Human Immunodeficiency Virus Type 1 Variants with Increased Replicative Capacity Develop during the Asymptomatic Stage before Disease Progression

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We examined the replicative properties of a series of sequential isolates and biological clones of human immunodeficiency virus type 1 (HIV-1) obtained from an individual who progressed from seroconversion to AIDS in approximately 5 years. HIV-1 isolated soon after seroconversion replicated slowly and to low levels in cultures of peripheral blood mononuclear cells; however, subsequent isolates obtained during asymptomatic infection showed a marked increase in replication kinetics. This was examined in more detail by using a panel of 35 biological clones of HIV-1 generated from sequential patient peripheral blood mononuclear cell samples. Each clone was evaluated for replication in primary macrophages and CD4⁺ T lymphocytes and for the ability to induce syncytium formation in MT-2 cell cultures. Consistent with earlier observations, we found that all of the clones isolated just after seroconversion were slowly replicating and non-syncytium inducing (NSI). However, NSI variants with increased replication kinetics in macrophages were identified soon thereafter. These variants preceded the appearance of NSI and syncytium-inducing variants, with rapid replication in both macrophages and CD4⁺ T lymphocytes. To determine whether changes in the rate of replication could be traced to the early stages of the virus life cycle, PCR assays were used to evaluate entry and reverse transcription of selected biological clones in macrophages and CD4⁺ T lymphocytes. We found there was no inherent block to entry or reverse transcription for the slowly replicating variants; however, this does not preclude the possibility that small differences in the rate of entry may account for larger differences in the replication kinetics over many cycles. Overall, our results demonstrate that rapidly replicating variants of HIV-1 emerge during the asymptomatic period in a patient who subsequently progressed clinically, suggesting that these variants may play an important role in HIV-1 pathogenesis.

Isolates of human immunodeficiency virus type 1 (HIV-1) can be distinguished by differences in replication rate, cell tropism, and cytopathic effects *in vitro* (3). In longitudinal studies, changes in the biological properties of sequential isolates have been associated with clinical progression and the onset of AIDS. Virus isolated from individuals shortly after seroconversion and during asymptomatic infection is predominantly macrophage tropic and non-syncytium inducing (NSI) *in vitro* (6, 29, 34, 35, 38, 39, 44). The emergence of T-cell-line-tropic, syncytium-inducing (SI) variants occur in about 50% of symptomatic patients and has been associated with rapid CD4⁺ T-cell decline and the development of AIDS (38–40). HIV-1 isolates from asymptomatic individuals also replicate more slowly in culture than do isolates from patients with AIDS (1, 12, 39), suggesting that selection for variants with increased growth potential may occur during the course of infection. It is possible that more rapidly replicating variants contribute to the development of immune dysfunction, or alternatively, these viruses may only emerge later as a result of immune system compromise and declining CD4⁺ T cells.

In vivo, HIV-1 is primarily targeted to CD4⁺ T lymphocytes and CD4⁺ cells of monocyte/macrophage lineage. Determinants which govern cell tropism have been mapped predominantly to the *env* gene (4, 18, 20, 24, 36, 41, 42). Exchanging regions of *env*, notably the V3 region of the surface glycoprotein gp120, between viruses with distinct tropisms has been

shown to influence entry into susceptible target cells, including macrophages (24) and CD4⁺ T cells (2, 5, 19). In addition, several regions of gp120, including sequences within the V2 (15) and V3 (8, 14) loops, contain known determinants of SI capacity. The *env* gene of HIV-1 is subject to a high degree of genetic diversity (23, 37), and it is reasonable to assume that immune and other selection pressures exerted on this region during infection may drive the evolution of variants with expanded tropism and increased cytopathicity. It is not clear, however, whether these changes also affect the efficiency of viral entry into susceptible target cells and contribute to increased rates of viral replication.

In this study, we examined in detail the phenotypic properties of a large series of sequential isolates and biological clones of HIV-1 derived from a single individual throughout the course of infection. We observed a progressive increase in the replication kinetics of sequential isolates in peripheral blood mononuclear cells (PBMC), with the most dramatic changes occurring during the period when this patient was clinically asymptomatic, with normal CD4⁺ T-cell levels. Predominantly slowly replicating, NSI viruses were isolated shortly after seroconversion; however, NSI variants with increased replication kinetics in macrophages were identified soon thereafter. These variants preceded the appearance of both NSI and SI clones with increased replication in macrophages and primary CD4⁺ T lymphocytes. We did not detect significant differences in the rate of entry and reverse transcription among biological clones with distinct replicative capacities, although this does not rule out the possibility that small differences in the efficiency viral penetration amplified over multiple rounds of

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FIG. 1. Dates with HIV-1 isolates were obtained from patient B, corresponding CD4⁺ T-cell counts, and syncytium-inducing phenotypes of P1 isolates.

replication may account for the observed changes in phenotype.

MATERIALS AND METHODS

Generation of primary isolates and biological clones. Patient B was selected from a large cohort of homosexual men evaluated at the New York Blood Center as part of a prospective AIDS study (6). His clinical course was marked by a very rapid decline in CD4⁺ T cells which occurred approximately 3 years after seroconversion for HIV-1 (Fig. 1). Samples of plasma and PBMC were collected at regular intervals throughout infection and stored in liquid nitrogen.

Sequential isolates of HIV-1 were obtained by a quantitative coculture method as previously described (16). Briefly, cryopreserved patient PBMC were thawed and the cells were serially diluted fourfold (from 10^6 cells down to approximately 10^3) in replicates of 4 to 10. Phytohemagglutinin (PHA)-stimulated normal donor PBMC (2×10^6) were added in 1.5 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and interleukin-2 (10 U/ml). The cocultures were incubated for 24 h at 37°C and washed three times with fresh medium. Samples of the culture supernatants were assayed for HIV-1 p24 antigen on days 7, 14, and 21, using a commercial enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories, North Chicago, Ill.). A culture was considered positive if the p24 value was above a cutoff value of 30 pg/ml. Supernatant from the first well of each titration was pooled from replicate wells, and the virus was propagated by a single short-term passage (5 to 7 days) in PBMC. This virus was designated P1.

Biological clones were generated by short-term propagation of the virus present in the last positive well of each titration (7). In general, 4 to 10 biological clones were obtained from each PBMC sample. The P1 isolates and biological clones were titrated on normal donor PBMC to determine the infectious titer, and the 50% tissue culture infective dose (TCID₅₀) was calculated by the method of Reed and Muench (10). Selected biological clones were titrated separately, using PBMC from

two to three different donors to confirm the results. The input inoculum for all experiments was standardized by the number of infectious units determined by TCID₅₀ and not by the amount of HIV-1 p24 antigen.

Cell isolation and culture. PBMC from healthy, HIV-1-seronegative donors were isolated by standard Ficoll-Hypaque density centrifugation. The cells were stimulated with PHA for 24 to 48 h in RPMI 1640 with 10% fetal calf serum and antibiotics and then switched to growth medium containing interleukin-2. Monocyte/macrophages were isolated from fresh PBMC by plastic adherence prior to PHA stimulation. PBMC were first cultured for 2 h at 37°C to allow the monocytes to adhere. The nonadherent cell population was then removed and transferred to a new flask, and the remaining adherent cells were cultured overnight and removed from the flask the next day by gentle scraping. The adherent cells were cultured at a density of 2×10^6 per well in 24-well plates for an additional 7 to 10 days in the absence of any added growth factors. At the end of this period, >95% of the cells had the morphology of mature macrophages. The nonadherent cells were depleted of CD4⁺ T cells by immunomagnetic separation (Dynal, Inc., Great Neck, N.Y.), yielding a population that was enriched for CD4⁺ T lymphocytes. These cells were stimulated for 48 h with PHA and cultured in 1.5 ml of growth medium at a density of 3×10^6 cells per well in 24-well plates.

Phenotype characterization. The P1 isolates and biological clones were first assessed for infectivity and replication in either PBMC, macrophages, or CD4⁺ T lymphocytes. The cells were inoculated with a standardized input of each virus, incubated for 24 h at 37°C, and washed twice with fresh medium. Samples of culture supernatants were taken immediately after washing (day 0) and on designated days thereafter and assayed for the presence of HIV-1 p24 antigen by ELISA. To evaluate syncytium formation, MT-2 cells (10^6) were inoculated with each virus. Syncytium formation was evaluated in the cultures on days 7, 10, and 14 by standard light microscopy. Samples of the culture supernatants were tested in parallel for HIV-1 p24 antigen. All cultures that were positive for syncytium formation also had p24 values of 1,000 pg/ml or greater. Conversely, all cultures that lacked syncytium formation were negative for p24 antigen. We have previously noted this correlation (6), suggesting that MT-2 cell tropism and syncytium formation may be linked properties. Cultures that were positive for both syncytium formation and p24 antigen were designated SI, whereas cultures negative for syncytium formation and p24 were designated NSI.

PCR analysis. PCR assays were used to evaluate early events taking place after inoculation of primary cells with selected biological clones. All virus-containing supernatants were first treated with DNase (25 U/ml) for 30 min at 37°C and then filtered (0.45-µm-pore-size filter) prior to addition to cell cultures. Primary cell cultures consisting of either macrophages or CD4⁺ T lymphocytes were prepared as outlined above. Where indicated, the cells were treated with zidovudine (AZT; 20 mM) for 30 min at 37°C prior to addition of virus. The cells were then mixed with a standardized amount of each virus. At designated times thereafter, the cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed in 500 µl of DNA lysis buffer (10 mM Tris-HCl [pH 8.0], 0.1 M EDTA [pH 8.0], 0.5% sodium dodecyl sulfate, 100 µg of proteinase K per ml, 20 µg of RNase per ml). To prepare DNA from macrophage cultures, the adherent cells were washed directly in the plates by aspiration and lysed with 500 µl of DNA lysis buffer. DNA was recovered from cell lysates by sequential extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform followed by ethanol precipitation,

using standard methods (31). DNA from each sample (1 μ g) was then evaluated by PCR to determine the extent of viral entry and reverse transcription. HIV-1 sequences were amplified by using primers M667 (GGCTAACTAGGGAAC CCACTG) and M661 (CCTGCGTCGAGAGACTCTGG), which hybridize within the long terminal repeat (LTR)/*gag* region of the HIV-1 genome (43), and primers RC-1 (GCCT CAATAAAGCTTGCCTTG, positions 69 to 89 [28]) and RC-2 (CCACTGCTAGAGATTTCCAC, positions 185 to 165 [28]), which hybridize within the R/U5 region of the LTR. One primer from each pair (M667 or RC-1) was 32 P end labeled by the method of Pang et al. (25). This allowed direct visualization and quantitation of PCR products following electrophoresis on 8% polyacrylamide gels. HIV-1 sequences were amplified in a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin, with 0.2 mM each dATP, dCTP, dGTP, and dTTP, 40 ng of each primer, and 2.5 U of recombinant *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), in a final volume of 50 μ l. A total of 32 cycles was run, using primers M667 and M661: 2 cycles at 94°C for 2 min, 63°C for 30 s, and 72°C for 2 min, and 30 cycles at 94°C for 1 min, 63°C for 30 s, and 72°C for 1 min. Identical conditions were used for primers RC-1 and RC-2 except that the annealing temperature was lowered to 55°C. Following electrophoresis, the radiation content of designated bands was measured with a Betascope 603 image analyzer (Betagen Corp., Waltham, Mass.). The HIV-1 DNA copy number for each sample was determined by comparison with a standard curve generated by using DNA from 8E5 cells, which contain one integrated copy of HIV-1 per cell (13). Parallel reactions were also run with primers PC03 and PC04 (human β -globin gene) (30) as a control to ensure that equivalent amounts of DNA were analyzed in each sample.

RESULTS

Sequential P1 isolates replicate with increasing kinetics in PBMC. In initial experiments, sequential P1 isolates from patient B were tested for the ability to replicate in PHA-stimulated normal donor PBMC (Fig. 2). The cells were infected with a standardized inoculum of each virus, and the culture supernatants were tested at regular intervals for the presence of HIV-1 p24 antigen. The earliest isolate of HIV-1, obtained shortly after seroconversion (May 1985), replicated slowly in PBMC cultures, reaching peak levels on day 10 after infection. By comparison, the subsequent isolate (November 1986) replicated slightly faster and to higher levels, while the next three isolates (June 1988, November 1988, and July 1990) each replicated rapidly to high levels between days 4 and 10 of culture. The most dramatic increase in replication kinetics occurred between May 1985 and June 1988, when patient B was clinically asymptomatic and had CD4⁺ T-cell counts above 500/mm³ (Fig. 1).

Biological clones of HIV-1 can be distinguished by differences in replication rate and syncytium formation. To assess the phenotypic properties of sequential HIV-1 isolates in more detail, a panel of biological clones was generated by limiting dilution culture of PBMC from patient B. A total of 35 clones was obtained from five successive time points. Each of the biological clones was evaluated for growth in primary cultures of macrophages and CD4⁺ T lymphocytes and for the ability to induce syncytium formation in MT-2 cell cultures. As seen in Table 1, all of the biological clones obtained shortly after seroconversion (May 1985) and 18 months later (November 1986) had an NSI phenotype. However, both NSI and SI clones

FIG. 2. Replication kinetics of sequential HIV-1 isolates. PHA-stimulated PBMC from normal donors were infected with 10^3 TCID₅₀ of sequential HIV-1 isolates. After overnight incubation at 37°C, the cells were washed with fresh medium, and virus replication was assessed by measuring HIV-1 p24 antigen in culture supernatants on days 4, 7, 10, and 14, using a commercial ELISA (Abbott Laboratories).

were identified in the subsequent sample obtained approximately 3 years after seroconversion (June 1988) and just prior to when patient B experienced a rapid decline in CD4⁺ T cells. Similarly, NSI and SI clones were isolated 5 months later (November 1988). Late in the course of infection (July 1990), only NSI clones were identified despite the SI phenotype of the P1 isolate at that time (Fig. 1). It is possible that variants with an SI phenotype made up only a minor component of the virus present at that time point. Nevertheless, these variants were sufficient to confer an SI phenotype on the bulk P1 isolate, since the SI property is scored as a dominant phenotype in the assay.

Evaluation of the replication kinetics of each of the biological clones revealed patterns consistent with those observed with the P1 isolates. To minimize differences in replication due to donor cell effects, all of the biological clones were assayed on the same day, using macrophages and CD4⁺ T lymphocytes derived from the same donor PBMC. As seen in Table 1, all of the clones from May 1985 replicated slowly and to low levels in primary cell cultures, while two of the four clones from November 1986 replicated more rapidly and to higher levels in macrophages. Variants with increased replication in both macrophages and CD4⁺ lymphocytes were identified in subsequent isolates from June 1988 and November 1988. Many of the biological clones from the final time point, July 1990, had slightly reduced replication kinetics compared with those from the previous two time points. Overall, the appearance of variants with increased replication in primary cells occurred when patient B was clinically asymptomatic. Among the biological clones, NSI variants with increased replication kinetics in macrophages were identified first, while clones with an SI phenotype and rapid replication in both macrophages and CD4⁺ T lymphocytes appeared later. Slowly replicating, NSI viruses were found immediately after seroconversion and persisted throughout the course of infection, at times appearing concurrently with more rapidly replicating SI variants.

Biological clones isolated during asymptomatic infection can have distinct replication patterns in macrophages and CD4⁺ T lymphocytes. Six biological clones were selected for

TABLE 1. Evaluation of viral replication and syncytium-inducing properties of sequential biological clones of HIV-1

Date	Clone	Replication ^a in:		Syncytium-inducing capacity ^b
		Macrophages	Lymphocytes	
May 1985	2-1	+	+	NSI
	2-2	+	+	NSI
	2-3	+	+	NSI
	2-5	+	+	NSI
	2-6	+	+	NSI
	2-7	+	+	NSI
	2-8	+	+	NSI
November 1986	3-1	++	+	NSI
	3-2	+	+	NSI
	3-3	+	+	NSI
	3-4	++	+	NSI
June 1988	4-1	+++	+++	SI
	4-3	+	+	NSI
	4-5	++	++	NSI
	4-6	+	+	NSI
	4-7	+	+	NSI
	4-9	+++	+	SI
	4-10	+	+	NSI
November 1988	5-1	+	+	NSI
	5-2	+	+	NSI
	5-3	+	+	NSI
	5-4	+++	+++	SI
	5-5	+	+	NSI
	5-6	+++	+	SI
	5-7	+++	+++	SI
	5-8	+++	+++	SI
	5-10	++	+	NSI
	6-3	+	+	NSI
July 1990	6-4	++	+	NSI
	6-5	+	+	NSI
	6-6	++	+	NSI
	6-7	++	+	NSI
	6-8	++	+	NSI
	6-9	++	+	NSI
	6-10	++	+	NSI

^a Assessed in primary cultures of macrophages and CD4⁺ T cells by measuring the levels of HIV-1 p24 antigen in culture supernatants on days 4, 7, and 10 after infection. Replication was characterized as slow (+), intermediate (++), or rapid (+++). Examples of the replication patterns for selected clones can be seen in Fig. 3.

^b Evaluated in MT-2 cell cultures on days 7 and 10 after infection.

further evaluation on the basis of differences in their replication kinetics in macrophages and CD4⁺ T lymphocytes (Fig. 3). Clones 2-5 and 2-6 replicated slowly and to low levels in both macrophages (Fig. 3A) and CD4⁺ T lymphocytes (Fig. 3B). Clone 3-3 also had limited replication in both cells types, whereas clone 3-4 isolated from the same PBMC sample replicated faster and to higher levels in macrophages (Fig. 3A). The replication of this clone in CD4⁺ T lymphocytes was only marginally elevated (Fig. 3B). Clone 4-3 had a pattern of replication similar to that of the biological clones from earlier time points, whereas clone 4-1 replicated efficiently to high levels in both macrophages (Fig. 3A) and CD4⁺ T lymphocytes (Fig. 3B). All of the clones were NSI except 4-1, which had an SI phenotype (Table 1).

Restricted replication in macrophages is not due to a major block at the level of entry and reverse transcription. PCR analysis was used to determine whether differences in replication in macrophages could be traced to changes in the early stages of the virus life cycle. Cells were infected with an equal inoculum of each virus, and at designated times after infection, DNA was extracted and analyzed by PCR using primers that

FIG. 3. Replication of biological clones in macrophages (A) and CD4⁺ T lymphocytes (B). Cultures of macrophages and CD4⁺ T lymphocytes were incubated with 10³ TCID₅₀ of selected biological clones for 24 h at 37°C and then washed with fresh medium. HIV-1 p24 antigen was measured in culture supernatants on days 3, 7, 10, and 14 after infection. Biological clones 2-5 (■) and 2-6 (●) were isolated by endpoint dilution cocultures, using patient PBMC from May 1985. Clones 3-3 (▲) and 3-4 (◆) were isolated by the same method from PBMC samples obtained in November 1986. Clones 4-1 (□) and 4-3 (○) were isolated from PBMC samples obtained in June 1988.

detect complete or nearly complete HIV-1 DNA sequences (43). Preliminary experiments indicated that reverse transcription occurred much more slowly in macrophages than in PHA-stimulated PBMC (data not shown). Full-length HIV-1 DNA was not detected in macrophages until 24 to 48 h after infection (Fig. 4), whereas signals were detected as early as 8 to 12 h after infection of PBMC. Increases in the PCR signal from 24 to 48 h could be blocked by the addition of AZT, confirming the presence of newly synthesized HIV-1 DNA. Forty-eight hours after infection, full-length HIV-1 DNA was detected in macrophages infected with each of the biological clones, indicating that these viruses were able to enter and reverse transcribe their genomes. However, 72 h after infection, the level of HIV-1 DNA had increased in macrophages infected with the rapidly replicating clones (3-4 and 4-1), whereas there was almost no change in the level of HIV-1 DNA detected in cells infected with the slowly replicating viruses (clones 2-5, 2-6, 3-3, and 4-3) (Fig. 4).

This finding was confirmed by using a second pair of PCR primers which detect early products of reverse transcription.

FIG. 4. PCR analysis of viral entry and reverse transcription in macrophages infected with slowly and rapidly replicating biological clones. Macrophages were infected with 10^3 TCID₅₀ of six biological clones with different replicative capacities. DNA was extracted from the cells at 24, 48, and 72 h after infection and analyzed by PCR using primers M667 and M661 (LTR/gag) (43). Control cultures were treated with AZT (20 μ M) for 30 min at 37°C prior to infection.

Twenty-four hours after infection, similar levels of HIV-1 DNA could be detected in macrophages infected with each of the biological clones, as judged from quantitation of DNA copy number (Table 2). The amount of early HIV-1 DNA increased roughly twofold between 24 and 48 h and corresponded with the appearance of full-length HIV-1 DNA. However, 72 h after infection, the amount of early, reverse-transcribed DNA had decreased by about 50% in cells infected with each of the slowly replicating clones, whereas approximately two- to fourfold-higher levels were detected in macrophages infected with the rapidly replicating clones. Decreases in the PCR signal seen with the slowly replicating viruses may reflect degradation of partially reverse transcribed viral DNA products, analogous to what has been observed in resting T lymphocytes (43). Similar processes may also take place in cells infected with rapidly replicating viruses; however, these changes may be masked by more efficient initiation of secondary rounds of infection, which would effectively increase the levels of early reverse-transcribed DNA.

In separate experiments, macrophages were infected with equivalent amounts of either slowly (clones 2-5 and 2-6) or rapidly (clones 3-4 and 4-1) replicating biological clones. HIV-1 p24 antigen levels were measured in the culture super-

TABLE 2. Evaluation of reverse transcription in macrophages infected with slowly and rapidly replicating biological clones

Clone	HIV-1 DNA copy no. ^a					
	24 h		48 h		72 h	
	Early	Complete	Early	Complete	Early	Complete
2-5	196	<10	401	114	189	127
2-6	182	<10	386	112	177	176
3-3	NT	<10	NT	61	NT	90
3-4	210	<10	441	89	903	778
4-1	160	<10	351	140	1,246	1,043
4-3	248	<10	392	93	193	114

^a Determined by comparison with a standard curve generated by using DNA from 8E5 cells, which contain one integrated copy of HIV-1 per cell. The limits of sensitivity of the assay were ≥ 10 copies. Early DNA products of reverse transcription were measured by using PCR primers RC-1 and RC-2, which hybridize to the R/U5 region of the HIV-1 LTR. Complete or nearly complete HIV-1 DNA was measured by using primers M667 and M661, which recognize sequences in the LTR/gag region of the genome (43). NT, not tested.

FIG. 5. Replication and spread of virus in macrophage cultures infected with selected biological clones. Macrophages were infected with 10^3 TCID₅₀ of either slowly replicating clones 2-5 and 2-6 or rapidly replicating clones 3-4 and 4-1. (A) p24 antigen levels in culture supernatants measured on days 3, 5, and 7 after infection; (B) PCR analysis of DNA extracted on day 7 after infection, using primers M667 and M661 (43).

natants at regular intervals up to day 7, at which time the cells were harvested and analyzed by PCR for full-length HIV-1 DNA (Fig. 5). The amount of p24 antigen measured in culture supernatants was roughly equivalent on day 3 after infection. However, by day 7, the p24 levels had increased rapidly in cultures infected with clones 3-4 and 4-1, while those infected with clones 2-5 and 2-6 showed only modest increases in p24 (Fig. 5A). PCR analysis of DNA extracted from the cells on day 7 revealed significantly higher levels of HIV-1 DNA in macrophages infected with the two rapidly replicating clones (Fig. 5B), indicating more efficient spread of these viruses in the culture.

Restricted replication in CD4⁺ T lymphocytes cannot be overcome by increasing inoculum. PCR was also used to evaluate entry and reverse transcription of selected biological clones in cultures enriched for CD4⁺ T lymphocytes. The cells were infected with equivalent inocula of clones 2-5, 2-6, 3-3, 3-4, 4-1, and 4-3. At designated times after infection, the cells were harvested and analyzed by PCR to determine the level of full-length HIV-1 DNA (Table 3). Eight hours after infection, low levels of HIV-1 DNA could be detected in CD4⁺ T lymphocytes infected with each of the biological clones. By 24 h, the amount of HIV-1 DNA had increased among all clones, indicating that the viruses were able to mediate entry and reverse transcription. Increases in the PCR signal from 8 to 24 h could be inhibited by the addition of AZT, confirming the presence of newly synthesized viral DNA.

To test whether the poor replication of clones 2-5, 2-6, and 3-4 in CD4⁺ T lymphocytes was related to the quantity of inoculum, cells were infected with increasing amounts of each virus (10^2 , 10^3 , and 10^4 TCID₅₀). At high input virus concentrations (10^4 TCID₅₀), clone 4-1 rapidly replicated to high levels (Fig. 6). Decreasing the inoculum 10- and 100-fold resulted in a delay in p24 production (9); however, virus

TABLE 3. Evaluation of reverse transcription in CD4⁺ T lymphocytes infected with slowly and rapidly replicating biological clones

Clone	HIV-1 DNA copy no. ^a		
	8 h	24 h	24 h + AZT ^b
2-5	90	1,085	30
2-6	117	761	72
3-3	75	818	21
3-4	120	785	62
4-1	198	1,396	95
4-3	73	752	40

^a Determined as described in the footnote to Table 2 and in Materials and Methods.

^b AZT was added at a concentration of 20 μ M to cultures 30 min before inoculation with HIV-1.

replication eventually reached comparably high levels. In contrast, replication of clones 2-5, 2-6, and 3-4 reached only low levels regardless of the inoculum (Fig. 6). Even when 100-fold more virus was added (10^4 TCID₅₀ compared with 10^2 TCID₅₀ for clone 4-1), replication of clones 2-5, 2-6, and 3-4 never reached the levels seen with clone 4-1.

PCR analysis at 24 h indicated that the amount of virus entering the cells was directly related to the input inoculum (Fig. 7). Increasing the inoculum by 10- and 100-fold resulted in an increase in the levels of HIV-1 DNA detected by PCR. Inoculation of CD4⁺ T lymphocytes with 10^4 TCID₅₀ of clones

FIG. 7. Effects of increasing inoculum on entry and reverse transcription in CD4⁺ T lymphocytes. Cultures enriched for CD4⁺ T lymphocytes were inoculated with an increasing number of TCID₅₀ of clones 2-5, 2-6, 3-4, and 4-1. DNA was extracted from the cells 24 h after infection and analyzed by PCR using primers M661 and M667 (43).

2-5, 2-6, and 3-4 resulted in higher levels of virus entering the cells; however, this was not sufficient to overcome the restriction in virus replication. The low level of HIV-1 DNA present after 24 h in cells inoculated with 10^2 TCID₅₀ of clone 4-1 presaged a much higher level of virus replication (Fig. 6) than did similar amounts of DNA present in cells inoculated with 10^2 TCID₅₀ of clones 2-5, 2-6, and 3-4 (Fig. 7).

DISCUSSION

Previously, we characterized quantitative changes in HIV-1 which occurred throughout the course of infection in three patients with rapid disease progression and one clinically stable patient (6). Increased viral burden was found to correlate

FIG. 6. Effects of increasing inoculum on virus replication in CD4⁺ T lymphocytes. Cultures enriched for CD4⁺ T lymphocytes were inoculated with either 10^2 ($\blacktriangle$), 10^3 ($\bullet$), or 10^4 ($\blacksquare$) TCID₅₀ of clone 2-5, 2-6, 3-4, or 4-1. Viral replication was assessed by measuring HIV-1 p24 antigen in culture supernatants on successive days after infection.

temporally with changes in the cytopathic properties of the virus, including a switch from NSI to SI phenotype which preceded CD4⁺ T-cell decline. Using paired viruses taken before and after this switch in phenotype, we observed in each case that the SI isolate replicated more rapidly than the paired NSI isolate in cultures enriched for CD4⁺ T lymphocytes. These results suggested that variants of HIV-1 with increased replicative capacity may evolve during the course of infection and may play a role in disease progression. Here we demonstrate that the rate of virus replication increases during infection, most notably during the asymptomatic period, in a patient who later progressed rapidly to AIDS.

Differences in the rate of viral replication have previously been found to correlate with the stage of disease in HIV-1-infected individuals (1, 12, 39). In longitudinal studies, isolates from patients who progressed to AIDS had a higher rate of replication compared with isolates from individuals who remained asymptomatic (39). Rapid CD4⁺ T-cell decline was associated with rapidly replicating, SI virus; however, in some cases, disease progression also occurred in patients with rapidly replicating, NSI virus (39).

In the present study, we examined in detail the replicative properties of a series of virus isolates derived from a single patient soon after seroconversion and subsequently throughout the course of infection. We found a progressive increase in the ability of sequential isolates to replicate in primary cultures of PBMC, macrophages, and CD4⁺ lymphocytes. The most dramatic increase in replication kinetics occurred during the period when this patient was clinically asymptomatic, with normal CD4⁺ T-cell counts. We have since confirmed this finding in two additional patients with rapid disease progression and have consistently found only slowly replicating virus in one long-term survivor of HIV-1 infection (5a). These results support a growing body of evidence that HIV-1 replication is active during asymptomatic infection (11, 16, 26, 27). Increased virus replication during this period may contribute to disease progression either directly by increasing the viral burden in tissues (11, 26) and peripheral blood (6, 16, 27) or indirectly by giving rise to more cytopathic variants.

Using a panel of sequential biological clones of HIV-1 derived from the same individual, we studied the evolution of phenotypically distinct variants throughout infection. Consistent with previous reports (29, 34, 35, 44), we found predominantly macrophage-tropic, NSI virus soon after seroconversion. Although many of the NSI viruses grew slowly in primary cell cultures, variants with increased capacity to replicate in macrophages were identified early on. This change in phenotype preceded the appearance of more cytopathic SI variants. The temporal evolution of these changes suggests that there may be a continuum of phenotypic properties in which slowly replicating NSI virus gives rise first to rapidly replicating NSI and then to SI variants (39). While the appearance of SI virus is strongly associated with subsequent CD4⁺ T-cell decline (40), changes in the underlying replication rate of NSI viruses, especially during asymptomatic infection, may be an important determinant of disease progression.

The emergence of viral variants with increased replicative capacity in macrophages has been reported recently in studies using the simian immunodeficiency virus macaque model (21). Specific pathogenic manifestations, including AIDS encephalitis and granulomatous pneumonia, were associated with the presence of macrophage-tropic variants. Our results are analogous, in that we identified sequential variants of HIV-1 with increased replication kinetics in macrophages. The ability of the simian immunodeficiency virus variants to replicate in macrophages was mapped to a small number of changes in the

env gene (22); however, surprisingly, these mutations did not affect virus entry (21). Within the limits of our quantitative PCR assay, we found relatively little difference in the ability of slowly and rapidly replicating variants of HIV-1 to mediate entry and reverse transcription in cultures of primary macrophages. These findings are in agreement with several recent reports (17, 21, 33). However, subtle differences in rates of entry and reverse transcription among our viral variants may escape detection in our assay system.

It is as yet unclear which stage of replication is impaired in the slowly growing viruses. Increased replication of HIV-1 variants may reflect subtle differences in entry events or possibly more efficient processing and incorporation of the envelope glycoproteins into progeny virions. This effect would not be evident during the first round of replication in our assays, since the inoculum was adjusted for an equivalent number of infectious units. However, upon subsequent cycles of infection, this may increase the efficiency of CD4 binding and enhance virus-cell fusion and entry. Alternatively, rapidly replicating viruses may mediate cell-cell fusion more readily at a given level of surface-expressed envelope, leading to increased virus spread in culture (32). Consistent with earlier studies by Tersmette et al. (38), we found relatively inefficient spread of the slowly replicating viruses in culture despite early release of p24 into the supernatant (Fig. 5). Further evaluation of sequential biological and molecular clones of HIV-1, which are likely to be highly related at the genetic level yet phenotypically distinct, should provide important clues as to the nature of the determinants which mediate these processes.

Changes in the *in vitro* replication rate of sequential virus isolates may reflect the emergence of variants with increased infectivity *in vivo*. Although recovery of rapidly replicating virus from HIV-1-infected patients is strongly associated with advanced disease, our results indicate that even during asymptomatic infection, the virus is subtly changing its biological properties. We have identified early changes in the replication rate of NSI viruses, particularly in macrophages, which precede the appearance of more virulent variants. Further longitudinal studies are now needed to determine whether these changes are significantly associated with subsequent disease progression.

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REFERENCES

1. Åsjö, B., L. Morfeldt-Mäson, J. Albert, G. Biberfeld, A. Karlsson, K. Lidman, and E. M. Fenyö. 1986. Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. *Lancet* ii:660-662.
2. Cann, A. J., J. A. Zack, A. S. Go, S. J. Arrigo, Y. Koyanagi, P. L. Green, Y. Koyanagi, S. Pang, and I. S. Y. Chen. 1990. Human immunodeficiency virus type 1 T-cell tropism is determined by events prior to provirus formation. *J. Virol.* 64:4735-4742.
3. Cheng-Mayer, C., D. Seto, M. Tateno, and J. A. Levy. 1988. Biological features of HIV-1 that correlate with virulence in the host. *Science* 240:80-82.
4. Cheng-Mayer, C., T. Shioda, and J. A. Levy. 1991. Host range, replicative, and cytopathic properties of human immunodeficiency virus type 1 are determined by very few amino acid changes in Tat and gp120. *J. Virol.* 65:6931-6941.

5. Chesebro, B., J. Nishio, S. Perryman, A. Cann, W. O'Brien, I. S. Y. Chen, and K. Wehrly. 1991. Identification of human immunodeficiency virus envelope gene sequences influencing viral entry into CD4-positive HeLa cells, T-leukemia cells, and macrophages. *J. Virol.* **65**:5782-5789.
- 5a. Connor, R. I. Unpublished data.
6. Connor, R. I., H. Mohri, Y. Cao, and D. D. Ho. 1993. Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. *J. Virol.* **67**:1772-1777.
7. Connor, R. I., D. W. Notermans, H. Mohri, Y. Cao, and D. D. Ho. 1993. Biological cloning of functionally diverse quasiespecies of HIV-1. *AIDS Res. Hum. Retroviruses* **9**:541-546.
8. DeJong, J.-J., J. Goudsmit, W. Keulen, B. Klaver, W. Krone, M. Tersmette, and A. de Ronde. 1992. Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. *J. Virol.* **66**:757-765.
9. Dimitrov, D. S., R. L. Willey, H. Sato, L.-J. Chang, R. Blumenthal, and M. A. Martin. 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. *J. Virol.* **67**:2182-2190.
10. Dulbecco, R. 1988. End-point method-measurement of the infectious titer of a viral sample, p. 22-23. *In* R. Dulbecco and H. S. Ginsberg (ed.), *Virology*, 2nd ed. J. B. Lippincott, Philadelphia.
11. Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature (London)* **362**:359-362.
12. Fenyö, E. M., L. Morfeldt-Måson, F. Chiodi, B. Lind, A. von Gegerfelt, J. Albert, E. Olausson, and B. Åsjö. 1988. Distinctive replicative and cytopathic characteristics of human immunodeficiency virus isolates. *J. Virol.* **62**:4414-4419.
13. Folks, T. M., D. Powell, M. Lightfoote, S. Koenig, A. S. Fauci, S. Hogan, S. Venatesan, and M. A. Martin. 1986. Biological and biochemical characterization of a cloned Leu-3-cell surviving infection with the acquired immunodeficiency syndrome retrovirus. *J. Exp. Med.* **164**:280-290.
14. Fouchier, R. A. M., M. Groenink, N. A. Kootstra, M. Tersmette, H. G. Huisman, F. Miedema, and H. Schuitemaker. 1992. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J. Virol.* **66**:3183-3187.
15. Groenink, M., R. A. M. Fouchier, S. Broersen, C. H. Baker, M. Koot, A. B. van't Wout, H. G. Huisman, F. Miedema, M. Tersmette, and H. Schuitemaker. 1993. Relation of phenotype evolution of HIV-1 to envelope V2 configuration. *Science* **260**:1513-1516.
16. Ho, D. D., T. Moudgil, and M. A. Alam. 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N. Engl. J. Med.* **321**:1621-1625.
17. Huang, Z.-B., M. J. Potash, M. Simm, M. Shahabuddin, W. Chao, H. E. Gendelman, E. Eden, and D. J. Volsky. 1993. Infection of macrophages with lymphotropic human immunodeficiency virus type 1 can be arrested after viral DNA synthesis. *J. Virol.* **67**:6893-6896.
18. Hwang, S. S., T. J. Boyle, H. K. Lyster, and B. R. Cullen. 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**:71-74.
19. Kim, S., K. Ikeuchi, J. Groopman, and D. Baltimore. 1990. Factors affecting cellular tropism of human immunodeficiency virus. *J. Virol.* **64**:5600-5604.
20. Liu, Z.-Q., C. Wood, J. A. Levy, and C. Cheng-Mayer. 1990. The viral envelope gene is involved in macrophage tropism of a human immunodeficiency virus type 1 strain isolated from brain tissue. *J. Virol.* **64**:6148-6153.
21. Mori, K., D. J. Ringler, and R. C. Desrosiers. 1993. Restricted replication of simian immunodeficiency virus strain 239 in macrophages is determined by *env* but is not due to restricted entry. *J. Virol.* **67**:2807-2814.
22. Mori, K., D. J. Ringler, T. Kodama, and R. C. Desrosiers. 1992. Complex determinants of macrophage tropism in *env* of simian immunodeficiency virus. *J. Virol.* **66**:2067-2075.
23. Myers, G., A. B. Rabson, J. A. Berzofsky, T. F. Smith, and F. Wong-Staal. 1990. Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N. Mex.
24. O'Brien, W. A., Y. Koyanagi, A. Namazie, J.-Q. Zhao, A. Diagne, K. Idler, J. A. Zack, and I. S. Y. Chen. 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. *Nature (London)* **348**:69-73.
25. Pang, S., Y. Koyanagi, S. Miles, C. Wiley, H. V. Vinters, and I. S. Y. Chen. 1990. High levels of unintegrated HIV-1 DNA in brain tissue of AIDS dementia patients. *Nature (London)* **343**:85-89.
26. Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature (London)* **362**:355-358.
27. Piatak, M., Jr., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K.-C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* **259**:1749-1754.
28. Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* **313**:277-283.
29. Roos, M. T. L., J. M. A. Lange, R. E. Y. de Goede, R. A. Coutinho, P. T. A. Schellekens, F. Miedema, and M. Tersmette. 1992. Viral phenotype and immune response in primary human immunodeficiency virus type 1 infection. *J. Infect. Dis.* **165**:427-432.
30. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullins, and H. A. Erlich. 1988. Primer-directed enzyme amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487-491.
31. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed., p. 9.14. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. Sato, H., J. Orenstein, D. Dimitrov, and M. Martin. 1992. Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. *Virology* **186**:712-724.
33. Schmidtmayerova, H., C. Bolmont, S. Baghdiguian, I. Hirsch, and J.-C. Chermann. 1992. Distinctive pattern of infection and replication of HIV-1 strains in blood-derived macrophages. *Virology* **190**:124-133.
34. Schuitemaker, H., M. Koot, N. A. Kootstra, M. W. Dercksen, R. E. Y. de Goede, R. P. van Steenwijk, J. M. A. Lange, J. K. M. E. Schattenkerk, F. Miedema, and M. Tersmette. 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic populations. *J. Virol.* **66**:1354-1360.
35. Schuitemaker, H., N. A. Kootstra, R. E. Y. de Goede, F. de Wolf, F. Meidema, and M. Tersmette. 1991. Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture. *J. Virol.* **65**:356-363.
36. Shioda, T., J. A. Levy, and C. Cheng-Meyer. 1991. Macrophages and T-cell line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. *Nature (London)* **349**:167-169.
37. Starcich, B. R., B. H. Hahn, G. M. Shaw, P. D. McNeely, S. Modrow, H. Wolf, E. S. Parks, W. P. Parks, S. F. Josephs, R. C. Gallo, and F. Wong-Staal. 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell* **45**:637-648.
38. Tersmette, M., R. E. Y. de Goede, B. J. M. Al, I. N. Winkel, R. A. Gruters, H. T. Cuypers, H. G. Huisman, and F. Miedema. 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J. Virol.* **62**:2026-2032.
39. Tersmette, M., R. Gruters, F. de Wolf, R. E. Y. de Goede, J. M. A. Lange, P. T. A. Schellekens, J. Goudsmit, H. G. Huisman, and F. Meidema. 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential isolates. *J. Virol.* **63**:2118-2125.
40. Tersmette, M., J. M. A. Lange, R. E. Y. de Goede, F. de Wolf,

- J. K. M. Eefink Schattenkerk, P. T. A. Schellekens, R. A. Coutinho, H. G. Huisman, J. Goudsmit, and F. Meidema.** 1989. Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. *Lancet* **i**:983–985.
41. **Westervelt, P., D. B. Trowbridge, L. G. Epstein, B. M. Blumberg, X. Li, B. H. Hahn, G. M. Shaw, R. W. Price, and L. Ratner.** 1992. Macrophage tropism determinants of human immunodeficiency virus type 1 in vivo. *J. Virol.* **66**:2577–2582.
42. **York-Higgins, D., C. Cheng-Mayer, D. Bauer, J. A. Levy, and D. Dina.** 1990. Human immunodeficiency virus type 1 cellular host range, replication, and cytopathicity are linked to the envelope region of the viral genome. *J. Virol.* **64**:4016–4020.
43. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **61**:213–222.
44. **Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho.** 1993. Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. *Science* **261**:1179–1181.