A Novel Assay Allows Genotyping of the Latent Reservoir for Human Immunodeficiency Virus Type 1 in the Resting CD4⁺ T Cells of Viremic Patients

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A latent reservoir for human immunodeficiency virus type 1 (HIV-1) consisting of integrated provirus in resting memory CD4⁺ T cells prevents viral eradication in patients on highly active antiretroviral therapy (HAART). It is difficult to analyze the nature and dynamics of this reservoir in untreated patients and in patients failing therapy, because it is obscured by an excess of unintegrated viral DNA that constitutes the majority of viral species in resting CD4⁺ T cells from viremic patients. Therefore, we developed a novel culture assay that stimulates virus production from latent, integrated HIV-1 in resting CD4⁺ T cells in the presence of antiretroviral drugs that prevent the replication of unintegrated virus. Following activation, resting CD4⁺ T cells with integrated HIV-1 DNA produced virus particles for several days, with peak production at day 5. Using this assay, HIV-1 *pol* sequences from the resting CD4⁺ T cells of viremic patients were found to be genetically distinct from contemporaneous plasma virus. Despite the predominance of a relatively homogeneous population of drug-resistant viruses in the plasma of patients failing HAART, resting CD4⁺ T cells harbored a diverse array of wild-type and archival drug-resistant viruses that were less fit than plasma virus in the context of current therapy. These results provide the first direct evidence that resting CD4⁺ T cells serve as a stable reservoir for HIV-1 even in the setting of high levels of viremia. The ability to analyze archival species in viremic patients may have clinical utility in detecting drug-resistant variants not present in the plasma.

Highly active antiretroviral therapy (HAART) for human immunodeficiency virus type 1 (HIV-1) infection can decrease levels of free virus in the plasma to below the limit of detection of the most sensitive clinical assays (25, 27, 49). However, integrated, replication-competent viral genomes accumulate in resting CD4⁺ T cells and persist there in a reversibly nonproductive form that protects the virus from decay, host immunity, and therapeutic intervention (7, 10, 12, 23, 70). This latent reservoir can serve as a source of rebound viremia if therapy is stopped (8, 20, 33, 55, 76). Longitudinal studies of patients on HAART with undetectable levels of viremia for up to 7 years have shown that the frequency of latently infected cells is extremely stable, with a half-life of approximately 44 months (22, 59). Even though the pool of latently infected cells in the body is relatively small (7), it would take over 70 years for this population to decay to less than one latently infected cell, due to the extremely long half-life (22). In patients with low CD4 counts, viral latency mandates life-long HAART to prevent progression of HIV-1 infection to AIDS (22, 62). Prolonged

exposure to antiretroviral drugs, however, can lead to severe toxicities (29), and suboptimal adherence can result in the evolution of drug resistance (2, 43, 48, 66), leading to treatment failure (13, 28, 38). It is therefore critical to develop therapeutic strategies to eliminate the resting CD4⁺ T-cell reservoir.

The genetic composition of at least a component of this reservoir is very stable in patients with suppression of viral replication on HAART (23, 26, 41, 51, 55, 62, 70, 77). The latent HIV-1 population stored in resting CD4⁺ T cells is a diverse collection of archival viral species exhibiting various degrees of divergence from the most recent common ancestor (37, 44, 51, 55, 67). Longitudinal studies have demonstrated a lack of temporal structure and evolutionary change in the viral species residing in this reservoir in most patients with undetectable plasma HIV-1 RNA measurements (3, 26, 41, 52, 55, 77). The extreme genetic stability of the latent reservoir in aviremic patients may simply be due to the lack of viral replication necessary for evolution (14), or it may indicate an intrinsic property of the resting CD4⁺ T-cell reservoir that would hold true in the setting of high levels of viral replication that result in viral evolution and immune activation. To distinguish between these possibilities, it is important to examine the genetic composition of the latent reservoir in viremic patients who have high levels of viral replication and immune activa-

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tion. Under these conditions, there might be a higher turnover of latently infected resting CD4⁺ T cells, with the entry of newly evolved viral species replacing viruses that exit due to activation of resting, memory cells. Turnover of the viral population in resting CD4⁺ T cells would alter its genetic composition to more closely resemble the plasma viral population.

Previous studies that have attempted to characterize the latent reservoir in viremic patients have not excluded the unintegrated viral DNA that predominates in the setting of high levels of viral replication (18, 34, 56). HIV-1 can enter resting CD4⁺ T cells and undergo reverse transcription, but it cannot enter the nucleus to undergo integration into the host genome (6, 53, 63, 71). Therefore, the resting CD4⁺ T cells in viremic patients harbor labile, unintegrated HIV-1 DNA (4, 53, 63, 72). Studies using PCR to examine the HIV-1 DNA in CD4⁺ T cells from viremic patients are incapable of distinguishing the quantitatively dominant unintegrated forms of HIV-1 (6, 7), which represent actively replicating virus, from the stably integrated, latent proviruses. PCR methods also fail to provide information on the replication competence of the virus amplified. Replication-competent viruses can be rescued from resting CD4⁺ T cells by cellular activation, but this approach does not distinguish preintegration and postintegration latency. Unintegrated forms of HIV-1 in the cytoplasm of resting CD4⁺ T cells can continue through the viral life cycle upon cellular activation (6, 72). The half-life of preintegration latency in resting CD4⁺ T cells is approximately 1 day, and several days of culture are required for preintegration forms of latent virus to decay (53, 61, 71). Therefore, in viremic patients, available methods potentially overestimate the turnover of the viral population in resting CD4⁺ T cells and do not give a clear view of its genetic composition.

For these reasons, we developed a novel assay to analyze stably integrated, replication-competent viral genomes in the resting CD4⁺ T cells of viremic patients. This method excludes unintegrated viral species by stimulating the expression of latent HIV-1 in the presence of antiretroviral drugs that block the replication of viruses in the preintegration state of latency. This assay was used to compare the latent viral species residing in the resting CD4⁺ T cells of viremic patients with the contemporaneous plasma viral population. The results provide the first direct demonstration of a stable latent reservoir of archival integrated virus in the resting CD4⁺ T cells of viremic patients.

MATERIALS AND METHODS

Assessment of antiretroviral drug toxicity by treatment of primary CD4⁺ T cells. Peripheral blood mononuclear cells (PBMCs) were purified from HIV-1-negative donors via Hypaque-Ficoll gradient centrifugation and depleted of monocytes by adherence. Resting CD4⁺ T cells were purified either by negative selection alone or by a combination of positive and negative selection. For negative selection, CD8⁺ T cells, B cells, monocytes, NK cells, and activated CD4⁺ T cells were removed using mouse monoclonal antibodies to appropriate cell-surface markers (CD19, CD14, CD16, CD8, CD25, CD69, and HLA-DR) and magnetic beads conjugated with antibodies to mouse immunoglobulin G (Caltag Laboratories, Becton-Dickinson, and Dynal Biotech) (10). Activated CD4⁺ T cells were depleted using antibodies to both early (CD25 and CD69) and late (HLA-DR) activation markers. Alternatively, unactivated CD4⁺ T cells were positively selected using anti-CD4-coupled magnetic beads followed by bead detachment per the manufacturer's instructions (CD4 positive isolation kit; Dynal Biotech). Positively selected CD4⁺ T cells were then depleted of activated cells and remaining unwanted cell populations by magnetic bead depletion as described above. The purified resting CD4⁺ T cells were cultured without any

antiretroviral drugs, with dimethyl sulfoxide used for reconstitution of the antiretroviral drugs, or in the presence of different combinations and concentrations of antiretroviral drugs. Cultured cells were counted daily, and their viability was determined by trypan blue exclusion or by flow cytometric analysis of propidium iodide exclusion.

Assessment of antiretroviral drug potency by infection of primary CD4⁺ T cells. PBMCs purified from HIV-1-negative donors via Hypaque-Ficoll gradient centrifugation were activated by the addition of 0.5 µg of phytohemagglutinin (PHA)/ml and cultured for 3 days in medium containing interleukin-2 (IL-2) and cytokine-rich supernatant from activated T cells. On day 4, CD4⁺ T cells were positively selected using anti-CD4 coupled to magnetic beads followed by bead detachment per the manufacturer's instructions (CD4 positive isolation kit; Dynal Biotech). These activated primary CD4⁺ T cells were infected with a vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped, envelope-deleted, HIV-1 reporter virus (53) at a multiplicity of infection of 0.1 by spin inoculation at 1,800 × g at 30°C for 2 h (45). Infections were carried out in the absence and presence of the reverse transcriptase inhibitors (RTIs) lamivudine (3TC; 8.6 µM), 9-[2-(R)-(phosphonomethoxy)propyl] adenine (PMPA; 470 µM), and efavirenz (EFV; 0.5 µM) and/or with the integrase inhibitors L731988 (L7; 40 µM) or compound 5 (C5; 20 µM). Integrase inhibitors were kindly provided by Michael Miller, Merck Research Laboratories. Fresh drugs were added to cultures of the infected cells daily, and culture supernatants were sampled on days 2, 3, 5, 7, and 9 postinfection for analysis of virion production by p24 enzyme-linked immunosorbent assay (ELISA; Beckman-Coulter).

Selection of study participants. Patients were selected based on detectable viremia, a history of treatment failure, and CD4 counts of >200 cells/µl. The latter criterion ensured a yield of resting CD4⁺ T cells high enough to permit ready analysis of latent, integrated HIV-1. Patients who maintained detectable plasma HIV-1 RNA levels were sampled longitudinally every 3 months. Informed consent was obtained from all patients.

Plasma collection and purification of resting CD4⁺ T cells. The purification of resting CD4⁺ T cells has been previously described (10). In brief, PBMCs were purified from 120 to 180 ml of whole blood via Hypaque-Ficoll gradient centrifugation. For this assay, resting CD4⁺ T cells can also be purified from PBMCs that have been cryopreserved in liquid nitrogen (data not shown). The plasma layer was collected and frozen in 1.5-ml aliquots at -80°C for subsequent RNA isolation and reverse transcriptase PCR (RT-PCR). PBMCs were negatively selected to remove CD8⁺ T cells, B cells, monocytes, NK cells, and activated CD4⁺ T cells using mouse monoclonal antibodies to appropriate cell surface markers and magnetic beads conjugated with antibodies to mouse immunoglobulin G (Caltag Laboratories, Becton-Dickinson, and Dynal Biotech). Activated CD4⁺ T cells were depleted using antibodies to both early (CD25 and CD69) and late (HLA-DR) activation markers. Further purification of resting CD4⁺ T cells was accomplished by sorting for small lymphocytes with high CD4 and low HLA-DR surface expression on a MoFlo cell sorter (DakoCytomation) using phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR (Becton-Dickinson). The resulting resting CD4⁺ T-cell populations were generally >99% pure. Aliquots of highly purified resting CD4⁺ T cells were lysed and frozen for DNA isolation and PCR to allow comparison with HIV-1 *pol* clones obtained by RT-PCR of the plasma and culture supernatants. DNA was isolated using the Puregene DNA isolation kit (Gentra), and nested PCR was performed as previously described (51).

Culture assay for postintegration latent virus. Highly purified resting CD4⁺ T cells were cultured in the presence of four antiretroviral drugs for 24 h in order to allow the prodrugs to be processed to their fully active forms prior to cellular activation. Integration is unlikely to occur during this period because of the block to the virus life cycle prior to integration in resting CD4⁺ T cells (6, 54, 64, 72). The four antiretroviral drugs used were one nucleoside reverse transcriptase inhibitor (NRTI; 8.6 µM 3TC), one nucleotide reverse transcriptase inhibitor (NRTI; 470 µM PMPA), one nonnucleoside reverse transcriptase inhibitor (NNRTI; 0.5 µM EFV), and one integrase inhibitor (either 40 µM L7 or 20 µM C5). The cells were then activated in the presence of the four antiretroviral drugs by adding 0.5 µg of PHA/ml and irradiated PBMCs from an HIV-1-negative donor to stimulate virus production from integrated HIV-1. Activated cells were cultured in medium containing IL-2 and cytokine-rich supernatant from activated T cells. The culture supernatant containing HIV-1 produced from activated cells was collected daily, and fresh medium and drugs were added to the culture. Culture supernatants were frozen in 1.5-ml aliquots at -80°C for RNA isolation and RT-PCR. The ultrasensitive Roche Amplicor HIV-1 Monitor test was used to determine the concentration of HIV-1 RNA in the culture supernatants. A sample of the cultured cells was stained with trypan blue and counted daily to assess viability and the amount of cellular proliferation following activation. Controls included cultures of unactivated resting CD4⁺ T cells in the

presence and absence of antiretroviral drugs and cultures of activated cells in the absence of antiretroviral drugs. The nonparametric sign test was used to determine the significance of differences in virion production between cultures of activated resting CD4⁺ T cells in the presence of antiretroviral drugs and control cultures.

HIV-1 RNA isolation and RT-PCR. Virus from 1.5 to 10.5 ml of plasma or culture supernatant was pelleted at $23,600 \times g$ for 2 h at 4°C as previously described (19). Viral pellets were lysed, and the viral RNA was isolated using a QIAmp viral RNA mini kit per manufacturer instructions (QIAGEN). For samples with large amounts of viral RNA, 140 μ l of plasma was lysed directly without ultracentrifugation. Eluted viral RNA was protected with 30 U of porcine RNAGuard (Amersham Biosciences) and treated for 15 min at room temperature with 7.5 U of DNase (Invitrogen). The DNase reaction was stopped with 2.5 mM EDTA and incubation at 65°C for 10 min.

Eight separate RT-PCRs were set up for each patient sample, including six reactions with RT and two RT-negative control reactions to test for DNA contamination. One control reaction without sample RNA was also performed to test for RNA contamination. Annealing of the RT primer, 3' outer *pol* (CCTT GCCCTGCTTCTGTATTCTGC; 3 μ M), was carried out at 50°C for 10 min. The 5' outer *pol* primer (GCCCTAGGAAAAAGGGCTGTTGG) and additional 3' outer *pol* primer were added to the reaction mixtures at 0.4 and 0.2 μ M, respectively. RT was performed in the presence of 42 U of RNAGuard using the Superscript one-step RT-PCR for long templates (Invitrogen) at 50°C for 30 min and was followed immediately by the first round of nested PCR under the following reaction conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 90 s; and a final extension at 68°C for 5 min. Platinum *Taq* HiFi alone was used for the two RT-negative reactions. Nested PCR was performed with 1:40 dilutions of the first-round PCR products using 2.5 U of Platinum *Taq* HiFi or Platinum *Pfx* (Invitrogen). Nested PCR with Platinum *Taq* HiFi used 1 μ M 5' inner *pol* primer (TGAAAGATTGTACTGAGAGACAGG), 1 μ M 3' inner *pol* primer (CATGTACCGGTTCTTTAGAAATCTCCCTGTT), 0.2 mM deoxynucleoside triphosphates (dNTPs), and 2 mM MgSO₄ under the same reaction conditions as in the first-round PCR (30). Nested PCR with Platinum *Pfx* used primers incorporating the *Apa*I and *Age*I sites required for cloning into the pNL4-3-derived vector utilized in the phenotypic assay. Nested PCR with Platinum *Pfx* was carried out with 0.4 μ M 5' inner *pol* primer (TGCAAGGCC CCTAGGAAAAAGGGCTG), 0.4 μ M 3' inner *pol* primer (CATGTACCGGT TCTTTAGAAATCTCCCTGTT), 0.3 mM dNTPs, and 1 mM MgSO₄ under the following reaction conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 90 s; and a final extension at 68°C for 5 min (75).

RT-PCR in which the protease (PR) and RT regions of HIV-1 *pol* were amplified separately with a sensitivity of 1 to 5 RNA copies per reaction mixture was used for samples with very low amounts of viral RNA (35, 52). RT-PCR for amplification of *PR* was carried out with the Superscript one-step RT-PCR system at 50°C for 30 min followed by the first round of nested PCR with 0.4 μ M 5' outer PR primer (GCCCTAGGAAAAAGGGCTGTTGG), 0.4 μ M 3' outer PR primer (GCTTTTATTTCTCTCTGTCAATGGCC), and 75 U of RNAGuard under the following reaction conditions: 94°C for 3 min; 32 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 30 s; and a final extension at 68°C for 5 min. Nested PCR using 1:40 dilutions of the first-round PCR products was performed with 0.5 mM 5' inner PR primer (TGAAAGATTGTACTGAGAGACAGG), 0.5 mM 3' inner PR primer (CCTGGCTTTAATTTTACTGGTACAG), 0.2 mM dNTPs, 2 mM MgCl₂, and 2.5 U Platinum *Taq* HiFi as follows: 94°C for 3 min; 32 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s; and a final extension at 68°C for 5 min. RT-PCR for amplification of *RT* was carried out with Superscript one-step RT-PCR at 52.5°C for 30 min followed by the first round of nested PCR with 0.5 μ M 5' outer RT primer (GTCCTRTTGAAACYGTRC), 0.5 μ M 3' outer RT primer (GGCTGTACTGTCCAYTTA), 3 mM MgSO₄, and 30 U of RNAGuard under the following reaction conditions: 94°C for 3 min; 32 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s; and a final extension at 68°C for 5 min. Nested PCR of 1:40 dilutions was performed with 0.5 mM 5' inner RT primer (AYGGCCCAARAGTYAAC), 0.5 mM 3' inner RT primer (TTATCAGGATGGAGYTCA), 0.2 mM dNTPs, 2 mM MgCl₂, and 2.5 U of Platinum *Taq* HiFi as follows: 94°C for 3 min; 32 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 45 s; and a final extension at 68°C for 5 min.

Cloning and sequence analysis. Products of 1.5 kb containing all of HIV-1 *PR* and the first 915 nucleotides of *RT* were obtained for 50 to 100% of the six reactions. The reaction products were gel purified (QIAquick gel extraction kit; QIAGEN) and cloned using the Zero Blunt TOPO cloning kit for sequencing (Invitrogen) or by digestion with *Age*I and *Apa*I followed by ligation with pNL4-3 vector (75). Bidirectional sequencing was performed using the following primers: 5' PR (CAACTCCCTCTCAGAAGCAGGAGCCG), 3' PR (CTTCC CAGAAGTCTTGAG), 5' RT (CCTGTCAACATAATTGGA), and 3' RT (A

TTGCYTTACTTTAATCC). The gel-purified 572-bp HIV-1 *PR* PCR products and the gel-purified 663-bp HIV-1 *RT* PCR products were cloned using the Zero Blunt TOPO cloning kit and sequenced bidirectionally using vector primers T3 and T7. Sequencing was performed via a fluorescent dideoxy terminator method of cycle sequencing on an Applied Biosystems 3730 automated DNA sequencer following the manufacturer's protocols.

Sequences were manually inspected and aligned using Sequencher (Gene Codes, Inc.). Clones obtained from different PCRs were considered independent. Clones obtained from the same PCR were considered independent if they differed by two or more nonsynonymous mutations and one or more synonymous mutations. These criteria were based on the predicted frequency of PCR-induced mutations using an estimated error rate of 4.5×10^{-5} substitutions per nucleotide per cycle, a conservative estimate compared to the manufacturer's reported error rates of 1.8×10^{-6} for Platinum *Taq* HiFi and 0.44×10^{-6} for Platinum *Pfx* (Invitrogen) (60).

Neighbor-joining phylogenetic trees were generated to determine the patient specificity of independent clones using PAUP* version 4b10 (Sinauer Associates, Sunderland, Mass.). Distances for the tree were calculated using the HKY+G model, with transition/transversion ratio and shape parameter estimated empirically. The tree was visualized in TreeView (Rod Page). The commonly used HIV-1 laboratory strains HXB2, LAI, and NL4-3 were included to rule out contamination. The robustness of phylogenetic clustering was assessed using bootstrap analysis with 1,000 resamplings of the sequence data.

Net mean distances between groups, the within-group diversity, and the divergence from an inferred most recent common ancestor (MRCA) were calculated for the sequences (MEGA version 2.1) (36a). The MRCA for each patient was inferred using PAUP* version 4b10. The location of each MRCA near the patient-specific branch point was confirmed by inclusion on the neighbor-joining phylogenetic tree. To reduce the impact of artifactual substitutions on the analysis, sporadic substitutions were removed using VarPlot (Stuart Ray) as previously described (35, 60). Standard errors of the genetic distances were calculated by bootstrap analysis with 100 resamplings. The parametric one-sample *t* test was used to determine whether the plasma and culture viral populations were significantly different. The nonparametric sign test was used to determine whether the plasma diversity and divergence were significantly different from the culture diversity and divergence.

Analysis of phenotypes of patient HIV-1 *pol* clones. The replication capacity of recombinant HIV-1 carrying patient-derived *gag-pol* sequences was assessed using a phenotypic assay as previously described (75). Briefly, recombinant HIV-1 vectors were made by replacing the 1.5-kb *Apa*I/*Age*I fragment of pNL4-3- Δ E-GFP with patient-derived *gag-pol* sequences amplified with primers containing *Apa*I and *Age*I sites. Virus pseudotyped with VSV-G was produced from the recombinant vectors via cotransfection of 293T cells in the presence or absence of protease inhibitors. Supernatants containing the recombinant virus were purified by passage through a 0.2- μ m filter and standardized based on the number of green fluorescent protein (GFP)-positive 293T cells per unit volume at 24 h posttransfection. Jurkat cells were infected using viral supernatants in the presence or absence of RT inhibitors and analyzed by fluorescence-activated cell sorter (FACS) for GFP expression 48 h postinfection after fixation with 1% paraformaldehyde. Replication capacity, quantified as the percentage of GFP-positive Jurkat cells, was examined in the absence of antiretroviral drugs and at the minimum and maximum plasma drug concentrations (*C*_{min} and *C*_{max}, respectively) for the antiretroviral drugs in the HAART regimen at the time of sampling.

RESULTS

Culture assay for characterizing latent, integrated HIV-1 in resting CD4⁺ T cells from viremic patients. We developed a novel culture method to stimulate the production of virus from latently infected resting CD4⁺ T cells carrying integrated HIV-1 DNA while simultaneously preventing the replication of virus in the preintegration state of latency. Resting CD4⁺ T-cell populations with purities of generally >99% were obtained from the blood of viremic patients as previously described (10), using magnetic bead depletion of unwanted cell types followed by FACS for CD4⁺ HLA-DR⁻ cells (Fig. 1). Highly purified resting CD4⁺ T cells were activated in the presence of HIV-1 RT and integrase inhibitors under conditions that induce virtually 100% of resting T cells to undergo

FIG. 1. Purification of resting CD4⁺ T cells from HIV-1-infected patients with viremia. Flow cytometric analysis results are shown for unsorted PBMCs (top panel) and highly purified resting CD4⁺ T lymphocytes (bottom panel) stained with phycoerythrin-conjugated anti-CD4 and FITC-conjugated anti-HLA-DR antibodies. Numbers indicate the percentages of cells in each quadrant.

blast transformation (31, 47, 53). Culture supernatants were collected, and fresh medium and antiretroviral drugs were added daily. The amount of virus in culture supernatants was quantified using the ultrasensitive Roche Amplicor HIV-1 Monitor test, and the HIV-1 *pol* gene was amplified via RT-PCR and cloned for genetic analysis.

The critical feature of this assay is the use of a combination of antiretroviral drugs to block the replication of the unintegrated, latent virus that predominates in patients with high levels of viremia. Four antiretroviral drugs were used to block the viral life cycle prior to integration, thereby preventing the rescue of viruses in the preintegration state of latency that would otherwise occur when infected resting CD4⁺ T cells from viremic patients are activated in culture (6, 72). Because reverse transcription is very slow in resting CD4⁺ T cells, many recently infected resting cells contain incomplete reverse transcripts (36, 53, 72). RT inhibitors can prevent the rescue of virus from these cells (53). Thus, the chain-terminating NRTIs

3TC and PMPA (tenofovir), along with the potent NNRTI EFV, were used to block the replication of any viruses that had not yet completed reverse transcription. In addition, an integrase inhibitor, either L7 or C5, was used to prevent any completely reverse transcribed viral DNA from integrating into the host DNA upon cellular activation. 3TC and PMPA were used in the culture assay at concentrations equivalent to their in vivo C_{max} values of 8.6 and 470 μ M, respectively. EFV proved to be too toxic at its C_{max} (13 μ M) and was used at a concentration of 0.5 μ M, greatly reducing its toxicity without significantly changing its in vitro potency (99% inhibitory concentration, 0.1 μ M). The concentrations of the integrase inhibitors, 40 μ M for L7 and 20 μ M for C5, were selected based on manufacturer recommendations and experiments characterizing potency and toxicity (data not shown). The inclusion of a combination of four antiretroviral drugs for the duration of the culture also prevents multiple rounds of infection, ensuring that no viral evolution or selection occurs in culture.

Potent inhibition of HIV-1 replication by RT and integrase inhibitors. To confirm that these inhibitors prevent the replication of viruses in the preintegration state of latency, primary CD4⁺ T lymphoblasts were infected in vitro with a VSV-G-pseudotyped HIV-1 reporter virus at a high multiplicity of infection (0.1) in the absence or presence of the relevant antiretroviral drugs. In comparison with the no-drug control, all drug combinations potentially inhibited virus replication in these activated cells as assessed by p24 production (Fig. 2A). Both four-drug combinations, including the three RTIs and an integrase inhibitor (L7 or C5), showed a 97% inhibition of p24 production 9 days postinfection. The three RTIs alone showed a similar level of inhibition of p24 production (98%). The integrase inhibitors alone also potentially inhibited p24 production, with L7 showing 94% inhibition and C5 showing 96% inhibition at 9 days postinfection. Thus, even under experimental conditions where a high fraction of cells are infected and all of the virus is in the preintegration state, the antiretroviral drugs suppressed p24 production to near-background levels. Therefore, any virus rescued from patient samples was likely to have come from integrated provirus.

The potency of these antiretroviral combinations was also evident in a comparison of the amount of virus produced upon activation of resting CD4⁺ T cells from viremic patients in the presence and absence of the drugs. Total virion production, calculated from daily measurements of HIV-1 RNA in the supernatant over 7 days in culture, was greatly decreased by the addition of antiretroviral drugs (Fig. 2B). The mean inhibition was 90% (range, 69 to 99%). In contrast to the in vitro infections described above, incomplete inhibition was expected here because there is also integrated, latent virus in resting CD4⁺ T cells from infected individuals. These viral genomes can produce virions following cellular activation, even in the presence of the antiretroviral drugs. The significant decrease in the total virion production in the presence of antiretroviral drugs is consistent with the drugs being effective in preventing virus production by cells in the preintegration state of latency following cellular activation. In fact, the greatest inhibition of total virion production (99%) was seen for the patient who had the highest plasma HIV-1 RNA measurement and likely the highest number of recently infected resting CD4⁺ T cells with unintegrated virus (sample 102t2; 17,155 copies/ml) (Fig. 2B).

FIG. 2. RT and integrase inhibitors block the rescue of virus in the preintegration state of latency. (A) Ability of antiretroviral drug combinations to prevent unintegrated virus from completing the viral life cycle, as determined by in vitro infection. Activated primary CD4⁺ T cells were infected with VSV-G-pseudotyped HIV-1 at a high multiplicity of infection (0.1) in the presence or absence of the indicated drugs. Fresh drugs were added daily, and supernatants were assayed for HIV-1 p24 antigen by ELISA. Day 9 p24 values are shown. (B) Effects of antiretroviral drug combinations on activation-induced virus production from resting CD4⁺ T cells of viremic patients. Highly purified resting CD4⁺ T cells from four patient samples were activated in the presence (+) or absence (–) of four antiretroviral drugs. Culture supernatants were sampled daily, and the concentration of HIV-1 RNA was determined by RT-PCR. The number of virions produced in culture each day was calculated by multiplying the number of HIV-1 RNA copies per milliliter by the culture volume (5 ml) and dividing by 2 RNA copies/virion. The total virion production for 7 days in culture was standardized per million CD4⁺ T cells activated in culture.

These results support the conclusion that the potent drug combination used in the culture assay prevents the replication of viruses in the quantitatively dominant preintegration state of latency, allowing detection of the postintegration form. Further support for this conclusion comes from the evidence presented below that this drug combination allows the isolation of wild-type virus, even in patients whose circulating virus populations are highly resistant (see Fig. 5, 6, 7, 8, and 10, below). These results also provide a dramatic illustration of the fact that analysis of the stable pool of integrated virus in the resting

CD4⁺ T cells of viremic patients cannot be done without special methods to prevent the detection of unintegrated forms.

Activation dependence and kinetics of virion production. Highly purified resting CD4⁺ T cells from the peripheral blood of patients who have suppression of viremia on HAART do not produce virus without cellular activation (10, 11, 31, 40). However, in patients who are viremic, the low-level release of virus from populations of resting CD4⁺ T cells can be detected (11, 78). This may result from abnormal immune activation affecting a subset of the infected cells. In the assay system described here, mitogen activation is used to induce resting CD4⁺ T cells from viremic patients to undergo blast transformation and become permissive for virus production. It was therefore important to demonstrate that virion production in this culture system was dependent on cellular activation. Resting CD4⁺ T cells from viremic patients were cultured in the presence of antiretroviral drugs with or without activation on day 2. Efficient activation was induced by adding PHA and irradiated PBMCs from an HIV-1-negative donor. Although cultures of unactivated resting CD4⁺ T cells from viremic patients showed low levels of virion production, cellular activation significantly increased total virion production for all seven patient samples tested (Fig. 3A). Activated resting CD4⁺ T cells from viremic patients produced a median of 15-fold more virions per million cells over 7 days in culture than unactivated cells (range, 2- to 27-fold). Interestingly, as shown in Fig. 3B, virion production by unactivated cells correlated with the plasma HIV-1 RNA levels at the time the sample was taken ($R^2 = 0.998$), consistent with the notion that active viral replication in vivo increases the permissiveness of resting CD4⁺ T cells for virus production (11). The same precise correlation was not observed following activation in the presence of drugs ($R^2 = 0.547$) (Fig. 3B). In this situation, virion production reflects the activation of a stable reservoir of integrated virus that does not vary directly with plasma virus levels. In summary, activation of resting CD4⁺ T cells from viremic patients in the presence of RT and integrase inhibitors results in an increase of total virion production that is not correlated to the plasma HIV-1 RNA levels.

Activation of resting CD4⁺ T cells from viremic patients also caused a change in the rate of virion production. The virion production rate increased in all 16 cultures of activated CD4⁺ T cells from viremic patients (Fig. 4A). Daily virion production in activated cultures increased by up to 2 logs over time in culture. The geometric mean ratio of day 7 virus production to day 1 virus production for activated cultures was 29 (range, 2 to 332). This is consistent with the induction of latent virus following cellular activation. All seven cultures of unactivated resting CD4⁺ T cells from viremic patients showed no significant change or a decrease in virion production over time in culture. The geometric mean ratio of virus production on day 7 to virus production on day 1 for unactivated cultures was less than 1, indicating a decrease in the rate of virus production. This result is consistent with the turnover of resting CD4⁺ T cells that were stimulated in vivo to produce virus.

To determine the optimal time for genetic analysis of latent HIV-1, the kinetics of virion production were examined in 16 cultures of resting CD4⁺ T cells activated in the presence of antiretroviral drugs. The kinetics were similar in all cultures (Fig. 4B). Daily virion production began to increase a median

FIG. 3. Activation of resting CD4⁺ T cells from viremic patients increases HIV-1 virion production. (A) Effect of cellular activation on virus production from resting CD4⁺ T cells with integrated HIV-1 DNA. Highly purified resting CD4⁺ T cells from seven viremic patients were cultured in the presence of four antiretroviral drugs with or without activating stimuli (PHA and irradiated allogeneic PBMCs). The number of virions produced over 7 days normalized per million cells at day 2 in culture is shown. (B) Correlation between plasma viral levels and total virion production by unactivated (left panel) and activated (right panel) resting CD4⁺ T cells in the presence of drugs from viremic patients over 7 days in culture.

of 3 days after activation (range, 2 to 5 days), around the same time the cells started to proliferate. The maximum daily HIV-1 virion production occurred between 4 and 7 days after cellular activation for all 16 cultures (median, 5 days). In this system, the drugs prevent the spread of infection to other cells, and therefore the results provide a direct picture of the kinetics of virus production by individual latently infected cells following activation. Given that most productively infected cells in vivo turnover rapidly, with a half-life of about 1 day (32, 50, 69), it is interesting that at least in vitro, latently infected cells produce virus for several days following activation. In cultures followed past day 9, virus production reached a plateau and declined. The culture supernatant sample from the day with the greatest virion production was used for HIV-1 RNA amplification to maximize the number of viral species for genotypic and phenotypic characterization.

Latent, integrated HIV-1 in resting CD4⁺ T cells is genetically distinct from plasma virus in patients failing HAART. Using the assay described above, we compared latent, integrated HIV-1 recovered from highly purified resting CD4⁺ T cells to actively replicating plasma virus in 16 blood samples obtained from 12 patients (Table 1). Patients were chosen for the study based on recent detectable viremia and a history of treatment failure. The selection of patients with a history of treatment failure allowed us to compare the frequency of wild-type and drug-resistant viruses in the latent reservoir and the plasma. Patients with CD4 counts of >200 cells/ μ l were selected to maximize the yield of resting CD4⁺ T cells and thereby maximize the amount of latent, integrated HIV-1 that could be obtained from each patient for genetic characterization. At the time of sampling, patients had a broad range of plasma virus levels from <50 to 63,010 copies/ml, with a mean

FIG. 4. Kinetics of HIV-1 virion production following activation of resting CD4⁺ T cells from viremic patients. (A) Activation increases the rate of virion production from the resting CD4⁺ T cells of viremic patients. The geometric mean ratio of the virion production rate on day 7 to the virion production rate on day 1 for unactivated and activated cultures is shown. Error bars depict the standard errors of the means. (B) Kinetics of virion production following cellular activation. Purified resting CD4⁺ T cells from viremic patients were activated with PHA and irradiated allogeneic PBMCs on day 2 in the presence of four antiretroviral drugs. The ratio of virion production on the indicated day to virion production on day 1 is indicated. The heavy black line indicates the geometric mean ratio for all 16 cultures.

of 7,682 copies/ml. The plasma virus levels for three patients were undetectable at the time of sampling despite previous measurements of >50 copies/ml. The CD4 counts at the time of sampling ranged widely from 230 to 766 cells/ μ l (mean, 452 cells/ μ l). The patient population was also heterogeneous with respect to antiretroviral treatment history and degree of virologic suppression. All of the study participants had a history of prior treatment failure, with a mean time of 5 years of viral replication in the presence of antiretroviral therapy (range, 8 to 128 months). Eleven of the 12 patients were on HAART at the time of sampling. These patients had been on their current therapy for a mean of 9 months (range, 0.5 to 20 months) and had been failing their current therapy for a mean of 7 months (range, 0.5 to 20 months).

The HIV-1 *pol* gene was amplified by RT-PCR from supernatants of resting CD4⁺ T-cell cultures activated in the pres-

ence of RT and integrase inhibitors and from contemporaneous plasma. A 1.5-kb region of *pol* including *PR* and the first 915 nucleotides of *RT* was amplified for most samples. More sensitive RT-PCR assays that separately amplified *PR* and a 663-bp region of *RT* were used for samples with lower amounts of HIV-1 RNA. A mean of eight independent clones from plasma and eight independent clones from resting CD4⁺ T cells was obtained for each of the 16 samples.

Sequencing independent viral clones from the plasma and from resting CD4⁺ T cells revealed striking differences between the two compartments. Representative examples are shown in Fig. 5. A majority of independent viral clones from the plasma of viremic patients contained mutations known to confer resistance to the current antiretroviral regimen, while wild-type clones predominated in resting CD4⁺ T cells. For example, in patient 137, all of the independent clones obtained

TABLE 1. Characteristics of the patient cohort^h

Patient data				Infection history							Sample information		
Pt no.	Sex	Race	Age (yrs)	Infected ^a (mos)	ART ^b (mos)	VL ^{+c} (mos)	ART VL ^{+d} (mos)	Current ART ^e (mos)	Current ART VL ^{+f} (mos)	Previous VL ^g (c/ml)	VL (c/ml)	CD4/ μ l	Current ART
001	M	W	38	153	107	106	60	30	30	62,705	30,498	364	None
096	F	B	59	105	97	47	39	18	11	82	32	685	AZT, 3TC, TDF, EFV
100	M	B	47	132	131	129	128	6	6	403	72	455	AZT, 3TC, ABC, TDF, LPV/r
102	M	W	42	85	80	30	25	6	6	528	792	766	AZT, 3TC, TDF, NVP, SQV, RTV
			42	88	83	33	28	9	9	792	17,155	506	AZT, 3TC, TDF, NVP, SQV, RTV
			42	91	86	36	31	1	1	214	43	512	AZT, 3TC, ABC, TDF, LPV/r
105	M	B	46	171	83	171	83	20	20	5,697	9,063	283	AZT, 3TC, ABC
			47	174	86	174	86	1	1	16,019	1,368	230	3TC, ddI, TDF, EFV, LPV/r
			47	176	88	176	88	3	3	427	38	303	3TC, ddI, TDF, EFV, LPV/r
110	M	W	42	196	54	167	25	18	9	120	38	498	AZT, 3TC, ABC, TDF, EFV
112	M	W	42	149	99	149	99	9	9	23,000	63,010	291	AZT, 3TC, ABC
122	M	W	49	53	43	41	31	8	8	2,795	<50	532	AZT, 3TC, ABC, TDF, LPV/r
126	M	W	56	209	151	155	97	1	1	239	<50	340	AZT, 3TC, TDF, NVP, LPV/r
127	M	W	37	50	21	37	8	13	7	639	436	279	AZT, 3TC, ABC
137	M	W	44	215	119	201	105	8	8	518	<50	699	ABC, TDF, EFV, LPV/r
138	M	B	53	223	81	175	33	9	9	409	211	488	TDF, NVP, SQV, RTV
Median			45	151	86	139	50	9	8	523	142	472	
Mean			46	142	88	114	60	10	9	7,162	7,682	452	

^a Time from diagnosis of HIV-1 infection to sampling date, in months.

^b Time patients have been on antiretroviral therapy, not necessarily consecutively.

^c Time patients have had detectable plasma HIV-1 RNA measurements (either >400 or >50 copies/ml).

^d Time patients have had detectable plasma HIV-1 RNA measurements while on antiretroviral therapy.

^e Time patients have been on their current antiretroviral regimen.

^f Time patients have had detectable plasma HIV-1 RNA measurements on their current antiretroviral regimen.

^g Plasma HIV-1 RNA measurement at visit prior to sampling date.

^h Abbreviations: Pt, patient; M, male; F, female; W, white; B, black; ART, antiretroviral therapy; VL, viral load; c, copies.

from the plasma contained a set of mutations associated with broad resistance to NRTIs. At the same time point, sequences from resting CD4⁺ T-cell cultures were wild type (Fig. 5A). For patient 105, clones detected in the plasma all contained an extensive set of five to seven mutations conferring resistance to NRTIs (Fig. 5B). In contrast, clones from resting CD4⁺ T-cell cultures were either wild type or displayed archival patterns of resistance with two mutations. It is important to note that in viremic patients, the labile, unintegrated virus in recently infected resting CD4⁺ T cells will resemble the plasma virus. Thus, the ability of this assay to retrieve viral species from resting CD4⁺ T cells that are not found in contemporaneous plasma provides strong confirmation that the method is able to access stably integrated viral genomes.

Further evidence that the assay was capable of excluding unintegrated virus was provided by a comparison of the viral species obtained from cultures of resting CD4⁺ T cells of viremic patients in the presence and absence of drugs. The culture of cells without the use of antiretroviral drugs to block the rescue of unintegrated viral genomes resulted in the isolation of virus that was uniformly drug resistant, resembling actively replicating virus from the plasma. For example, in patient 102, all of the viral species from the plasma and from cultures without drugs contained drug resistance mutations, whereas none of the viral species from activated cultures with drugs were resistant (Fig. 6). Similar results were obtained in two other samples.

Interestingly, virus spontaneously released from unactivated resting CD4⁺ T cells from viremic patients also contained drug-resistant virus that resembled the actively replicating vi-

rus in the plasma (Fig. 6). This genetic similarity and the correlation between levels of virus production in unactivated cultures and viremia (Fig. 3B) suggest that HIV-1 spontaneously produced from resting CD4⁺ T cells in viremic patients does not represent the release of archival virus, but rather recent infection subject to the current drug-selective pressure. It is likely that these cells have received some stimulus making them permissive to viral replication without changing their surface expression of activation markers (11, 57, 65, 78).

Latent, integrated HIV-1 in resting CD4⁺ T cells is phylogenetically distinct from plasma virus in viremic patients. Phylogenetic analysis was used to further examine whether virus from resting CD4⁺ T cells was genetically distinct from the plasma virus population in viremic patients. Neighbor-joining phylogenetic trees of independent clones demonstrated patient-specific clustering, confirming the expected origin of the sequences. As is shown in Fig. 7, viral sequences from resting CD4⁺ T-cell cultures clustered separately from contemporaneous plasma virus in 10 out of 12 patients (all patients except 001 and 112). Plasma typically contained the most resistant and most divergent viral species. Latent integrated HIV-1 in resting CD4⁺ T cells was primarily wild type, although some viral species contained archival patterns of drug resistance (patients 096, 100, 102, 105, 126, and 138). Virus with high-level resistance was also found in the resting CD4⁺ T cells of some patients, consistent with the entry of recently evolved viral species into these cells (patients 096, 105, and 126). The differences between the culture and plasma virus of most viremic patients studied suggest that the turnover of integrated HIV-1 in resting CD4⁺ T cells is very slow, resulting

A.

Pt#137	< 50 c/ml	TDF										ABC										EFV										LPV/r									
Source	Clone	RT																				PR																			
		2 0 6 6 6 7 7 7 1 1 1 1 1 2 2 2 3 5 7 1 1 2 3 1 3 6 6 7 9 3																																							
		K K S T K V F F E D I Q D R L V I S L I V I																																							
Plasma	1	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	2	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	3	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	4	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	5	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	6	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	7	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	8	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	9	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	10	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
	11	-	R	G	I	R	I	L	Y	K	E	-	M	E	K	F	I	-	D	P	L	I	L																		
Culture	1	-	-	-	-	-	-	-	-	K	E	T	-	E	-	F	I	V	D	P	L	I	L																		
	2	-	-	-	-	-	-	-	-	K	E	T	-	-	-	F	I	V	D	P	L	I	L																		
	3	-	-	-	-	-	-	-	-	K	E	T	-	-	-	F	I	V	D	P	L	I	L																		
	4	-	-	-	-	-	-	-	-	K	E	T	-	-	-	F	I	V	D	P	L	I	L																		
	5	-	-	-	-	-	-	-	-	K	E	T	-	-	-	F	I	V	D	P	L	I	L																		
	6	-	-	-	-	-	-	-	-	K	E	T	-	-	-	F	I	V	D	P	L	I	L																		
	7	-	-	-	-	-	-	-	-	K	E	T	-	-	-	F	I	V	D	P	L	I	L																		
	8	-	-	-	-	-	-	-	-	K	E	T	-	-	-	F	I	V	D	P	L	I	L																		

B.

Pt#105	9,063 c/ml	AZT										ABC										3TC																			
Source	Clone	RT																				PR																			
		3 3 4 4 5 6 6 9 1 1 1 1 2 2 2 2 2 2 2 2 3 3 3 6 6 6 6 7																																							
		5 9 1 4 8 0 0 7 9 8 2 5 8 9 0 0 1 1 1 1 1 1 5 9 3 3 3 6 0 1 3 2																																							
V T M E S I V D T A E A M G E Q L R L T K																				V M S D Q L I																					
Plasma	1	I	A	L	D	T	-	I	N	-	S	K	S	V	E	K	E	W	K	F	Y	N	I	I	N	E	E	P	T												
	2	I	A	L	D	T	-	I	N	-	S	K	S	V	E	K	E	W	K	F	Y	N	I	I	N	E	E	P	T												
	3	T	M	L	D	T	-	I	-	-	S	K	S	V	E	K	E	W	K	F	Y	N	I	I	N	E	E	P	T												
	4	T	M	L	D	T	-	I	-	A	S	K	S	V	-	K	E	W	K	F	Y	-	I	I	N	E	E	P	T												
	5	T	M	L	D	T	-	I	-	-	S	K	S	V	E	K	E	W	K	F	Y	-	I	I	N	E	E	P	T												
	6	T	M	L	D	T	-	I	-	-	S	K	S	V	E	K	E	W	K	F	Y	-	I	I	N	E	E	P	T												
	7	T	M	L	D	T	-	I	-	-	S	K	S	V	E	K	E	W	K	F	Y	-	I	I	N	E	E	P	T												
	8	T	M	L	D	T	-	I	-	-	S	K	S	V	E	K	E	W	K	F	Y	-	I	I	N	E	E	P	T												
	9	T	M	L	D	T	-	I	-	-	S	K	S	V	E	K	E	W	K	F	Y	-	I	I	N	E	E	P	T												
Culture	1	T	-	-	-	T	-	-	-	-	K	S	V	-	-	E	-	K	F	Y	-	I	I	N	E	E	P	T													
	2	T	-	-	-	T	-	-	-	-	K	S	V	-	-	E	-	K	F	Y	-	I	I	N	E	E	P	T													
	3	T	-	-	-	-	V	-	-	-	K	S	V	-	-	E	-	K	F	Y	-	I	I	N	-	-	P	T													
	4	T	-	-	-	-	V	-	-	-	K	S	-	-	-	-	-	K	F	-	-	I	I	N	-	-	P	T													
	5	T	-	-	-	-	V	-	-	-	K	S	-	-	-	-	-	K	F	-	-	I	I	N	-	-	P	T													
	6	T	-	-	-	-	V	-	-	-	K	S	-	-	-	-	-	K	F	-	-	I	I	N	-	-	P	T													
	7	T	-	-	-	-	V	-	-	-	K	S	-	-	-	-	-	K	F	-	-	I	I	N	-	-	P	T													
	8	T	-	-	-	-	V	-	-	-	K	S	-	-	-	-	-	K	F	-	-	I	I	N	-	-	P	T													

Drug Resistance Key

■ = NRTI

■ = 3TC

■ = NNRTI

■ = PI

FIG. 5. Latent, integrated HIV-1 in resting CD4⁺ T cells is genetically distinct from contemporaneous plasma virus in patients failing HAART. (A and B) A 1.5-kb region of HIV-1 *pol* was amplified using RT-PCR from the supernatants of cultures of resting CD4⁺ T cells activated in the presence of antiretroviral drugs and from contemporaneous plasma. The genotypes of independent clones from two representative patients are shown and depict amino acid substitutions in RT and PR, including drug resistance mutations and polymorphisms. Mutations known to confer significant resistance to antiretroviral drugs are highlighted in color. RT and PR are linked when separated by a dashed line and are independent clones when separated by a solid line. Antiretroviral drug abbreviations: TDF, tenofovir disoproxil fumarate; ABC, abacavir; EFV, efavirenz; LPV/r, lopinavir boosted with ritonavir; AZT, zidovudine; 3TC, lamivudine.

in the maintenance of a viral population that is distinct from actively replicating virus in the plasma.

The two patients that showed commingling of culture and plasma virus had the highest plasma viral levels at the time of sampling. Patient 001 had a plasma HIV-1 RNA measurement of 30,498 copies/ml and was not on HAART at the time of sampling. Despite a history of treatment failure, only wild-type sequences were found in the plasma and culture. Patient 112 had a plasma HIV-1 RNA measurement of 63,010 copies/ml and had a high level of resistance in the viral species from both compartments. The similarity of viral populations in the plasma and resting CD4⁺ T cells in patients with higher plasma viral levels may indicate that the turnover of virus in resting

CD4⁺ T cells increases at higher levels of viral replication. Alternatively, the culture assay may not be able to exclude all unintegrated viral species at very high plasma viral levels.

To quantify the differences observed between culture and plasma virus, the net average phylogenetic distance between viral species in the two populations was calculated. A distance significantly greater than zero indicates that the two populations are different. Twelve out of 16 samples had a net average distance between viral species in the resting CD4⁺ T cells and the plasma that was significantly greater than zero, confirming quantitatively that there was a difference between the two populations (Table 2). The median net average distance between the two viral populations was 0.005 substitutions per site

FIG. 7. Latent, integrated HIV-1 in resting CD4⁺ T cells is phylogenetically distinct from contemporaneous plasma virus in patients failing HAART. Neighbor-joining phylogenetic trees of independent clones from the plasma and culture of all 12 patients were generated using a 637-nucleotide region of *RT*. The scale is indicated by the bar representing 0.01 substitutions per nucleotide. The robustness of clustering was assessed using bootstrap analysis with 1,000 resamplings. Percentages of permuted trees supporting the indicated clade by >80% are shown. Resting CD4⁺ T-cell clones are represented by a circle, and plasma clones are represented by an asterisk. Clones that contain drug resistance mutations are highlighted in red.

It is interesting that although the plasma virus in viremic patients failing HAART was more drug resistant than viral species in resting CD4⁺ T cells in the context of the current regimen, only 4 out of 13 plasma samples contained virus with intermediate- or high-level resistance to all of the drugs in the current antiretroviral regimen. In addition, drug resistance mutations conferring resistance to protease inhibitors were found in only one of nine patients on protease inhibitor-containing regimens. This is in accordance with previous studies showing

that viral species containing mutations which confer resistance to only part of the current antiretroviral regimen can replicate in at least some patients on HAART (17, 28). To directly assess the comparative fitness of culture and plasma viral species, *gag-pol* clones from the resting CD4⁺ T cells and the plasma of patient 105 were inserted into a previously described recombinant HIV-1 reporter virus encoding GFP (75). Recombinant viruses were used to infect CD4⁺ T cells in the presence of the antiretroviral regimen the patient

TABLE 2. Net average phylogenetic distance between plasma and culture HIV-1 in viremic individuals

Patient no.	Sample		Taxa (n)		Net avg between-groups mean ^c		
	VL (c/ml)	Cells (millions)	Plasma	Culture ^a	Mean ^b	SEM ^c	>2 SEM ^d
001	30,498	4.4	10	12	0.000	0.000	No
096	32	2.0	4	12	0.005	0.002	Yes
100	72	5.2	5	11	0.004	0.002	No
102t1	792	1.0	9	7	0.008	0.002	Yes
102t2	17,155	4.6	7	3	0.006	0.002	Yes
102t3	43	5.8	11	9	0.004	0.001	Yes
105t1	9,063	2.0	9	8	0.031	0.006	Yes
105t2	1,368	5.2	8	11	0.003	0.001	Yes
105t3	38	7.2	12	7	0.015	0.003	Yes
110	38	3.0	6	6	0.016	0.004	Yes
112	63,010	3.0	9	6	0.000	0.000	No
122	<50	6.0	6	8	0.003	0.002	No
126	<50	7.0	6	12	0.004	0.001	Yes
127	436	3.8	4	10	0.005	0.002	Yes
137	<50	4.6	11	8	0.025	0.005	Yes
138	211	6.0	6	4	0.013	0.004	Yes

^a Culture HIV-1 was obtained from the activation of resting CD4⁺ T cells from viremic patients in the presence of antiretroviral drugs.

^b Genetic distances were calculated for a 1.5-kb region of *pol* or a 637-nucleotide region of *RT* for independent clones cleaned of sporadic mutations, using the Kimura 2-parameter model with transition/transversion ratio and shape parameter estimated empirically and given as number of substitutions per site.

^c Standard error of the mean was calculated by bootstrap analysis with 100 repetitions.

^d Net average between-group distances greater than zero by more than two standard errors were considered indicative that the two populations were significantly different. Overall mean was 0.009 (standard deviation, 0.009).

^e The parametric one-sample *t* test was used to determine that the plasma and culture viral populations were significantly different ($T = 3.983$).

was taking at the time of sampling. This assay allows the inhibitory effects of drug combinations to be assessed under conditions mimicking drug suppression in vivo (75). While no inhibition of four different resistant clones from the plasma was observed by the combination of drugs at their in vivo concentrations, replication of the wild-type clone from the culture was inhibited by 3,000-fold (Fig. 8B). Similar results were obtained in three other patients. These results demonstrate the persistence of viral species in the resting CD4⁺ T cells of viremic patients that would be profoundly unfit under the ambient selective conditions.

Wild-type clones that persisted in the resting CD4⁺ T cells of patients failing HAART were not only less fit than plasma virus in the presence of the current antiretroviral regimen, but also less divergent from an inferred MRCA (Fig. 9A and B). Furthermore, the viral population in resting CD4⁺ T cells had a lower mean divergence than the plasma virus in 8 out of 13 samples and a higher mean diversity than the plasma virus in 9 out of 13 samples (data not shown). This result is consistent with the archiving of a variety of different viral species over time. The lower mean divergence and higher mean diversity of the viruses in resting CD4⁺ T cells are consistent with a formal phylogenetic definition of a reservoir proposed by Mullins and colleagues (44) and argue against the possibility that virus in the resting CD4⁺ T cells of viremic patients is replicating in a drug-privileged site under different selective conditions than the plasma virus. Instead, the genotypic differences observed between the two compartments reflect the persistence of archival viral species in resting CD4⁺ T cells.

In addition to the preservation of archival wild-type virus in resting CD4⁺ T cells of viremic patients, viral species containing mutations that confer resistance to previous antiretroviral regimens were observed. For example, viral species in the resting CD4⁺ T cells but not in the plasma of patient 138 contained an M184V mutation, which confers resistance to the

RT inhibitor 3TC (Fig. 9C). The patient had taken zidovudine (AZT) and 3TC initially after being diagnosed with HIV-1 infection but had been off 3TC for 65 months prior to sampling. This mutation makes the virus less fit than wild-type virus in the absence of 3TC (1, 21) and hypersensitive to other NRTIs, including tenofovir in the current HAART regimen (39, 42, 46, 64, 68). Not surprisingly, the M184V mutation was absent from actively replicating plasma virus. These data illustrate the persistence of archival viral species in the resting CD4⁺ T cells of viremic patients and the potential clinical utility of this assay in the detection of archived resistance.

Cross-sectional analysis of the patient histories indicate that wild-type and other archival viral species persisted in the resting CD4⁺ T cells of viremic patients for up to 33 months, with plasma viral levels up to 150,000 copies/ml (in patient 105 from the last interruption of antiretroviral therapy at month 59 to the last detectable plasma viral measurement at month 92) (Fig. 10A). Archival virus was seen to persist in longitudinal sampling of two patients over 6 months, with plasma viral levels up to 17,000 copies/ml (Fig. 10B). The stable preservation of replication-competent viral species in resting CD4⁺ T cells that are unfit under the ambient conditions indicates that these cells still serve as a reservoir for HIV-1 in viremic patients despite high levels of viral replication and immune activation.

DISCUSSION

We have developed a culture system to characterize latent, integrated HIV-1 from the resting CD4⁺ T cells of viremic patients. Previously available methods have not been able to exclude the unintegrated viral DNA that predominates in the setting of high levels of viral replication (6, 7). The use of standard PCR methods to examine HIV-1 DNA in resting CD4⁺ T cells cannot distinguish between integrated and un-

FIG. 8. HIV-1 that is relatively unfit in the context of current therapy persists in the resting CD4⁺ T cells of patients failing HAART. (A) HIV-1 from the resting CD4⁺ T cells of patients failing HAART contain fewer mutations that confer drug resistance to the current antiretroviral regimen than contemporaneous plasma virus. Mean percentages of viral clones in the plasma and culture that contained at least one mutation known to confer resistance to the current antiretroviral regimen are shown, with the standard error for the 13 samples in which drug-resistant viral species were found. (B) HIV-1 from the resting CD4⁺ T cells of patients failing HAART is relatively less fit than contemporaneous plasma virus in the context of current therapy. (Top panel) Fitness was determined in the context of the current antiretroviral regimen for four plasma clones and one clone from resting CD4⁺ T cells of patient 105. (Bottom panel) Fitness was measured as the percentage of target cells expressing GFP 48 h after infection with a GFP-containing, VSV-G-pseudotyped virus in the presence of zidovudine (AZT), lamivudine (3TC), and abacavir (ABC) at their in vivo trough (C_{min}) and peak (C_{max}) concentrations.

integrated viruses, and most of the virus amplified is replication defective (7). Replication-competent viruses can be rescued from resting CD4⁺ T cells by cellular activation, but virion production from unintegrated virus in the preintegration state of latency overwhelms virus produced from stably integrated, latent proviruses (Fig. 6) (6, 72). Therefore, previously available methods have overestimated the turnover of the viral population in resting CD4⁺ T cells and have not given a clear view of its genetic composition in viremic patients. The development of a culture system using antiretroviral drugs to exclude unintegrated forms of virus has allowed the first visual-

ization of latent integrated HIV-1 in the resting CD4⁺ T cells of viremic patients.

Using this assay, we have shown that HIV-1 in the resting CD4⁺ T cells of viremic patients is genetically distinct from actively replicating HIV-1 in the plasma. Resting CD4⁺ T cells in patients failing HAART continue to harbor wild-type HIV-1 and archival drug-resistant viral species that are less fit than the virus found in the plasma in the setting of the current drug regimen. The viral population found in the resting CD4⁺ T cells of viremic patients was generally more diverse and less divergent than the plasma viral population demonstrating its

FIG. 9. HIV-1 that persists in the resting CD4⁺ T cells of patients failing HAART is archival in nature. (A and B) HIV-1 from the resting CD4⁺ T cells of patients failing HAART has a lower divergence than contemporaneous plasma virus. Divergence calculated from an inferred MRCA is shown for two representative patients. Error bars depict the standard errors determined by bootstrap analysis with 100 repetitions. (C) Persistence of resistance mutations to a previously taken antiretroviral drug. (Top panel) The genotypes of independent clones from patient 138 demonstrate persistence of an M184V mutation that confers resistance to 3TC in resting CD4⁺ T cells, but not in plasma. (Bottom panel) The plasma HIV-1 RNA levels and treatment history for patient 138 show that the patient was last on a 3TC-containing regimen 65 months prior to sampling. Open symbols for the plasma HIV-1 RNA levels indicate measurements below the limit of detection (either <400 or <50 HIV-1 RNA copies/ml). The black line and arrow indicate the sampling time point. Antiretroviral drug abbreviations: AZT, zidovudine; 3TC, lamivudine; ddI, didanosine; d4T, stavudine; HU, hydroxyurea; NVP, nevirapine; SQV, saquinavir; RTV, ritonavir; TDF, tenofovir disoproxil fumarate; ABC, abacavir.

FIG. 10. Stable persistence of archival HIV-1 in the resting CD4⁺ T cells of viremic patients. (A) Treatment history of patient 105 demonstrates the persistence of wild-type and other archival viral species for up to 33 months with plasma viral levels of up to 150,000 copies/ml (from the last interruption of antiretroviral therapy at month 59 to the last detectable plasma viral measurement at month 92). Open symbols for the plasma HIV-1 RNA levels indicate measurements below the limit of detection (either <400 or <50 HIV-1 RNA copies/ml). The black lines and arrows indicate the sampling time points. (B) Longitudinal analysis demonstrates the persistence of wild-type virus over 6 months with plasma viral levels up to 17,000 copies/ml. Independent clones of virus from the resting CD4⁺ T cells and plasma for the first and last time points of patient 102 are shown. RT and PR are linked when separated by a dashed line and are independent clones when separated by a solid line. Mutations known to confer significant resistance to antiretroviral drugs are highlighted in color. Antiretroviral drug abbreviations: AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; NfV, neftinavir; ABC, abacavir; ddI, didanosine; TDF, tenofovir disoproxil fumarate; EFV, efavirenz; LPV/r, lopinavir boosted with ritonavir; NVP, nevirapine; SQV, saquinavir; RTV, ritonavir.

archival nature. Wild-type virus and archival drug-resistant viral species were found in the resting CD4⁺ T cells of patients who had been failing therapy for up to 33 months, with plasma HIV-1 RNA levels up to 150,000 copies/ml. Persistence of archival virus was seen over 6 months in patients followed longitudinally with plasma HIV-1 RNA levels of up to 17,000 copies/ml. Taken together, these findings demonstrate a slow turnover of viral species in resting CD4⁺ T cells that serve as a reservoir for HIV-1, even in the setting of active viral replication and immune activation present in viremic patients. This is the first direct demonstration of the existence of a stable latent reservoir of integrated HIV-1 in the resting CD4⁺ T cells of patients with viremia.

The slow turnover of virus in resting CD4⁺ T cells at high levels of viral replication suggests that the entry of viral species into the latent reservoir is a low-frequency event, despite the early establishment of this reservoir during acute infection (9). The slow turnover and persistence of archival, unfit viral species in the resting CD4⁺ T cell reservoir of viremic patients also argues against the hypothesis that the creation of new latently infected cells significantly contributes to the stability of this reservoir (54). Our data are consistent with the reservoir being maintained via a nonreplicative mechanism in the absence of drug-selective pressure, such as the intrinsic stability of the long-lived memory CD4⁺ T cells or lymphocyte homeostasis (5).

This study does not rule out the possibility that turnover of the resting CD4⁺ T-cell reservoir may increase at plasma HIV-1 RNA levels above 20,000 copies/ml. The plasma and resting CD4⁺ T-cell viral populations were indistinguishable in the two patients with plasma HIV-1 RNA levels above 30,000 copies/ml. It is unclear whether this is a limitation of the assay in excluding unintegrated virus at very high plasma viral levels or whether it reflects a higher turnover of the resting CD4⁺ T-cell reservoir at higher levels of viral replication. Further studies using the culture assay are required for quantitation of the turnover of viral species in the resting CD4⁺ T-cell reservoir for HIV-1 in viremic patients.

The ability to characterize the archival viral species stored in resting CD4⁺ T cells with the assay described here could assist clinicians facing difficult therapeutic decisions for patients failing HAART. Current resistance testing detects only mutations that are present in a majority of viral species in the plasma and therefore does not give a complete picture of all of the drug resistance mutations present in an infected individual. Knowing both current and archived drug resistance mutations would reveal whether there are remaining antiretroviral drugs that are likely to suppress replication of all the viral species present and would help clinicians choose the best alternate regimens. If no other regimen options are available, clinicians need to decide whether to keep the patient on their current antiretroviral regimen or whether to stop therapy (16). The best way to make this decision is to determine whether the current regimen offers any benefit by suppressing viral replication or by maintaining drug-resistant viral species that have a lower replicative capacity than the wild-type virus that would return if therapy were interrupted (1, 15, 16, 24, 74). This could be done with phenotypic assays that assess the fitness of drug-resistant viral species in patients failing HAART compared to the patient's own wild-type virus obtained from the resting CD4⁺ T

cells using the approach described here. Thus, the ability to access the resting CD4⁺ T-cell latent reservoir in viremic patients may prove to be valuable in making treatment decisions for patients failing HAART.

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