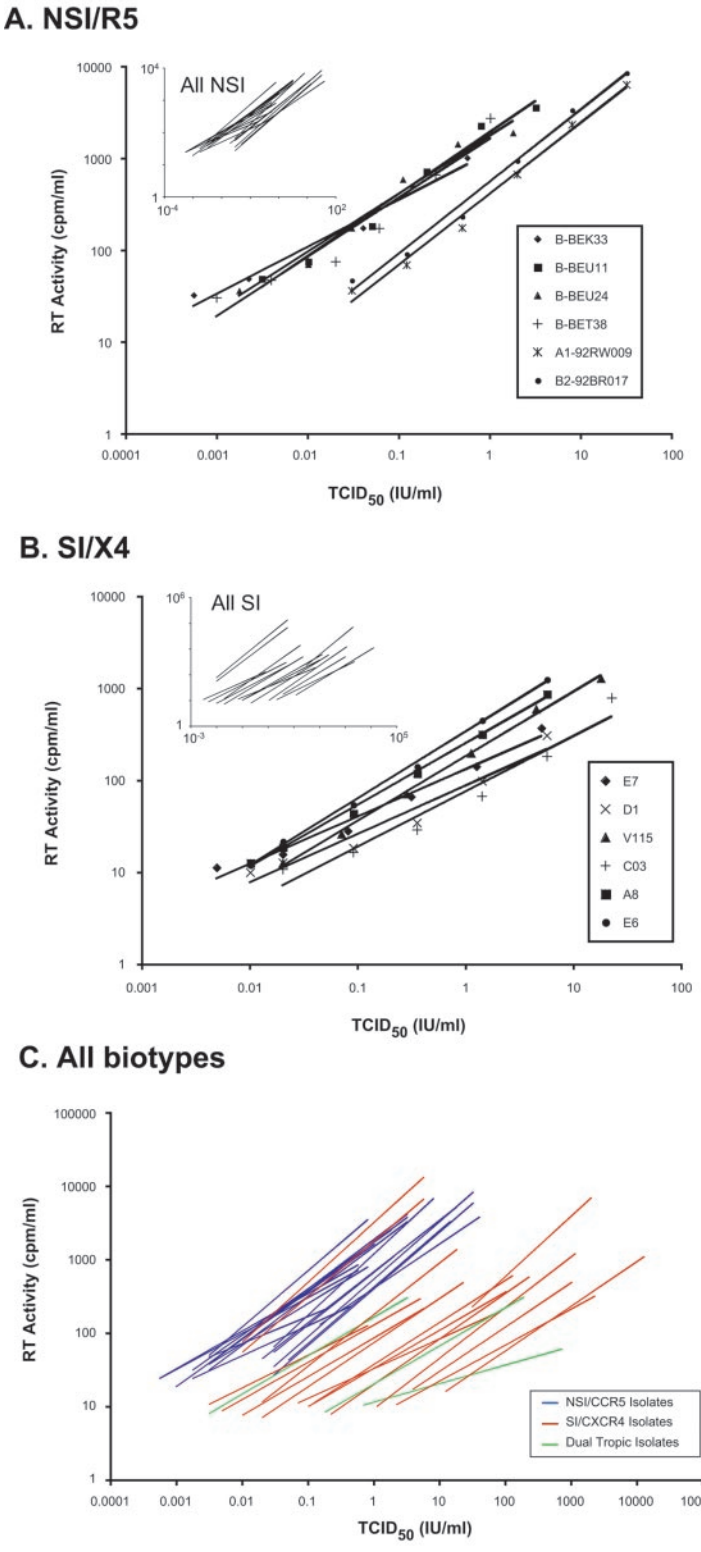
Comparing RT activities and infectious titers of HIV-1 stocks. As described above, it appears that RT activity may be a better predictor of the infectious HIV-1 titer than either the viral RNA level or p24 content. To further study the utility of the RT assay as a virtual $TCID_{50}$ measurement, we calculated the actual $TCID_{50}$ value, or infectious titer, for a total of 44 primary HIV-1 isolates of different subtypes (Fig. 3). Samples of virus stocks were then serially diluted (1/4 dilutions) in triplicate and subjected to a radioactive RT assay. The mean RT value for each dilution (<15% CV) was then plotted against the known $TCID_{50}$ values (Fig. 3A to C). Only linear regressions are shown in Fig. 3A to C, but a strong correlation was observed ($r > 0.85$) for nearly every data set.

The separation of the viruses based on phenotype and tropism (NSI/R5 [Fig. 3A] and SI/X4 [Fig. 3B]) revealed similar slopes of the linear regressions within groups. The median NSI/R5 and SI/X4 slope values derived from RT activities versus infectious titers (horizontal line), 25 and 75% confidence intervals, and outlier slopes are shown in Fig. 4. A student *t* test indicated that the slope values for the NSI/R5 and SI/X4 HIV-1 isolates were statistically different ($P < 0.001$). However, it is important to note that there were several outlying slope values within both the NSI/R5 and SI/X4 virus groups. In general, the slopes of the NSI/R5 isolates (RT activity versus infectious titer) clustered in a tighter group. A linear regression analysis that suggested a stronger correlation between RT activity and infectious titer was also observed when the SI/X4 viruses were removed from the data used for Fig. 1. These findings suggest that endogenous RT activity in an NSI/R5 HIV-1 stock is a strong predictor of the infectious HIV-1 titer. The RT activity in an SI/X4 HIV-1 stock is a weaker surrogate of the infectious titer, but it is still more significant than either the p24 antigen content or the viral RNA load.

Possible relationship between relative replication efficiency (fitness), infectious titer, and genetic diversity. Previous reports suggested that the relative replication efficiency, or fitness, of HIV-1 is not related to the infectious titer (3, 39). Although slower propagations of HIV-1 isolates from PBMC of HIV-infected patients often result in lower stock titers, these low titers appear more related to the relative viral load in that patient and are not necessarily due to the replicative capacity of the infecting isolate (3). Monoinfections are commonly employed to measure the replication kinetics of HIV-1 variants, but this technique is less sensitive for discerning differences in replication efficiencies than HIV-1 competitions (38). As previously described, wild-type HIV-1 (or all of the HIV-1 isolates utilized in this study) isolates replicate with equal efficiencies in monoinfections. However, it is quite common to find one primary HIV-1 isolate failing to replicate when it is competed against another primary HIV-1 isolate.

For this study, we derived relative fitness values for each NSI/R5 and SI/X4 HIV-1 isolate by competing these isolates against two reference strains of the same biotype (i.e., NSI/R5 isolates A1-92RW009 and B2-92BR017 and SI/X4 isolates E6-CMU06 and A8-92UG029) (Fig. 5A and B, respectively). Although each stock contained different infectious titers, the same amount of virus was used in each competition (0.0005 MOI or IU/cell). Significant differences in the relative fitness values for each HIV-1 isolate did not correspond with the original titer of each virus (Fig. 5). For example, SI/X4 C22 had a relatively high stock titer of $10^{6.1}$ IU/ml (or an RT activity of $10^{5.8}$ cpm/ml), but it could not compete against A8 or E6. In contrast, isolate D4 had one of the highest relative fitness values ($W = 3.0$) but the lowest titer of any SI/X4 isolate ($10^{3.5}$ IU/ml or $10^{4.5}$ cpm/ml). It is important to note that infectious titer in this study is the measure of the minimum dilution required for PBMC infection and is not a measure of replication kinetics. These results suggest that replication efficiency is not directly related to the infectious titer. Likewise, relative fitness (or competitive replication efficiency) was not related to a surrogate of infectious titer, i.e., RT activity. The relative RT activities contained in the virus inocula used in each competition were comparable to the infectious titers.

Monitoring HIV-1 infections by using RT activities and p24 contents. For the next set of experiments, four SI/X4 HIV-1 isolates (MOI of 0.001) were added to U87.CXCR4.CD4 (Fig.



D.

Virus	Country of Origin	Tropism	$TCID_{50}$ (Log 10)
A1*-92RW00902	Rwanda	NSI	3.5
A14-UG	Uganda	SI	3.25
A8-92UG029	Uganda	SI	2.75
B-BEK11	Belgium	NSI	2.5
B-BEK33	Belgium	NSI	1.75
B-BEK44	Belgium	NSI	2.5
B-BEK69	Belgium	SI	3.5
B-BEP48	Belgium	NSI	1.75
B-BEP72	Belgium	NSI	1.75
B-BEQ0	Belgium	NSI	3.75
B-BEQ13	Belgium	NSI	3.5
B-BEQ22	Belgium	NSI	3.25
B-BET0	Belgium	NSI	2.5
B-BET21	Belgium	NSI	2.5
B-BET38	Belgium	NSI	2
B-BET46	Belgium	NSI	2.5
B-BET49	Belgium	NSI	2.25
B-BEU0	Belgium	NSI	2.5
B-BEU11	Belgium	NSI	2.5
B-BEU24	Belgium	NSI	2.25
B-BEU49	Belgium	NSI	2.5
B2-92BR017	Brazil	NSI	3.5
B3-91US005	United States	NSI	3.5
B4-92US076	United States	SI	6.5
B6-91US714	United States	NSI	3.7
B9-92BR018	Brazil	NSI	3.7
C3-97ZA012	South Africa	NSI	4.2
C8-96USNG58	U.S./Nigeria	NSI	3.5
C18-ZMTCDD03	Zambia	SI	3.35
C19-ZMTCDD4	Zambia	Dual	4.85
C20-ZMTCDD08	Zambia	SI	4.35
C21-ZMTCDD13	Zambia	SI	5.05
C22-ZMTCDD22	Zambia	SI	6.1
C23-ZMTCDD23	Zambia	SI	5.35
C24-ZMTCDD25	Zambia	SI	5.6
C25-ZMTCDD28	Zambia	SI	4.1
C26-ZMTCDD29	Zambia	SI	3.85
C27-ZMTCDD30	Zambia	Dual	4.27
D1-92UG021	Uganda	SI	2.75
D4-93UG067	Uganda	SI	2.5
E6-CMU06	Cameroon	SI	2.75
E7-CMU02	Cameroon	SI	2.7
O12-BCF07	Cameroon	SI	4.0
O13-BCF11	Cameroon	Dual	2.5

*denotes subtype

FIG. 3. Comparing RT activities with infectious titers of 44 primary HIV-1 isolates. The plots in panels A and B show six representative regression lines of RT activity versus $TCID_{50}$ value for NSI/R5 and SI/X4 viruses (respectively) diluted 1:4 through 1:1,024. Inset graphs show all NSI/R5 and SI/X4 viruses examined in the same manner for panels A and B, respectively. All RT activity values and $TCID_{50}$ values of the diluted viruses are shown in panel C: NSI/R5 viruses are shown in blue, SI/X4 viruses are shown in red, and dual-tropic isolates are shown in green. A general description of each HIV-1 isolate used in these tests is outlined in panel D. The subtype of each isolate is provided by the first letter of each virus name.

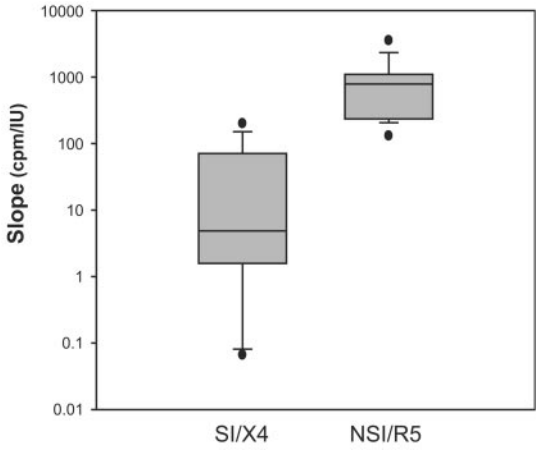


FIG. 4. Slopes derived from regression analyses of infectious titers versus RT activities. Slopes calculated from correlation lines for each isolate in Fig. 2 (RT activity versus $TCID_{50}$ value for each virus) are presented in this box-and-whisker plot. The median values (horizontal lines) and values falling within the 25 and 75% confidence intervals are enclosed by the gray boxes. Error bars display 95% confidence intervals. Outlier slope values are plotted separately for each viral biotype.

6B) and PHA- and IL-2-treated PBMC (Fig. 6C), whereas U87.CCR5.CD4 cells (Fig. 6A) were exposed to two NSI/R5 HIV-1 isolates. A small aliquot of cell-free supernatant (50 μl or 1/40 of the culture volume) was harvested every 2 days and analyzed for virus production by both the RT assay and the p24 antigen capture assay. It is important to note that all assays were performed in triplicate and that the CV was <10% for all values (error bars are not shown in Fig. 6). However, all p24 values were derived from triplicate analyses of three dilutions of supernatant (1:5, 1:250, and 1:12,500) in order to obtain absorption values in the range of the p24 kinetic standard. In contrast, all RT values were derived from assays performed in triplicate on undiluted culture supernatants. As mentioned above, the range in RT assay sensitivity was almost 3 $\log_{10}$.

Figure 5A provides an example of HIV-1 infections that did not reached peak viremia in culture. NSI/R5 HIV-1 isolates tended to replicate slower and have lower fitness levels than their SI/X4 counterparts (compare Fig. 6A and B) (3, 39). In this example, the p24 production mirrored the RT activity in the culture supernatant for both the subtype A (A1) and subtype B (B-BET49) viruses. Interestingly, at the low MOIs used, virus production from subtype C infections was difficult to monitor with the p24 assay (data not shown) (3), as predicted by the poor detection by the anti-p24 antibody (Table 1 and Fig. 2). These findings suggest that p24 levels as determined by EIA may provide good estimates of HIV-1 production but that diversity could affect the sensitivity of the assay. However, when we accounted for variations between enzyme-linked immunosorbent assays, the p24 content appeared to provide a good measure of total virus production in that it assessed infectious, noninfectious, and decaying virus particles. Dilution of the supernatant showed an accurate detection of p24 content when the assay was performed in the linear, sensitive range of the assay. However, as described in Table 1 and Fig. 1, the p24 content appears to show only a weak relationship to the amounts of infectious virus.

As shown in Fig. 6B, SI/X4 virus production in $CXCR4^+$ $CD4^+$ U87 cells, as measured by RT activity, peaked around day 7 and then rapidly decreased over the next 4 days. This peak was preceded by syncytium formation and followed by significant cell death at day 9. Cell death was nearly 70% at day 11 and 90% at day 13. This rapid cell destruction was due to the increased replication kinetics and cytopathogenicity of SI/X4 HIV-1 isolates compared to NSI/R5 HIV-1 strains in $CXCR4^+$ $CD4^+$ U87 cells. Thus, it was apparent that very few cells produced virus after day 9. Interestingly, the p24 antigen levels appeared to plateau between days 9 and 11, but they did not decrease. This result can be explained by the nature of both assays, i.e., the RT assay measures a specific enzyme function, which may be related to infectious titer, whereas the p24 EIA only requires the presence of an intact epitope in the capsid or $p55^{gag}$ protein. Nearly identical results were observed for PHA- and IL-2-treated PBMC infected with the same four SI/X4 HIV-1 isolates (Fig. 6C). Peak RT activities were slightly delayed (days 7 to 9) in PBMC and were not accompanied by the same level of cell death. This difference between the infected cultures of PBMC and $CXCR4^+$ $CD4^+$ U87 cells was likely due to the slower replication rate in PBMC, fewer susceptible cells in the mixed PBMC population, and decreased pathogenicity in PBMC. Otherwise, the peak in RT activity was followed by a precipitous drop over the next 7 days, whereas the p24 levels in the supernatant reached a plateau at 7 to 9 days in both PBMC and U87 cells infected with SI/X4 HIV-1 isolates (Fig. 6C). These findings further emphasize the inability of the p24 antigen capture assays to accurately assess infectious virion production but show their ability to instead provide a good measure of the total virus quantity.

During actual propagations in our laboratory, viruses were harvested at peak viremia as measured by the RT activities, which were offset from peak p24 levels by at least 2 days. As discussed below, the apparent discrepancy between peak RT activities and peak p24 levels may be related to the continual decay of infectious virus particles during virus production. Thus, the highest proportion of infectious virus particles may actually occur on day 7. In addition, the order of virus concentration at day 7 as measured by RT activity was B4 $\gg$ A8 $>$ E6 $>$ D4, whereas this order was different as measured by p24 levels, i.e., B4 = D4 $\gg$ A8 = E6. This difference in virus production as measured by RT activity and p24 content may be related to the variable sensitivity of the p24 assay (described above).

DISCUSSION

Infectious titers of HIV-1 stocks are commonly measured with a classic $TCID_{50}$ assay that involves exposing cells to limiting dilutions of HIV-1 and then scoring them for infection after prolonged incubations (7 to 15 days). This assay is typically well controlled within a laboratory, but $TCID_{50}$ values for the same HIV-1 stock of virus can vary between laboratories due to the use of (i) different cell lines or PBMC for infections and (ii) various assays for measuring HIV-1 production. There are also several formulas for the calculation of $TCID_{50}$ values (1, 12, 41). A surrogate assay for infectious titer should be

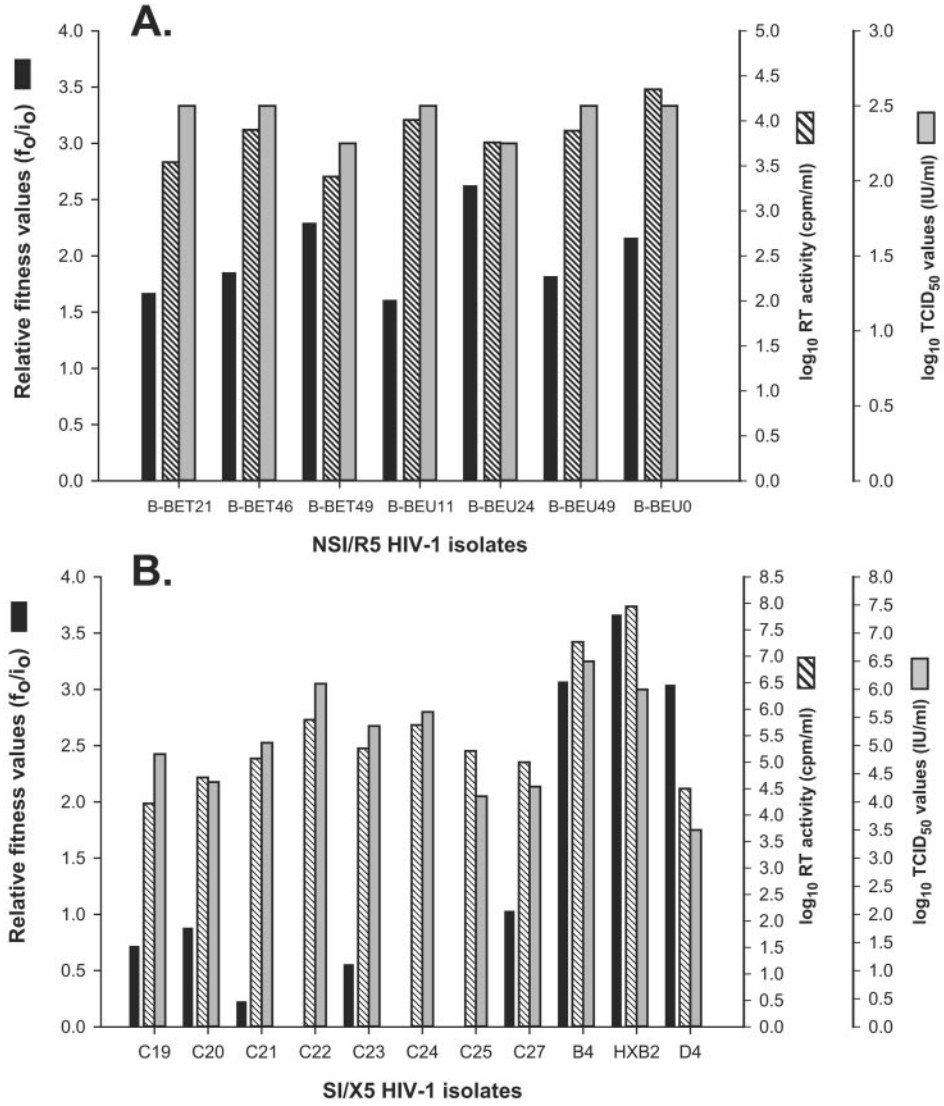


FIG. 5. Comparison of relative ex vivo fitness to titers of virus stocks. NSI/R5 HIV-1 isolates (A) were competed against two NSI/R5 reference HIV-1 strains, A1-92RW009 and B2-92BR017, whereas the SI/X4 HIV-1 isolates (B) were competed against the SI/X4 strains E6-CMU06 and A8-92UG029. PHA- and IL-2-treated PBMC were exposed to two viruses at an MOI of 0.005. The relative production of the primary HIV-1 isolates and the reference strain was detected and quantified in the competition after PCR and HTA (see Materials and Methods). Relative fitness values (W) were calculated from the fraction of the primary HIV-1 isolate after the competition (f_0) divided by the fraction of that virus initially added to the dual infection (i_0) (39). The y axis showing $\log_{10}$ RT activity (cpm/ml or 0.000152 × pmol of [α-^{32}P]TMP incorporation) (striped bars) is provided on the near right side of the charts. The far right y axis shows the $\log_{10}$ $TCID_{50}$ values (gray bars) of the various HIV-1 stocks.

accurate, rapid, less labor-intensive, and standardized. In this study, we compared the abilities of an RT assay, a p24 antigen capture assay-EIA, anti-p24 Western blotting, and a viral RNA load assay to meet these criteria. Previous experiments indicated that the viral RNA load assay as a surrogate for infectious titer may be the least accurate, most expensive, and least amenable to research studies of these methods (3; also data not shown). This hypothesis was supported by the present study. RT activity, not the detection of p24 capsid or viral RNA, was the best in vitro measure of the infectious titer. RT assays performed on serial dilutions of HIV-1 stocks also indicated that the relationship to infectious titer was segregated between SI/X4 and NSI/R5 HIV-1 isolates. The slopes derived from plots of RT activities and infectious titers of diluted HIV-1 stocks were significantly smaller with the SI/X4 isolates than with the NSI/R5 HIV-1 isolates. In addition, the RT activities per infectious unit of SI/X4 isolates were lower than those for NSI/R5 HIV-1 isolates.

The use of RT activity as a surrogate assay for infectious titer is supported by (i) the similar slope values and (ii) the direct relationship between RT activity and infectious titer for nearly all NSI/R5 HIV-1 isolates. Infectious titers have now been estimated for more than 30 NSI/R5 HIV-1 isolates by measuring the RT activities on 1/3 serial dilutions of patient HIV-1 isolates and of controls with known $TCID_{50}$ values. Subsequent measurements of $TCID_{50}$ values for these primary

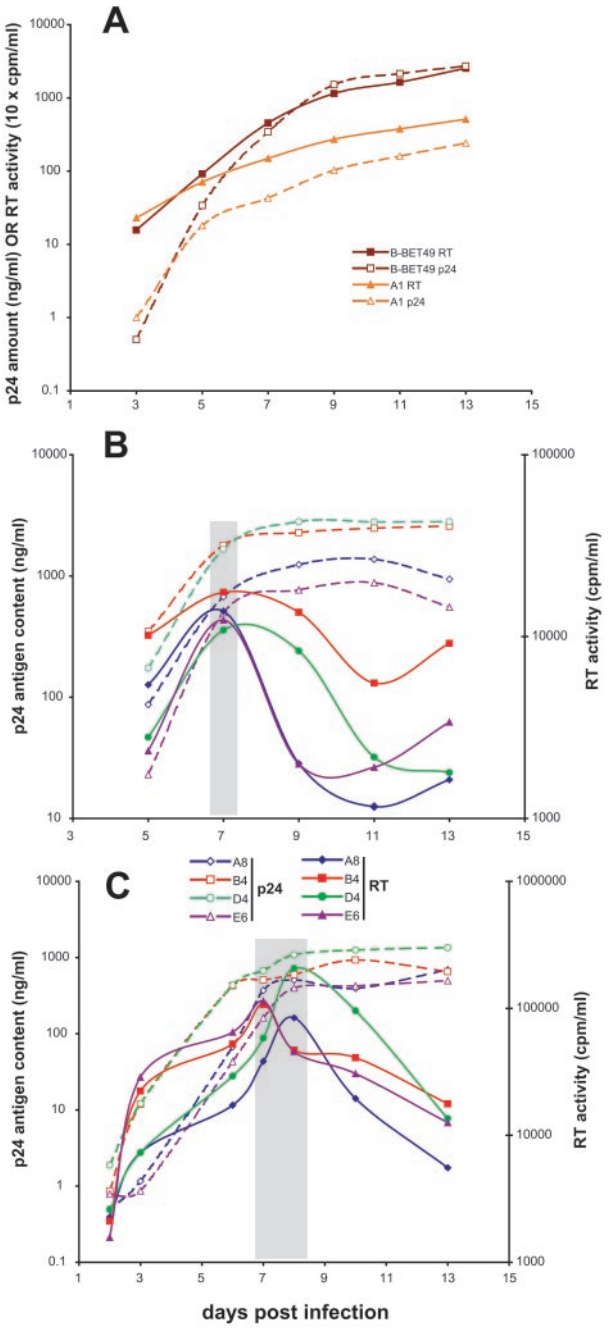


FIG. 6. Monitoring virus production with RT assays and p24 EIAs. U87.CD4.CCR5 cells (A) were exposed to two NSI/R5 HIV-1 isolates (A1 or B-BET49) at an MOI of 0.001. For panels B and C, U87.CD4.CXCR4 cells or PBMC were exposed to four SI/X4 isolates (A8, B4, D4, or E6) at an MOI of 0.001. Cell-free supernatants were harvested on days 3, 5, 7, 9, 11, and 13 to monitor RT activities (solid lines) and p24 antigen levels (dashed lines). Other symbols are described in the figure. The gray boxes in panels B and C highlight the RT activities and p24 levels near the peak RT activity.

NSI/R5 HIV-1 isolates has revealed a <25% difference between actual infectious titers and the virtual infectious titers derived from RT activities (data not shown). It is important to note that most $TCID_{50}$ values are only accurate to 0.10 to 0.25 log_{10} IU/ml, depending on the number of replicates. Thus, measuring the RT activities of NSI/R5 HIV-1 isolates may actually be a more refined measure of infectious titers. A standardized protocol for this virtual $TCID_{50}$ assay is available at www.cwru.edu/med/microbio/artslab/.

The relationship between infectious titers and RT activities is less accurate for SI/X4 than for NSI/R5 isolates. Variations between slope values derived from SI/X4 stocks may be related to the presence of NSI/R5 HIV-1 clones in the population of most primary SI/X4 HIV-1 stocks (R. Troyer and E. J. Arts, unpublished data). Even the minor presence of SI/X4 clones in a patient HIV-1 population typically results in an outgrowth of SI/X4 over NSI/R5 clones during virus propagation. In other words, viruses propagated from patient PBMC tend to be either populations of only NSI/R5 clones or mixtures of mostly SI/X4 clones with variable fractions of NSI/R5 clones. Since SI/X4 clones have a higher infectious potential (i.e., the increase in infectious titer is larger in proportion to increasing RT activities) than their NSI/R5 counterparts, an increased fraction of NSI/R5 clones in a predominantly SI/X4 HIV-1 population could decrease this infectious potential. As a hypothetical example, the slope derived from the plot of RT activity versus infectious titer for SI/X4 HIV-1 isolate E6 was 220 cpm/IU and was close to the slopes obtained with NSI/R5 HIV-1 isolates. In contrast, many SI/X4 HIV-1 isolates, such as HIV-1 B4, resulted in slope values of <2 cpm/IU. Thus, it is possible that a larger fraction of NSI/R5 clones was found in the E6 virus population, which could dampen the infectious potential. We are currently testing this hypothesis. Even though the RT activities of SI/X4 HIV-1 isolates may not be an accurate measure of infectious titers, it may be possible predict the biological phenotype from the relationship between $TCID_{50}$ values and RT activities. However, we acknowledge that testing coreceptor usage on coreceptor-restricted cell lines (e.g., U87.CD4.CCR5 or CXCR4) is just as informative and simple to perform.

The use of RT activity as a surrogate measure of infectious titer may be very useful in future studies focusing on the biological properties of non-subtype B HIV-1 isolates. To date, the majority of studies analyzing effects of HIV-1 mutations, gene products, and noncoding genomic sequences on HIV-1 replication have focused on subtype B laboratory strains (e.g., NL4-3 and HXB2) (10, 14, 17, 21, 22, 26, 27, 32, 33, 44, 49, 54, 55). The p24 antigen capture assay-EIA has been the most commonly used assay to equalize HIV-1 titers in these studies (14, 21, 26, 44, 49), yet even among clade B isolates, the p24 content in the cell-free supernatant appears to be a poor predictor of the infectious titer. With non-clade B isolates, there was no statistical relationship between infectious titer and p24 content as measured by EIA. This result was further confirmed by anti-p24 Western blot analyses. However, RT activity as a measure of infectious titer was nearly as accurate with non-clade B isolates as with clade B isolates. The poor performance of the p24 antigen capture assay in this regard may be related to variations in p24 which do not affect proper capsid structure and function but may disrupt binding to the monoclonal antibody. The RT assay as a measure of polymerase function is not affected by the conservative but significant RT sequence variation that maintains polymerase activity. Several reports have described similar polymerase activities between diverse HIV

RTs, e.g., those of HIV-1 group M, HIV-1 group O, and HIV-2 (18, 30, 42). In fact, one viral load assay based on endogenous RT activity (AMP-RT) is capable of measuring viral loads in individuals infected with HIV-2 and group O as well as group M (7, 25). Viral load assays, which are based on the amplification and quantitation of HIV-1 RNA, do not display the same sensitivity with diverse HIV isolates (e.g., group O, various group M subtypes, and HIV-2) (47). This standardized measure of RT activity as a rapid assay for determining the infectious titer is another controlled method to promote studies of non-clade B HIV-1 isolates.

It is now quite apparent that the relative replicative capacities of wild-type HIV-1 isolates can vary as much as 100-fold (3, 39). Although subtype C isolates are typically less fit than isolates of other group M subtypes (3), this difference can even be observed with primary HIV-1 isolates within a subtype (39). Most notably, subtype B NSI/R5 HIV-1 isolates from long-term survivors were at least 1,000-fold less fit than NSI/R5 HIV-1 strains isolated from patients progressing to AIDS (39). A slow propagation of HIV-1 isolates from a patient could result in lower HIV-1 stock titers ($TCID_{50}$ values or RT activities) and may be related to poor fitness. Indeed, we have noted that isolates that are more difficult to propagate from patients often have lower stock titers (3). It is important to note that the measurement of $TCID_{50}$ values during long incubation periods likely overrides rate-limiting steps, such as host cell entry, that have a profound effect on the relative fitness measured in competitions (A. J. Marozsan and E. J. Arts, unpublished). In addition, slow propagations from patient PBMC and the low titers of virus stocks may be more related to lower viral loads than to actual differences in relative replication efficiencies. Finally, this study confirms that p24 EIAs are as efficient as RT activity assays at measuring the total production of most subtype B HIV-1 isolates. However, RT activity peaks during virus propagations and then drops off during the decay of infectious virus particles. In contrast, p24 antigen capture assays cannot differentiate between noninfectious virus particles and virions.

In summary, the data presented herein suggest that the endogenous RT activities of HIV-1 isolates found in cell-free supernatants are an accurate measure of infectious titers. This relationship is highly significant for diverse NSI/R5 HIV-1 isolates (any group M subtype) but is less distinct between SI/X4 HIV-1 isolates. In contrast, viral RNA loads or p24 antigen contents, as measured by EIA, cannot be used to accurately predict infectious titers. Finally, the relative HIV-1 fitness (or replicative capacity) is not related to the titer of the HIV-1 stock. The HIV-1 competition assay measured relative replicative capacities rather than total infectivities of cells as determined by a $TCID_{50}$ assay. Due to the long incubation times and plus-minus scoring based on the evidence of infection, it is likely that $TCID_{50}$ values are not governed by the replication rate, but rather by the numbers of infectious particles.

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