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Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy

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Combination therapy for HIV-1 infection can reduce plasma virus to undetectable levels, indicating that prolonged treatment might eradicate the infection. However, HIV-1 can persist in a latent form in resting CD4+ T cells. We measured the decay rate of this latent reservoir in 34 treated adults whose plasma virus levels were undetectable. The mean half-life of the latent reservoir was very long (43.9 months). If the latent reservoir consists of only 1×10^5 cells, eradication could take as long as 60 years. Thus, latent infection of resting CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective anti-retroviral therapy.

Analysis of viral load in HIV-1 infection has contributed to our understanding of AIDS pathogenesis and has facilitated management of the disease[1–10]. Virus replication continues throughout the disease, even during the asymptomatic phase between primary infection and the development of AIDS (refs. 2,3). Potent inhibitors of HIV-1 reverse transcriptase and protease produce a rapid exponential decrease in plasma virus levels, reflecting the short half-lives of free virus ($t_{1/2}$, less than 6 hours) and of the cells that produce most of the plasma virus ($t_{1/2}$, about 1 day)(refs. 11–13). After the first few weeks of therapy, a second, slower phase of viral decay becomes apparent ($t_{1/2}$, about 14 days). This reflects the slower turnover of a distinct population of chronically infected cells[14]. After several weeks of combination therapy, plasma virus levels decrease to below the limit of detection in many patients[15,16], and it becomes difficult to culture virus from the blood. Viral burden in lymph node tissue is also correspondingly reduced[17]. Extrapolation of the second phase of decay to zero residual infected cells led to the suggestion that 2–3 years of fully suppressive therapy might be sufficient to achieve eradication[14]. However, this projection was made with the caveat that there might be other, more stable compartments in which the virus could persist.

One potential mechanism for viral persistence involves the establishment of a state of latent infection[18–23]. HIV-1 replicates well in activated CD4+ T cells[18,24], and latent infection is thought to occur only in resting CD4+ T cells[22]. A potentially stable latent reservoir may form when productively infected CD4+ lymphoblasts survive the cytopathic effects of HIV-1, evade the immune system, and return to a resting memory state carrying integrated provirus[21–23]. In these cells, there is minimal transcription from the HIV-1 long terminal repeat because of the absence of necessary host factors that are present only in activated T cells[18]. Because the biological function of memory cells is to persist for long periods of time to allow responses to previously encountered antigens, and because the viral DNA in these cells is stably integrated, these latently infected memory cells can potentially serve as a long-term reservoir for HIV-1.

Recent studies have directly demonstrated that HIV-1 establishes a state of latent infection in resting memory CD4+ T cells *in vivo*[22,23]. Using new culture methods, replication-competent HIV-1 can be recovered from these cells, even in patients on combination therapy who have no detectable plasma virus[25–27]. The half-life of this latent reservoir, an essential parameter in determining whether current therapy regimens can produce eradication, is unknown. Here we have used direct longitudinal measurements to assess the size and stability of the latent reservoir in patients who have been treated with current 'standard of care' anti-retroviral therapy. Our results challenge the idea that anti-retroviral therapy as it is now given can ever be expected to eradicate the infection.

We evaluated the latent reservoir by longitudinal and cross-sectional studies in 34 HIV-1-infected adults who had responded

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well to 'standard of care' anti-retroviral therapy[28,29]. Patients were selected on the basis of strict patient- and physician-reported adherence to three to five drug regimens that met or exceeded current guidelines[28,29]; viral load measurements that decreased to less than the limit of detection of a standard RT–PCR assay (200 copies/ml) within 1–4 months after initiation of therapy; and follow-up viral load measurements that were consistently less than 200 copies/ml. The patients were diverse in age, sex, race or ethnic group, risk factors and CD4 levels at the start of therapy (Table 1). Thus, this population reflects well the diversity of the epidemic. Various combination anti-retroviral regimens were used, all of which had at least one protease inhibitor (except for patient 7, who was on zidovudine (AZT), lamivudine (3TC) and nevirapine (NVP)).

To evaluate the latent reservoir, we isolated resting CD4+ T cells from the peripheral blood using a procedure that gives purities of greater than 99% (refs. 22,23). We evaluated the presence of cells with latent virus among these purified resting cells using a limiting dilution virus culture assay that detects only virus that is fully competent for replication[22,23].

Table 1 Characteristics of patients studied

Patient number	Age	Sex	Race/Ethnic group[a]	Risk group[b]	Status at start of Rx[c]	CD4 Nadir	Anti-retroviral drugs[d]
1	34	M	C	GM	Chronic	612	d4T,3TC,RTV,SQV
2	40	M	AA	Het/IDU	Chronic	49	RTV,SQV (d4T,NVP) ‡3TC
3	53	M	C	Het	Chronic	474	AZT,3TC,(RTV) ‡NFV,SQV
4	56	M	C	GM	Chronic	36	d4T,3TC,IDV
5	30	M	C	GM	Chronic	10	AZT,3TC,NFV
6[e]	46	F	AA	IDU	Chronic	440	AZT,3TC,RTV
7	49	F	AA	IDU/Het	Chronic	334	AZT,3TC,NVP
8	33	M	H	GM	Chronic	562	AZT,3TC,(IDV)‡NFV
9	32	M	AA	GM	Chronic	620	AZT,3TC,RTV,SQV
10	50	M	C	GM	Chronic	151	d4T,3TC,IDV
11	40	M	C	GM	Chronic	981	d4T,(RTV,SQV) ‡3TC,NFV
12	41	M	C	IDU	Chronic	133	d4T,3TC,(RTV)‡SQV
13	24	M	C	GM	Chronic	477	d4T,3TC,RTV
14[e]	35	F	AA	IDU/Het	Chronic	253	d4T,3TC,IDV
15	45	F	AA	IDU	Chronic	176	3TC, IDV,(ddI)‡EFV
16	34	M	C	GM	Chronic	472	d4T,3TC,RTV
17	25	F	C	Het	Acute (10)	504	AZT,3TC,RTV
18	44	M	C	GM	Chronic	477	AZT,3TC,d4T,IDV
19	29	F	C	TF	Chronic	8	AZT,3TC,RTV
20	31	M	C	GM	Acute (11)	916	AZT,3TC,RTV
21	52	M	C	GM	Chronic	246	AZT,3TC,IDV
22	38	M	C	GM	Chronic	244	AZT,3TC,IDV
23	30	M	C	GM	Acute (4)	1023	AZT,3TC,IDV
24	34	M	C	GM	Acute (<1)	289	AZT,3TC,NFV
25	22	M	C	GM	Chronic	504	ddI,IDV,HU
26	28	M	C	GM	Chronic	673	ddI,IDV,HU
27	33	M	C	GM	Chronic	630	ddI,IDV,HU
28[f]	35	M	C	GM	Chronic	350	AZT,3TC,(IDV)‡NFV
29[f]	34	M	C	GM	Chronic	240	AZT,3TC,(RTV)‡IDV
30[f]	29	M	C	GM	Chronic	426	AZT,3TC,IDV
31[f]	42	M	AA	GM	Chronic	338	AZT,3TC,IDV
32	39	F	AA	UN	Chronic	14	d4T,3TC,IDV
33	30	F	H	Het	Chronic	123	ddI,d4T,NVP,RTV,SQV
34[e]	52	M	AA	Het	Chronic	323	d4T,3TC,RTV,NFV
35	30	M	C	GM	Chronic	434	NVP,RTV,NFV

[a]C, Caucasian; AA, African American; H, Hispanic. [b]Risk groups: GM, gay male; IDU, intravenous drug user; Het, heterosexual; TF, transfusion recipient; UN, unknown. [c]Chronic, initiation of combination therapy in patients with well-established HIV-1 infection; acute, combination therapy initiated during primary HIV-1 infection, either before, during or shortly after seroconversion (numbers in parentheses indicate weeks between presentation with acute retroviral syndrome and initiation of treatment). [d]Rx, anti-retroviral therapy: AZT, zidovudine; d4T, stavudine; 3TC, lamivudine; ddI, didanosine; NVP, nevirapine; EFV, efavirenz; RTV, ritonavir; SQV, saquinavir; IDV, indinavir; NFV, nelfinavir, HU, hydroxyurea. Drugs used in the regimens that produced effective suppression of viral replication are listed; drug substitutions (‡) were made because of side effects, not virologic failure (drugs in parentheses were discontinued and replaced with drugs to the right of the arrow). [e]Removed from the study because of virologic failure and/or interuption of therapy as a result of drug toxicity or problems with adherence. [f]Also on daily subcutaneous low-dose IL-2.

Size of the latent reservoir

We determined the frequency of latently infected resting CD4+ T cells with replication-competent HIV-1 as a function of time on combination therapy (Fig. 1, summary plot). Of 79 cultures of resting CD4+ T cells from 34 treated patients in whom plasma virus was less than 200 copies/ml, replication-competent virus was isolated 83% of the time (Fig. 1, filled symbols). This high rate of virus isolation was important because it allowed quantitative analysis of decay rates. Only 2 of these 34 patients (6%) were uniformly negative in repeated assays. Despite the wide dynamic range of the culture assay (more than 6 logs: 10,000–0.01 infectious units per million, IUPM), measurements showed a distinct propensity to cluster in the range of 0.1–1.0 IUPM (Fig. 1). The geometric mean frequency for all positive determinations after 3 months was 0.82 IUPM. These results confirm the persistence of HIV-1 in a small latent reservoir in most patients, including those with plasma virus levels less than 200 copies/ml.

Longitudinal analysis of the decay rate of the latent reservoir

To address the essential issue of the decay rate of the latent reservoir, we did longitudinal analysis of the frequencies of latently infected cells in individual patients. Included in the data set (Fig. 1) are longitudinal measurements on 20 patients whose plasma virus levels had decreased to and remained less than 200 copies/ml (Fig. 2). In these patients, the frequencies of latently infected cells were measured two to seven times over the course of 7–21 months (mean follow-up, 14.4 months). Although there was some fluctuation in the levels in individual patients, consistent with the relatively wide 95% confidence intervals for individual determinations, the data demonstrate that this compartment has an extremely slow decay rate, with slopes nearly zero in most cases (Fig. 2).

To provide a more accurate estimate of half-life, we did statistical analysis using a random-effects regression model[30] for decay with first-order kinetics (Table 2). This analysis used all the positive longitudinal and cross-sectional measurements on patients who had plasma virus levels less than 200 copies/ml, taking into account correlation between repeated observations on the same

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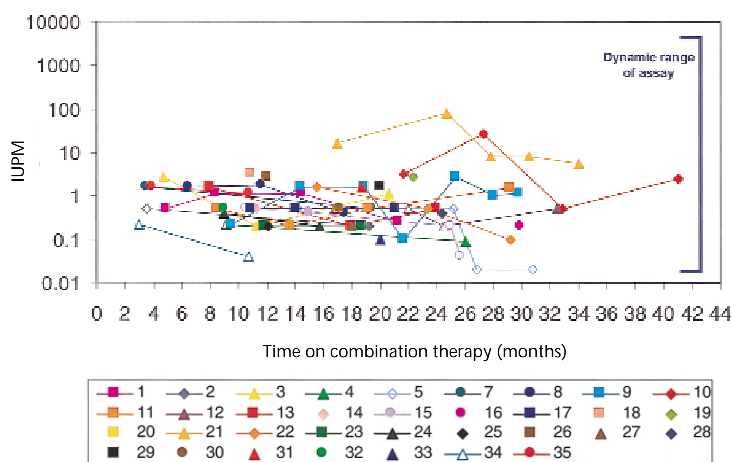

Fig. 1 Frequency of latently infected cells in 34 HIV-1-infected adults on combination therapy who had plasma virus levels less than 200 copies/ml. Frequencies were measured using a culture assay that detects replication-competent HIV-1 persisting in resting $CD4^+$ T cells. Results are expressed as infectious units per million (IUPM) resting $CD4^+$ T cells. Colored lines, repeat measurements in individual patients (numbers in key, patient numbers); filled symbols, successful detection of resting $CD4^+$ T cells with replication-competent HIV-1 at the indicated frequencies; open symbols, unsuccessful virus isolation (for these, the upper bound on the infected cell frequency, estimated based on the number of input cells, is plotted). Initial time points on patients 1–22 have been previously reported[25] and are included here for reference and to provide initial values for the measurement of decay rates. Blue bar (right), dynamic range of the assay.

patient. The result was an extremely slow mean decay rate, with a slope of –0.00686 $\log_{10}$ IUPM/month, consistent with a $t_{1/2}$ of 43.9 months (Table 2). This slope is not statistically different from zero ($P = 0.406$). The lower 95% confidence bound for the slope (–0.0234) gave a half-life of 12.86 months. The upper confidence bound for the slope was positive; thus, the half-life could not be calculated. Using an estimate of 1×10^6 cells as the total size of the reservoir[23] and the mean decay rate calculated using random effects regression model, we predict that 73 years of therapy would be required for eradication of this reservoir. Even using the lower 95% confidence bound for the slope, 21.4 years on therapy would be required. If the reservoir contains only 1×10^5 cells, 60.8 years of therapy would be required in the average case, and 17.8 years would be required, if the lower 95% confidence bound is used (Fig. 3). If the analysis is restricted to patients from whom multiple determinations are available, then the estimated decay rate is even slower (Table 2). These results conclusively demonstrate that in a patient population that reflects the diversity of the epidemic, this reservoir does not decay in a clinically relevant time frame with current 'standard of care' therapy.

There was some variation in the slopes in individual patients, ranging from no decay at all (zero or positive slopes) in five patients (9, 11, 12, 17 and 23) to a fairly rapid decay rate in one patient (22, $t_{1/2} = 3.5$ months). Although decay rates were measured only in patients who had viral loads that were undetectable by a standard RT–PCR assay (less than 200 copies/ml), a subset of the patients (9, 10 and 21) had plasma RNA levels in the range of 20–200 copies/ml by an 'ultrasensitive' assay on more than one occasion. However, these 'spikes' did not seem to result in a slower decay rate. In two of these three patients, the decay rate was actually faster than the mean decay rate for the remaining patients, who consistently had less than 20 copies/ml.

In 3 of 34 patients (5, 15 and 34), virus isolation was sometimes or always unsuccessful. In the absence of culturable virus in the sample of resting cells (Fig. 1, open symbols), the value plotted represents an upper bound on the infected cell frequency. Virus isolation was most difficult in two patients (5 and 15) who were started on therapy late in the course of disease with very low CD4 nadirs. The only other patient in whom multiple attempts to detect latent HIV-1 were unsuccessful had a low level of latently infected cells before therapy began (data not shown) and is therefore also a 'special case'. Two attempts to detect these cells after the initiation of treatment were unsuccessful. Nevertheless, the persistence of virus in this patient is indicated by the fact that the patient experienced a 'rebound' in plasma virus after becoming noncompliant with therapy.

Establishment of the latent reservoir in primary HIV-1 infection

Given the extremely slow decay rate of the latent reservoir in resting $CD4^+$ T cells, we also determined whether early treatment could affect the size and stability of the reservoir. Included in the data set are four patients (17, 20, 23 and 24) who were started on combination therapy during primary HIV-1 infection and were then followed for 16–22 months, during which time they had suppression of viremia to less than 200 copies/ml. Latently infected cells were detected in each (Figs. 1 and 2), despite the fact that in two of these patients, treatment was started even before seroconversion (patients 23 and 24). For patient 24, therapy was started 48 hours after the patient presented with acute retroviral syndrome, but persistent, latently infected cells were nevertheless readily detectable. Longitudinal studies in three patients (17, 23 and 24) showed only minimal evidence for decay (two of three slopes were zero; Fig. 2).

Table 2 Decay of latently infected $CD4^+$ T cells

Method of analysis[a]	Slope (m)[b]	s.e.m. (m)	*P* value for difference from m = 0[c]	95% confidence intervals for m	Half-life (months)	95% confidence interval for half-life (months)	Time to eradicate 10^5 cells (years)	Time to eradicate 10^6 cells (years)
A	–0.00686	0.00812	.406	–0.0234, 0.00968	43.94	(12.86 (§)[d]	60.8	73.0
B	–0.00582	0.00786	.466	–0.0218, 0.0102	51.92	(13.84 ()	71.9	86.2
C	–0.00434	0.00890	.629	–0.0224, 0.0137	69.50	(13.43 ()	96.2	115.4

Longitudinal and cross-sectional data were analyzed using a random-effects regression model[31] for decay with first-order kinetics effects. Three patients (5, 15 and 34) from whom attempts to measure latently infected cells were generally unsuccessful were not included in the analysis; these patients represent 'special cases' because of extremely low CD4 nadirs (5 and 15) or low pre-treatment levels of latently infected cells (34). [a]A, all longitudinal and cross-sectional data points after 3.5 months were included except for the upper bound estimates for patients 2, 4 and 12; B, upper bound estimates were included as data; C, as described for A but restricted to only those patients providing at least two data points. [b]The slope (m) of the decay latently infected cells was determined from a plot of $\log_{10}$ IUPM versus time in months. [c]*P* values determine whether the slope (m) is statistically different from zero. [d]Upper confidence interval limit is positive and therefore a half-life cannot be calculated.

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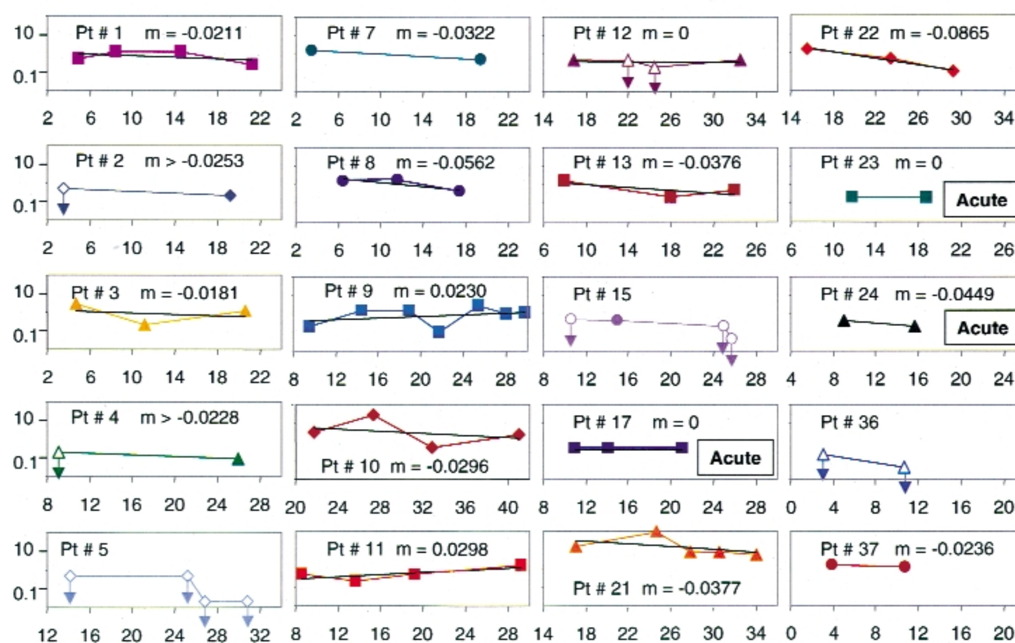

Fig. 2 Decay rates of the latent reservoir in individual patients. Frequencies in IUPM are plotted on a $\log_{10}$ scale (vertical axes) as a function of time in combination therapy in months (horizontal axes). Black lines, the slope of a least-squares regression line (when more than two determinations were made). Acute, patients started on combination therapy during acute infection. For patients 2 and 4, the initial measurements were negative and only an upper bound on the infected cell frequency could be determined. Subsequent determinations were positive. The slope is therefore a minimal estimate, which may underestimate the actual half-life. Pt #, patient number; m, slope.

Discussion

Recent advances in anti-retroviral therapy have proven to be very effective in reducing viral load in patients with HIV-1-infection, leading to demonstrated reductions in morbidity and mortality[31] and providing a basis for the first attempts, at predicting HIV-1 eradication since the beginning of the epidemic[14]. Despite this progress, our study indicates that latent HIV-1 in resting CD4+ T cells will be a principal obstacle to virus eradication. Through direct longitudinal analysis of the decay rate of the latent reservoir, we have demonstrated that the reservoir is very stable, with a half-life of more than 43 months in the average patient on current 'standard of care' therapy. Even with conservative estimates of the total body number of latently infected cells, an average of at least 60.8 years of treatment will be required to eradicate this compartment in most patients. Our findings are based on the isolation from this reservoir of viruses that are fully replication-competent and are therefore likely to be capable of 'rekindling' the infection in patients who stop therapy. The extremely slow decay rate of this reservoir raises the disturbing prospect that in some patients, the time required for HIV-1 eradication with current combination regimens may be so long that other intervening problems, such as cumulative toxicities of anti-retroviral drugs[32], may make eradication difficult.

In interpreting the data presented here, the strengths and limitations of the culture assay used must be considered. The 95% confidence intervals about individual determinations are +/- 0.7 logs; therefore random fluctuations could either overestimate or underestimate the decay rate on an individual level if only a few observations are used. To overcome this variability, we have collected observations on many patients over a long time and analyzed the data using a random-effects regression model. Based on the number of patients measured here, and the time that they have been followed, we have provided both a point estimate of the half-life (43.9 months) and a 95% confidence interval for the half-life. Although our study does not have sufficient statistical 'power', with these data, to discern small differences in decay rates, such as whether the half-life is closer to 40 months or 50 months, this study is able to exclude very rapid decay. The lower bound of the 95% confidence interval (12–13 months) indicates that our data would not be consistent with a more rapid mean decay rate (for example, less than 12 months) for the patient population studied. The upper bound on the mean slope is positive, consistent with no decay at all.

Because the assay involves actual virus isolation, it directly measures the form of the virus that is of the most concern: replication-competent virus persisting in a latent form in resting CD4+ T cells. Thus, when virus is isolated from a culture of 1 million resting CD4+ T cells, there is no other explanation than that the virus has persisted in latently infected cells present at a frequency of at least 1 latently infected cell per million. Therefore, the results presented here argue in a very definitive way for long-term persistence of the virus.

The decay rates measured here are best thought of as the observed decay rates for this compartment in patients treated with current 'standard of care' therapy, in contrast to the true intrinsic decay rate of the reservoir. The considerable stability of the latent reservoir is consistent with the fact that the reservoir is composed at least in part of memory T cells carrying integrated HIV-1 DNA (ref. 23). The biological function of memory T cells is to persist and provide protection against previously encountered microorganisms. The half-life of memory T cells in normal humans has not been studied extensively, but some estimates of an intermitotic half-life in the range of 5–6 months have been reported[33]. The mean half-life of the latent reservoir is much longer than the reported mean intermitotic half-life of memory CD4+ T cells, indicating that the latent reservoir may be renewed by occasional proliferation of the infected cells or by entry of new cells into the reservoir as a result of a low level of ongoing viral replication[34]. A subset of our patients had occasional 'spikes' in plasma RNA levels into the range of 20–200 copies/ml. The importance of this very low level of ongoing replication is unclear, and in two of three patients, the decay rate was actually faster than that seen in patients who had consistent suppression of viral replication to less than 20 copies/ml. Additional studies will probably be needed to determine whether suppression to less

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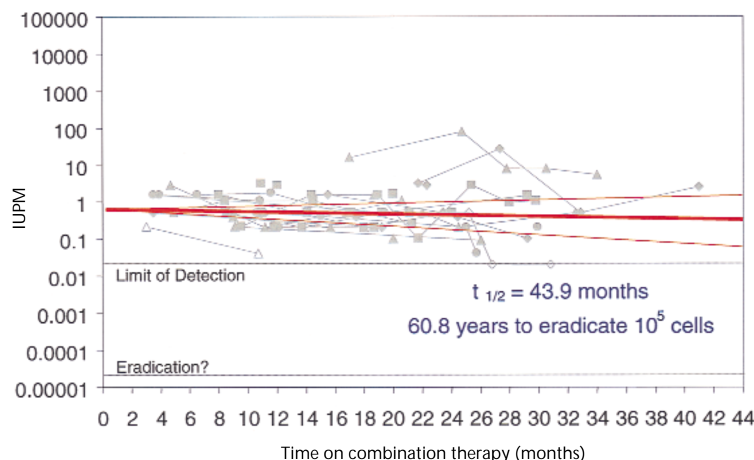

Fig. 3 Mean rate of decay of the latent reservoir. Elimination of a reservoir of 1×10^5 cells will require 5 logs of decay, therefore decay data are plotted on an expanded axis (vertical). The mean slope (thick red line) and the 95% confidence intervals about the mean slope (thin red lines) were calculated as described in Table 2, Analysis A. IUPM, infectious units per million.

than 20 copies/ml gives a faster decay than suppression to less than 200 copies/ml. If ongoing viral replication contributes to the stability of the reservoir, then the true intrinsic decay rate may be more rapid than that measured here. In this situation, the development of even more potent anti-retroviral regimens may stop all residual replication and show the true intrinsic decay rate of the latent reservoir, permitting eradication if this rate is sufficiently rapid and if there are not other substantial reservoirs.

If low-level ongoing replication contributes new infected cells to the latent pool, then selection for drug-resistant virus in this pool may eventually become evident. Sequencing of the reverse transcriptase and protease regions of the *pol* genes of viruses isolated from the latent reservoir has so far shown little evidence for the evolution of drug resistance[25,27]. Thus, our results so far are consistent with the idea that the long half-life of the reservoir is mainly due to its intrinsic stability. Nevertheless, it is possible that low-level ongoing replication is occurring and may eventually result in the emergence of drug resistance[26,35] during the extremely long time required for decay of the latent reservoir.

Given the considerable stability of the latent reservoir, there has been interest in whether early treatment can prevent the establishment of the reservoir, limit its size or accelerate its decay. The answer to the first question seems to be no. Latently infected cells are present in patients who have been treated during or shortly after seroconversion, as demonstrated here and in previous reports[25,26]. In the acute seroconvertors studied here, the size and decay rate of the latent reservoir were similar to those seen in patients who started therapy at later stages. However, it is possible that the size of the latent reservoir may be reduced by early treatment with new combination therapy regimens that include drugs like hydroxyurea that target the host cells (F.L., J.L, D.F. and R.F.S., unpublished results). In addition, a recent study indicates a more rapid decay rate in a subset of acute seroconvertors who started combination therapy within 90 days of infection (Zhang *et al.*, manuscript submitted).

In the analysis of 34 patients on combination therapy who had plasma HIV-1 RNA levels of less than 200 copies/ml, replication-competent virus was isolated from the latent reservoir in all but two. In one (patient 34), the persistence of a latent reservoir is indicated by the fact that the patient eventually failed therapy because of problems with adherence. These results emphasize that negative results in an assay for latently infected cells cannot be used to demonstrate that eradication has occurred. The lower end of the dynamic range of the assay depends on the number of cells from the patient that can be cultured. For patients with negative culture assays, it is very likely that virus isolation would be possible if more cells could be cultured. The only other patient from whom virus was never isolated despite repeated attempts was patient 5, who was in advanced stages of AIDS with a $CD4^+$ T-cell count of 10 when treatment was begun. It is possible that the few residual infected cells in this patient were simply 'diluted out' by newly generated $CD4^+$ T cells appearing after the initiation of therapy. Nevertheless, it is encouraging that even in very late-stage patients, immune reconstitution can occur without the production of detectable numbers of new latently infected cells.

Although decay of the latent reservoir is a very slow process, it is possible is that total eradication of the latent reservoir may not be necessary. In certain conditions, the immune system may be capable of controlling the small amount of virus released from the latent reservoir. Rare patients do not experience a rebound in plasma virus after stopping therapy even though they continue to harbor replication-competent virus in resting $CD4^+$ T cells (J.L. *et al.*, manuscript submitted). Long-term nonprogressors have persistent polyclonal HIV-1-specific $CD4^+$ T-cell responses that effectively orchestrate antiviral immune responses[36]. New immunotherapeutic approaches such as HIV-1 vaccination or cytokine therapy might enhance virus-specific immunity sufficiently to allow containment of any virus released from this very stable reservoir or might directly mobilize the reservoir[37,38]. However, the results presented here demonstrate that without specific interventions, the persistence of latently infected cells will probably represent a major barrier to virus eradication.

Methods

Patient population. The patients included 21 patients from a previously described cohort[25] who maintained long-term suppression of viral replication on combination therapy. These patients are numbered 1–22 in keeping with the previous numbering; patient 6 in this group failed therapy and was not considered. Thirteen additional patients selected according to the above criteria (patients 23–34) were also studied. In 17 patients, multiple viral load measurements made with an 'ultrasensitive' assay (sensitivity, 20 copies/ml) were available. These measurements were generally negative, except in patients 9, 10 and 21, who had more than one measurement in the range of 20–200 copies/ml. In most patients, substantial increases in CD4 counts with therapy were noted. All patients gave informed consent for phlebotomy.

Isolation of resting $CD4^+$ T cells. Resting $CD4^+$ T cells were purified from peripheral blood mononuclear cells by bead depletion and flow cytometry as described[22,23].

Quantitation of latently infected cells. Latently infected cells were measured using a limiting dilution culture assay[22,23,25]. The culture assay is based on the standard virus culture assay used by the AIDS Clinical Trials Group and the data are analyzed using their standard method. However, our assay has an important modification: a specific step to activate the resting cells. Resting cells were activated with the mitogen phytohemagglutinin and a more than tenfold excess of irradiated HIV-negative donor peripheral blood mononuclear cells, in conditions that induce activation

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of CD4+ T cells with high efficiency, allowing rescue of infectious virus from latently infected cells by co-culture[22,23,25]. Growth of virus was detected by measuring p24 antigen in culture supernatants by ELISA. The assay was set up as a duplicate fivefold-dilution series, from as many as 25×10^6 cells/well to as few as 320 cells/well. Control wells with no patient cells were assessed in each assay and were invariable negative. Sequence analysis of *pol* and *env* genes from virus isolates demonstrated that the isolates from each patient were unique and distinct from all reported isolates and laboratory strains[25]. Infected cell frequencies were determined by the maximum likelihood method[39] and were expressed as infectious units per million (IUPM) resting CD4+ T cells. The confidence intervals for individual determinations were +/- 0.7 log IUPM. Random-effects regression models[30] were used to estimate the mean decline in log IUPM over follow-up time from initiation of therapy. Half-life estimates were calculated assuming first-order decay kinetics. Only data obtained more than 3.5 months after the initiation of therapy were used in the analysis to avoid detection of a labile pre-integration form of latency observed in patients with high viral loads (refs. 23,40, 41, and J.B. and R.F.S., unpublished data).

Measurement of plasma virus. Standard viral load measurements were made by an RT–PCR assay with a sensitivity of 200 copies/ml. 'Ultrasensitive' measurements of plasma virus were made using a modification of the Roche HIV-1 Monitor test.

Acknowledgments

We thank C. Raines, S.Barnett, B. Perdue-Sabundayo and J. Keruly for coordinating patient visits and help with data analysis. We also thank L. Carruth, J. L'Esperance and C. Murray for help with the experiments. We thank Y. Afacan for providing patients and R. Brookmeyer for advice on statistical analysis of decay rates. This work was supported by NIH grant AI43222 to R.F.S.

RECEIVED 25 MARCH; ACCEPTED 2 APRIL 1999

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