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Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy

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ABSTRACT **Although highly active antiretroviral therapy (HAART) in the form of triple combinations of drugs including protease inhibitors can reduce the plasma viral load of some HIV-1-infected individuals to undetectable levels, it is unclear what the effects of these regimens are on latently infected CD4$^+$ T cells and what role these cells play in the persistence of HIV-1 infection in individuals receiving such treatment. The present study demonstrates that highly purified CD4$^+$ T cells from 13 of 13 patients receiving HAART with an average treatment time of 10 months and with undetectable (<500 copies HIV RNA/ml) plasma viremia by a commonly used bDNA assay carried integrated proviral DNA and were capable of producing infectious virus upon cellular activation *in vitro*. Phenotypic analysis of HIV-1 produced by activation of latently infected CD4$^+$ T cells revealed the presence in some patients of syncytium-inducing virus. In addition, the presence of unintegrated HIV-1 DNA in infected resting CD4$^+$ T cells from patients receiving HAART, even those with undetectable plasma viremia, suggests persistent active virus replication *in vivo*.**

The use of highly active antiretroviral therapy (HAART) in the treatment of HIV-infected individuals has provided considerable information regarding the dynamics of virus replication and has raised the possibility that HIV-1 can be eradicated in infected individuals (1–7). A mathematical model based on the kinetics of viral decay in the plasma of infected individuals shortly after initiation of HAART consisting of a potent HIV-1 protease inhibitor plus two reverse transcriptase inhibitors has indicated that 2.3–3.1 years of a regimen that completely inhibits virus replication would be required to eradicate the infection (3). However, the presence of HIV-1 DNA in peripheral blood mononuclear cells and evidence for a very small amount of viral replication in the lymphoid tissues have been demonstrated even in HIV-infected individuals in whom HAART has successfully controlled plasma viremia (3, 5). Given the assumption that productively infected CD4$^+$ T cells have an extremely short half-life (1–4), and that virus production in that population is suppressed in most infected individuals, it is highly likely that persistence of virus occurs predominantly in latently infected CD4$^+$ T cells and/or other long-lived infected cells (3). The importance of latent reservoirs for HIV-1 has been underscored by the observation that the initial rapid decline in levels of plasma viremia in patients receiving HAART was followed by a much slower decay, reflecting the slower turnover of long-term virus reservoirs of chronically or latently infected cells (3). In this regard, latently infected resting CD4$^+$ T cells that are capable of producing infectious virus on activation have been characterized in infected individuals receiving less potent conventional antiretroviral regimens (8, 9). Despite their extremely low frequency ($<10^7$ per individual), it is believed that this population of cells with a memory phenotype has a relatively long half-life (8, 10) and the viral reservoir in these cells (11, 12) is unlikely to be affected by HAART because of low cell turnover and/or low level of virus expression (13–19). Moreover, immune activation associated with HIV infection itself as well as with infection with opportunistic pathogens may induce viral replication in latently infected CD4$^+$ T cells (20–24). It has been established that HIV infection can progress even though only a small fraction of the susceptible cell populations is infected (22) and it is assumed that a cure can only be achieved if the latent reservoirs of replication-competent provirus are also eliminated. Thus, it is critical to measure the size of the latently infected cell population as well as the replication competency of the virus in patients receiving HAART not only to evaluate the true efficacy of therapy but also to better understand the pathogenesis of HIV-1 disease. In the present study, we examined the size and inducibility of the latently infected, resting CD4$^+$ T cell pool carrying integrated and unintegrated forms of HIV-1 DNA in 13 patients receiving HAART. CD4$^+$ T cells from 13 of 13 patients and resting CD4$^+$ T cells from 11 of these 13 patients receiving HAART carry HIV-1 DNA that is capable of producing infectious virus on stimulation *in vitro* even after an average treatment time of 10 months.

MATERIALS AND METHODS

Patient Population. Thirteen HIV-1 seropositive patients receiving HAART, 4 drug naive patients, and 1 patient who received 3TC only (Table 1) were subjected to apheresis to obtain peripheral blood mononuclear cells according to a National Institute of Allergy and Infectious Diseases Institutional Review Board approved protocol.

Isolation of Resting CD4$^+$ T Cells from Peripheral Blood of Patients. Resting CD4$^+$ T cells were isolated from HIV-1-infected patients by using a combination of magnetic bead depletion and fluorescent activated cell sorting as described (9).

Assays for Integrated and Total HIV-1 DNA. Genomic DNA from purified resting CD4$^+$ T cells was serially diluted resulting in tubes containing 200,000, 40,000, 8,000, 1,600, 320, 16, 2.4 cell equivalents. The first PCR was carried out by using nested primers, Alu-long terminal repeat (LTR) 5′ from conserved sequences of human Alu and Alu-LTR 3′ from

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Abbreviations: HAART, highly active antiretroviral therapy; LTR, long terminal repeat.

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Table 1. Profiles of HIV-1-infected patients

Patient*	$CD4^+$ T cell count prior to HAART, cells/μl	$CD4^+$ T cell count at time of study, cells/μl	Plasma HIV RNA on HAART,† copies/ml	Anti-retroviral treatment	Months receiving HAART‡	Year sero-conversion
1	189	367	<500	3TC, d4T, Indinavir	12.5	1995
2	232	422	<500	3TC, d4T, Indinavir	8.4	1991
3	359	532	<500	AZT, 3TC, Ritonavir	7.0	1990
4	290	478	<500	AZT, 3TC, Indinavir	8.0	1990
5	184	273	<500	3TC, d4T, Indinavir	10.0	1987
6	305	372	<500	3TC, ddI, Indinavir	8.6	1987
7	392	556	<500	3TC, d4T, Indinavir	13.0	1995
8	285	390	<500	AZT, 3TC, Indinavir	9.3	1996
9	219	309	<500	AZT, 3TC, Indinavir	9.2	1984
10	806	732	2,800	AZT, 3TC, Saquinavir	11.0	1988
11	72	221	140,900	AZT, 3TC, Indinavir	13.0	1986
12	15	348	814	ddl, d4T, Ritonavir, Saquinavir	14.7	1989
13	200	148	6,518	AZT, 3TC, Saquinavir	5.5	1986
14	NA	334	319,100	NA	NA	1987
15	NA	476	127,200	NA	NA	1993
16	NA	391	35,570	NA	NA	1986
17	NA	599	10,760	3TC	NA	1991
18	NA	464	25,330	NA	NA	1991

NA, not applicable. 3TC, lamivudine; d4T, stavudine; AZT, 3′-azido-3-deoxythymidine; ddI, didanosine.
*The informed consent was signed by all patients prior to apheresis. No patients had an opportunistic infection at the time of apheresis.
†Plasma HIV RNA was measured using the branched DNA assay (Chiron) with a detection limit of 500 copies/ml.
‡Indicates the number of months after the start date of protease inhibitor.

conserved HIV-1 LTR sequences. Sequences of the primers are as follows: Alu-LTR, 5′-TCCCAGCTACTCGGGAGGCTGAGG-3′; Alu-LTR 3′, 5′-AGGCAAGCTTTATTGAGGCTTAAGC-3′. For all PCRs, dNTPs (0.2 mM) and primers (25 pmol) were added to each tube followed by addition of Ampliwax (Perkin–Elmer). Tubes were heated to 75°C for 1 min and cooled to 4°C to form a solid wax layer in a 96-well formatted plate. dH_2O, 3.3× XL buffer II (Perkin–Elmer), 1.2 mM $Mg(OAc)_2$, 1.6 units of rTth DNA Polymerase, XL (Perkin–Elmer), and serially diluted genomic DNA (or other DNA templates) were added. Samples were then subjected to denaturation at 94°C for 3 min, 22 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 66°C, and 5 min extension at 70°C with 10 min at 72°C for the final extension step. In control reactions, an equivalent copy number of linearized plasmid template (pPstI-1481) mimicking the unintegrated form of HIV-1 DNA was also subjected to the same PCR parameters. Following the initial PCR, a second nested PCR amplification was carried out by using an aliquot equivalent to 1/400 of the 22-cycle PCR product. The second nested PCR, which allows amplification of a portion of the LTR region of HIV-1 DNA, was performed by using AmpliTaqGold (Perkin–Elmer), nested LTR primers, 10× PCR buffer containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, and 1.25 mM $MgCl_2$. For the second PCR reactions, primer NI-2 5′, 5′-CACACACAAGGCTACTTCCCT-3′, and NI-2 3′, 5′-GCCACTCCCCIGTCCCGCCC-3′ were used. Samples were then subjected to an enzyme activation step at 94°C for 12 min, 29 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 63°C, and 1 min extension at 72°C with 10 min at 72°C for the final extension step. In control reactions, genomic or plasmid DNA that had not been subjected to the first round of PCR was also amplified by using the second PCR primers. PCR products were analyzed by gel electrophoresis followed by Southern hybridization by using ^{32}P end-labeled probes (NI probe 5′-GGATGGTGCTTCAAGITAGTACC-3′). The frequency of cells carrying integrated HIV-1 DNA was determined from the limiting dilution PCR data by the statistical method of Myers *et al.* (25).

Total copy number of HIV-1 DNA was quantitated by using the second PCR primers and the probe as described above. PCR products were analyzed by gel electrophoresis followed by Southern hybridization by using ^{32}P end-labeled probe. After Southern hybridization, bands were quantified by PhosphorImager analysis by using a standard curve based on PCR of known copy numbers of serially diluted ACH-2 DNA.

Micro Coculture Assay for Replication-Competent HIV-1 DNA. To determine what fraction of resting $CD4^+$ T cells carrying HIV-1 DNA is replication-competent, a micro coculture assay was carried out *in vitro* as described (8).

Phenotypic Analysis of Induced HIV from Resting $CD4^+$ T Cells. To characterize the phenotype of the virus induced from replication-competent latently infected resting $CD4^+$ T cells of patients receiving HAART, MT-2 assays were performed as described (26).

RESULTS

Detection of Integrated and Total HIV-1 DNA in Resting $CD4^+$ T Cells from Patients Receiving HAART. We isolated resting $CD4^+$ T cells by using a combination of magnetic bead depletion and flow cytometric sorting techniques. The purity of resting $CD4^+$ T cells was generally greater than 99%. To determine what fraction of resting $CD4^+$ T cells carry the stable form of HIV-1 DNA, we utilized a previously described Alu-LTR PCR method (27), which was modified to allow a more quantitative measurement of the integrated form of HIV-1 DNA in resting $CD4^+$ T cells. Because approximately one million copies of Alu elements are present throughout the human genomic DNA (28, 29) and because we used DNA polymerase, which has a long-range amplification capacity with a long extension time, this PCR method allowed us to detect with single molecule sensitivity integrated HIV-1 DNA in chronically infected ACH-2 and U1 cell lines, which carry one and two copies of the integrated form of HIV-1 DNA, respectively (30, 31) (Fig. 1). In addition, this method did not amplify a plasmid-derived unintegrated form of HIV-1 DNA (pPstI-1481) even when equivalent copy numbers were used (Fig. 1*B*). The Alu-LTR PCR was applied to serially diluted genomic DNA from highly purified resting $CD4^+$ T cells from patients receiving HAART as well as from untreated patients (Figs. 1*C* and 2*A*). The integrated form of HIV-1 DNA was

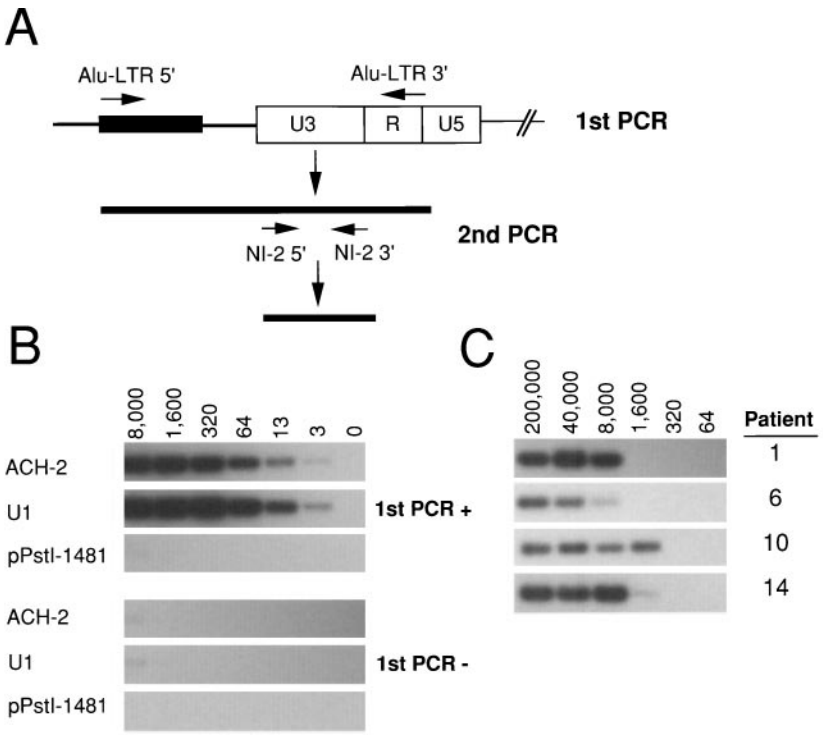

FIG. 1. Quantitative analysis of integrated HIV-1 DNA in resting CD4$^+$ T cells. (*A*) Detection of integrated HIV-1 DNA by using Alu-LTR PCR. Genomic DNA from resting CD4$^+$ T cells was serially diluted 5-fold starting with 200,000 cell DNA equivalents and subjected to PCR in duplicate by using nested 5′ primers from conserved Alu and 3′ primer from conserved HIV-1 LTR sequences. This dilution amplifies both cellular DNA upstream of the integration site and integrated HIV-1 LTR. An aliquot of the diluted first PCR product was further subjected to the second round of PCR by using nested HIV-1 LTR-specific primers. (*B*) Genomic DNA from ACH-2 and U1 cells carrying one and two copies of an integrated form of HIV-1, respectively, was serially diluted and subjected to Alu-LTR PCR followed by a second round of the nested PCR. As negative controls, first PCR-DNA was used for the second round of PCR and a plasmid mimicking unintegrated DNA was subjected to two rounds of PCR in the same manner. (*C*) Representative results of Alu-LTR PCR of genomic DNA from purified resting CD4$^+$ T cells from treated patients. The limiting dilution Alu-LTR PCR was carried out on the indicated number of cell equivalents of donor DNA. The product of two rounds of PCR was resolved in an agarose gel and bands were detected by Southern blot analysis by using an LTR-specific probe.

detected in all 13 treated patients. The geometric mean frequency of integrated HIV-1 DNA in resting CD4$^+$ T cells of patients receiving HAART with undetectable plasma HIV RNA was 324 copies/million resting CD4$^+$ T cells. This frequency was not significantly different from that in treated patients with detectable plasma RNA (geometric mean, 298 copies/million resting CD4$^+$ T cells). Moreover, levels of integrated HIV-1 DNA within resting CD4$^+$ T cells in HAART naive patients was not significantly higher (geometric mean, 370 copies/million resting CD4$^+$ T cells) than in treated patients. This finding suggests that resting CD4$^+$ T cells with integrated HIV-1 DNA do not decay rapidly in patients receiving HAART and thus represent a stable reservoir of HIV-1 DNA as suggested previously (8, 9). Alternatively, the reservoir could be continually replenished by a low, but undetectable, degree of ongoing viral replication. In this regard, measurements of total copy numbers of HIV-1 DNA indicated that levels of unintegrated HIV-1 DNA were approximately 28-fold higher than integrated HIV-1 DNA in resting CD4$^+$ T cells of treated patients with $<$500 copies of HIV RNA/ml plasma. This result is noteworthy because unintegrated HIV-1 DNA is felt to be relatively unstable *in vitro* (13, 18) with a short half-life *in vivo* (32), and therefore its continued presence in resting CD4$^+$ T cells of infected individuals receiving HAART for an average of 10 months is compatible with the possibility that viral replication is indeed ongoing despite the lack of detectable plasma viremia.

Detection of Replication-Competent HIV-1 in Resting CD4$^+$ T Cells from Patients Receiving HAART. Because a high proportion of HIV-1 DNA in cells of infected individuals exists as a defective form and only replication-competent HIV-1 provirus can give rise to infectious virus, we carried out a sensitive quantitative micro coculture assay (8) by using purified resting CD4$^+$ T cells to directly measure the frequency of resting CD4$^+$ T cells that are capable of producing infectious virus on cellular activation. Because resting CD4$^+$ T cells may harbor integrated as well as unintegrated HIV-1 DNA in the form of preintegration complexes (13, 18), purified resting CD4$^+$ T cells were preincubated in the absence of activating stimuli for 6 days to allow for the decay of unintegrated HIV-1 DNA. Serially diluted fresh (day 0) as well as preincubated (day 6) resting CD4$^+$ T cells were activated *in vitro* in duplicate as previously described (8), and supernatant from each culture was collected on day 14 for determination of HIV-1 p24 by ELISA. Infectious virus (Fig. 2*B*) from stimulated resting CD4$^+$ T cells from 11 of 13 patients was detected. Although patients 4 and 6 had undetectable infectious virus from 10^6 resting CD4$^+$ T cells assayed on day 0, an increased number of total CD4$^+$ T cells from those patients (2×10^6 cells) gave rise to infectious virus, indicating that replication-competent virus was indeed present and suggesting that infectious HIV-1 could have been detected in resting CD4$^+$ T cells if higher numbers of cells were activated. The geometric mean frequency of cells carrying infectious HIV-1 in resting CD4$^+$ T cells on day 0 and day 6 was 1.5 per 10^6 cells and 1.6 per 10^6 cells, respectively, in treated patients with undetectable plasma virus. The frequency was somewhat higher in treated patients with detectable plasma virus (8.5 per 10^6 and 2.4 per 10^6 resting CD4$^+$ T cells on day 0 and day 6, respectively). These data suggest that in treated patients with undetectable plasma viremia ($<$500 copies HIV RNA/ml) almost all infectious virus had been induced *in vitro* from resting CD4$^+$ T cells carrying integrated HIV-1 DNA. In contrast, in treated patients with detectable plasma viremia

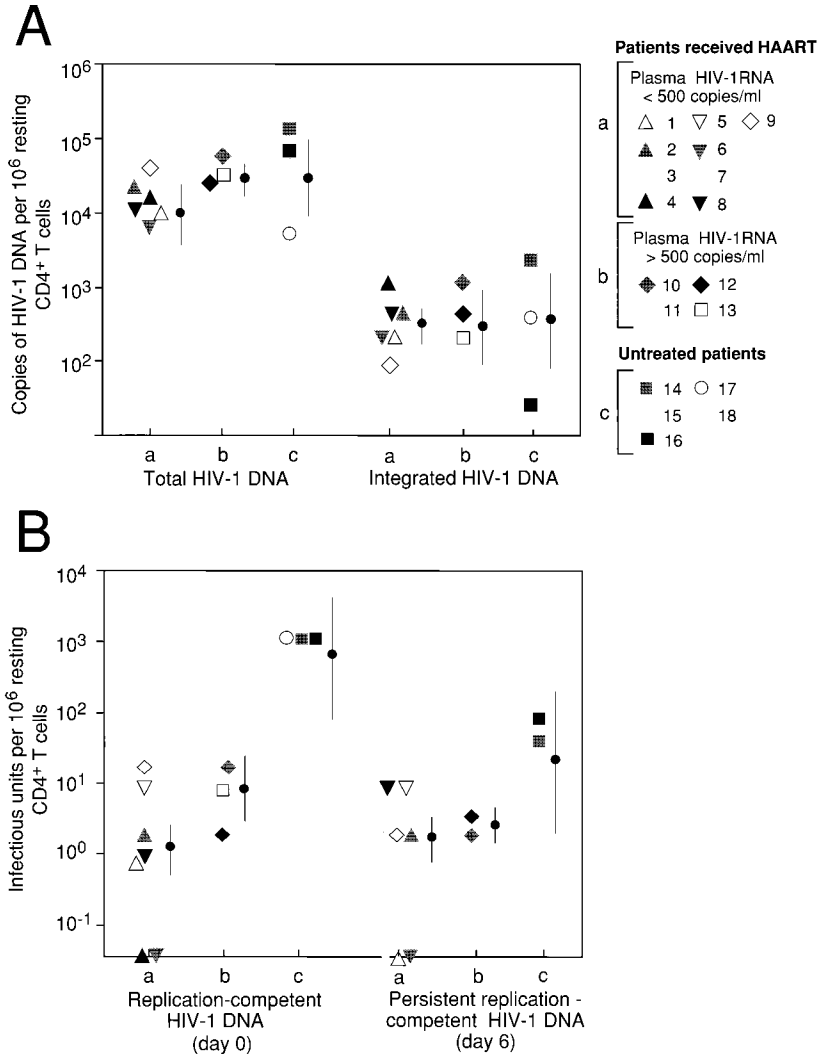

FIG. 2. Frequencies of resting CD4+ T cells carrying various forms of HIV-1 DNA in patients receiving HAART with undetectable and detectable plasma virus and drug naive patients. (*A*) Frequencies of resting CD4+ T cells carrying total and integrated HIV-1 DNA. The copy numbers of HIV-1 DNA including both unintegrated and integrated HIV-1 DNA in purified resting CD4+ T cells in patients were calculated as described in the text. The frequencies of resting CD4+ T cells with integrated HIV-1 DNA was determined by Alu-LTR PCR by using serially diluted genomic DNA from resting CD4+ T cells (Fig. 1). (*B*) Frequencies of resting CD4+ T cells carrying replication-competent HIV-1 DNA were determined by activating purified resting CD4+ T cells on day 0. Frequencies of resting CD4+ T cells carrying persistent replication-competent HIV-1 DNA were determined by activating 6 day pre-incubated cells in the absence of activating stimuli. For each assay, a statistical method was used by Myers *et al.* (25) to calculate copy numbers or infectious units per million resting CD4+ T cells. The geometric mean frequencies and corresponding 95% confidence intervals for each data set are plotted to the right of the individual donor values. The letters a, b, and c in each panel refer to the three separate groups of patients: a, HAART treated, <500 copies HIV RNA/ml; b, HAART treated, >500 copies HIV RNA/ml; c, untreated.

(>500 copies HIV RNA/ml) unintegrated as well as integrated HIV-1 DNA likely contributed to the infectious virus that had been induced from resting CD4+ T cells. Thus, replication-competent virus was induced from CD4+ T cells in all treated patients tested regardless of the presence or absence of detectable plasma viremia. Furthermore, the presence of unintegrated HIV-1 DNA in CD4+ T cells of treated patients suggests that virus replication, albeit at very low levels, was ongoing *in vivo* despite undetectable plasma viremia.

Detection of Syncytium-Inducing Virus from Induced Resting CD4+ T Cells. We carried out phenotypic analysis of infectious virus produced by purified resting CD4+ T cells from patients receiving HAART. Induced virus from purified resting CD4+ T cells from 3 of 13 patients (patients 2, 3, and 6) showed a syncytium-inducing viral phenotype (data not shown). Of note, all 3 patients had undetectable plasma viremia (<500 copies HIV RNA/ml), suggesting that activation of at least some latently infected resting CD4+ T cells can result in the production of highly cytopathic virus.

DISCUSSION

In the present study, we have provided definitive evidence for the presence of replication-competent HIV in latently infected, resting CD4+ T cells in patients receiving HAART who have successfully controlled their plasma viremia as measured by a commonly used bDNA assay. Although most active viral replication takes place in lymphoid tissue of infected individuals throughout all stages of disease (33–35), the fact that resting CD4+ T cells traffic continuously throughout the body (36) adds validity to the assumption that peripheral blood CD4+ T cells accurately reflect the total body pool (8). In a previous study, attempts to recover infectious virus from peripheral blood mononuclear cells of infected individuals after 8 weeks of HAART were unsuccessful (3). The discrepancy between the present study and others (3) might have resulted from the fact that we used highly enriched resting CD4+ T cell populations and amplified infectious virus in this population of cells in the absence of CD8+ T cells that have

been shown to markedly inhibit HIV-1 replication (37) by a number of mechanisms including the production of β-chemokines (38) as well as other yet uncharacterized soluble factors (37).

Replication-competent virus was induced from resting $CD4^+$ T cells from 11 of 13 patients receiving HAART and from the total $CD4^+$ T cell population of all 13 patients, including 9 of 13 in whom plasma viremia was undetectable by commonly employed bDNA assays. Of note, simultaneous with our observations, two other groups have demonstrated similar findings of an inducible reservoir of latently infected, resting $CD4^+$ T cells in patients receiving HAART whose plasma viremia was below the level of detection (39, 40). Furthermore, our data suggest that unintegrated HIV-1 DNA contributed to the inducible virus indicating that virus replication was likely ongoing and possibly contributing to the maintenance of the latently infected pool of resting $CD4^+$ T cells despite what appeared to be control of plasma viremia with HAART. It is likely that latently infected, resting $CD4^+$ T cells represent a heterogeneous population and that some of these cells have an extremely long half-life. Previous estimates of the half-life of infected cells (1, 2, 4) and projections of the length of time required for eradication of virus in patients receiving HAART have been derived from mathematical formulas based on the assumption of complete suppression of virus replication (3). The findings in the present study should elicit considerable caution in any predictions of virus eradication in individuals receiving HAART, even those individuals with undetectable plasma viremia by bDNA assays commonly used in clinical practice. The current demonstration of the presence of inducible and replication-competent virus in longer lived resting $CD4^+$ T cells in patients receiving HAART for extended periods of time suggests that the time required for virus eradication, if indeed this is possible, will be considerably longer than previously predicted (3). Long-term longitudinal studies of these latently infected resting $CD4^+$ T cell populations as well as other long-lived infected cells in patients receiving HAART will be required to address this question. Finally, more potent antiretroviral drugs need to be developed as well as strategies aimed at eradicating those minor populations of infected cells that serve as reservoirs of inducible and replication-competent HIV.

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