−80°C for quantitation of HIV RNA and p24 antigen. Virus isolations by the standard ACTG protocol (*17*) were performed with 10^7 unstimulated patient PBMCs along with 10^7 PBMCs from an HC donor, prestimulated for 24 to 48 hours with PHA (final concentration, 3 μg/ml). On the basis of CD4 percentage for these six patients, 10^7 PBMCs corresponded on average to 2.5×10^6 patient CD4 cells used for each standard culture (compared with 2×10^6 CD8-depleted/CD4-enriched cells used for each of the enhanced coculture conditions).

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19. In six of the seven individual coculture experiments, culture conditions incorporating either TNF-α or autologous macrophages or both (in addition to CD8 depletion and activation with CD3 and CD28 mAbs) resulted in the earliest and highest measured RNA concentrations (and p24 concentrations) by days 14 to 21. This occurred in two experiments with TNF-α alone, in three with macrophages alone, and in one with both TNF-α and macrophages.
20. RNA extractions from plasma and viral supernatants were performed using the guanidinium isothiocyanate–phenol system provided with the Amplicor Monitor (Roche Molecular Systems, Branchberg, NJ). Population sequencing was performed with the GeneChip system (Affymetrix, Santa Clara, CA) (*24*) with the following modifications of the RT and PCR procedures as described in the current protocol (Rev. 3, April 1997): Primers used were PRT T7 (5′-*GTAATACGACTCACTATAGGG*CCACTAACTTCTGTATGTCATTGACAGTCCA-3′) for reverse transcription, PRT T3 (5′-*AATTAACCCTCACTAAAG*GGCAGACCAGAGCCAACAGCCCCA-3′) for PCR sense primer, and PRT T7 for antisense primer (T7 RNA and T3 RNA promoter sequences, respectively, are in italics). Reaction conditions were according to the manufacturer, using AMV RT (Gibco-BRL) for reverse transcription and rTth XL polymerase (Perkin-Elmer) for PCR amplification. The labeled, transcribed RNA was fragmented and then hybridized per manufacturer's specifications, to oligonucleotide probes arrayed on the GeneChip. GeneChips were scanned using a confocal laser microscope. The GeneChip 2.0 software (Affymetrix) with the Rules algorithm was used for base-calling and analysis of sequence data. The region sequenced includes all of the HIV protease region and the first 242 codons of the HIV RT. In all cases, verification of sequence data by the GeneChip method involving the known resistance-conferring codon was done by automated dideoxynucleotide sequencing with an ABI 373A sequencer (Applied Biosystems). Sequences have been submitted to GenBank (accession numbers AF027708 to AF027721). Phylogenetic analysis with tree-building based on maximum likelihood analysis was performed on the complete nucleotide sequence data sets with DNAML from Phylip 3.5 (*25*) and trees were produced using the DRAWTREE utility as described (*25*). Separate analysis using parsimony analysis with bootstrapping (×100) performed with SEQBOOT, DNAPARS, and CONSENSE, all in Phylip, gave similar results (*18*). Sequences for laboratory strains of HIV used in the comparative analysis were obtained electronically from NLBI/GenBank.
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26. We thank the patients at the San Diego AIDS Treatment Center who participated in this study; K. Nuffer, B. Coon, and G. Dyak for blood collection and patient recruitment; L. Terry, N. Keating, S. Albanil, V. Alvarez, and J. Aufderheide for technical assistance; E. Emini, J. Sninsky, R. Kornbluth, A. J. Leigh-Brown, and N. Riggs for their cooperation and advice; J. Guatelli for advice and critical review of the manuscript; K.-E. Hellstrom for CD28 mAb clone 9.3; and D. Smith and S. Wilcox for administrative assistance. Supported by National Institute of Allergy and Infectious Diseases grants AI 01361 (J.K.W.), AI 27670 (D.D.R.), AI 38858 (D.D.R.), AI 36214 (D.D.R.), and AI 29164 (Center for AIDS Research, San Diego); the Research Center for AIDS and HIV Infection of the San Diego Veterans Affairs Medical Center (D.D.R.); a Merit Award of the Department of Veterans Affairs (C.A.S.); and Swiss National Science Foundation grants 823A-050434 (M.H.) and 84AD-046176 (H.F.G.).

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Identification of a Reservoir for HIV-1 in Patients on Highly Active Antiretroviral Therapy

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The hypothesis that quiescent $CD4^+$ T lymphocytes carrying proviral DNA provide a reservoir for human immunodeficiency virus–type 1 (HIV-1) in patients on highly active antiretroviral therapy (HAART) was examined. In a study of 22 patients successfully treated with HAART for up to 30 months, replication-competent virus was routinely recovered from resting $CD4^+$ T lymphocytes. The frequency of resting $CD4^+$ T cells harboring latent HIV-1 was low, 0.2 to 16.4 per 10^6 cells, and, in cross-sectional analysis, did not decrease with increasing time on therapy. The recovered viruses generally did not show mutations associated with resistance to the relevant antiretroviral drugs. This reservoir of nonevolving latent virus in resting $CD4^+$ T cells should be considered in deciding whether to terminate treatment in patients who respond to HAART.

Current understanding of HIV-1 infection has been greatly advanced by measurements of viral burden in infected individuals (*1*, *2*) and of changes in viral burden after treatment with potent new drugs that inhibit the HIV-1 protease or reverse transcriptase (*3*–*8*). Wei *et al.* (*3*) and Ho *et al.* (*4*) have shown that HIV-1 production in infected individuals is largely the result of a dynamic process involving continuous rounds of de novo virus infection of and replication in host cells with rapid turnover of both free virus and virus-producing cells. In patients treated with HAART, plasma virus falls to undetectable levels in 2 to 4 months, and it becomes difficult to culture the virus from the blood (*7*, *9*). Perelson *et al.* have used mathematical models of decay rates of plasma HIV-1 RNA and peripheral blood mononuclear cell (PBMC) infectivity in treated patients to make the first rational predictions of treatment times required for virus eradication, with the caveat that there may be more stable compartments or viral reservoirs that are not measurable by standard methods (*7*).

One potentially stable reservoir is composed of latently infected memory $CD4^+$ T cells carrying integrated provirus (*5*, *10*, *11*). Postintegration latency appears to result from the reversion of productively infected $CD4^+$ T lymphoblasts to a resting memory state in which there is minimal transcription of viral genes. Recently, inverse polymerase chain reaction (PCR) has been used to demonstrate that latently infected resting memory $CD4^+$ T cells with integrated HIV-1 DNA are present in infected individuals (*10*, *11*). The frequency of these cells is extremely low, but a fraction of them harbor replication-competent provirus (*10*, *11*). Because memory $CD4^+$ T cells can persist for months to years (*12*),

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resting memory $CD4^+$ T cells carrying replication-competent viral genomes, although rare, may represent an important long-term viral reservoir in patients on HAART. Our study was undertaken to determine whether replication-competent virus could persist in the resting $CD4^+$ T cells of patients on HAART who had no evidence of active virus replication.

The presence of latent virus in resting $CD4^+$ T cells was examined in 22 patients treated with HAART for up to 30 months (Table 1). We selected patients who met each of the following three criteria: (i) patient- and physician-reported strict adherence to aggressive HAART regimens (typically, three or four drugs, including a protease inhibitor), (ii) a rapid decline in plasma HIV-1 RNA to undetectable levels (<200 copies per milliliter) by quantitative-competitive (QC) reverse transcription PCR (RT-PCR) assay after the initiation of HAART, and (iii) continued undetectable levels of plasma HIV-1 RNA on repeated measurements throughout the course of the study. Plasma HIV-1 RNA measurements reached undetectable levels in 2 to 3 months and remained undetectable on repeated sampling, including the day blood was taken for analysis of latently infected cells (Fig. 1A). Patients were tested an average of four times each during the months after initiation of HAART. A subset of three more intensively studied patients (patients 16, 19, and 20) had monthly measurements of plasma HIV-1 RNA levels, which were uniformly undetectable. These results suggest that in all of the patients selected for study, long-term suppression of viral replication to undetectable levels was achieved. Consistent with the success of treatment were gradual increases in CD4 counts in most patients (Fig. 1B) and the lack of viral evolution (see below).

We next isolated highly purified populations of resting $CD4^+$ T cells from the peripheral blood of these donors. After depletion of monocytes by adherence, negative selection with monoclonal antibodies and magnetic beads was used to remove $CD8^+$ T cells, B cells, monocytes, and natural killer cells. In addition, activated $CD4^+$ T cells, which represented an average of 10% of the $CD4^+$ T cell populations in these individuals (Table 1), were removed by bead depletion with antibodies to CD25, CD69, and human lymphocyte antigen (HLA)–DR. Additional purification was achieved by flow cytometric sorting of small lymphocytes expressing CD4 but not HLA-DR, which is expressed on all activated T cells. This multistep purification gave populations of resting $CD4^+$ $HLA\text{-}DR^-$ T cells that were, on average, 97% pure (Table 1). Most importantly, the sorted populations were typically contaminated with <1% activated $CD4^+$ T cells.

Specialized PCR approaches have shown that a low frequency of resting $CD4^+$ T cells from untreated seropositive individuals carry HIV-1 DNA in an integrated form (*2*, *10*, *11*). Culture studies have demonstrated that the fraction of resting $CD4^+$ T cells harboring replication-competent provirus is even lower (*11*) and quite variable from patient to patient, reflecting the fact that much of the proviral DNA detected by PCR may be defective (*11*, *13*). Therefore,

Table 1. Characteristics of patients studied.

Patient*	Drug regimen†	Time on HAART (months)	Percent activated $CD4^+$ T cells (of total $CD4^+$ T cells)‡	Purity of sorted resting $CD4^+$ T cells§ (%)	HIV-1 DNA‖	Virus isolation¶	Cells with replication-competent provirus (per 10^6 resting $CD4^+$ T cells)#
1	d4T, 3TC, RTV, SQV	5.0	ND	98.5	+	+	0.5
		8.5	16.5	97.7	ND	+	1.1
2	d4T, NVP, RTV, SQV	3.5	ND	92.7	ND	−	<0.5
3	AZT, 3TC, RTV	4.5	ND	97.8	+	+	2.8
4	d4T, 3TC, IDV	9.0	ND	93.8	ND	−	<0.2
5	AZT, 3TC, NFV	14.0	ND	97.4	ND	−	<0.5
6	AZT, 3TC, RTV	1.7	ND	95.3	+	+	1.6
7	AZT, 3TC, NVP	3.5	ND	94.6	+	+	1.6
8	AZT, 3TC, IDV→NFV	6.5	ND	95.7	<40	+	1.6
9	AZT, 3TC, RTV, SQV	9.5	ND	98.0	+	+	0.2
10	d4T, 3TC, IDV	8.5	12.3	97.0	+	+	3.2
11	d4T, RTV, SQV	1.5	10.3	99.4	+	+	0.5
12	d4T, 3TC, RTV→SQV	17.0	ND	96.1	+	+	0.5
13	d4T, 3TC, RTV	8.0	6.3	97.8	<40	+	1.6
14	d4T, 3TC, IDV	10.5	ND	97.3	+	+	0.5
15	ddI, 3TC, IDV	10.0	20.9	95.4	ND	−	<0.5
16	d4T, 3TC, RTV	30.0	5.5	96.3	+	+	0.2
17	AZT, 3TC, RTV	11.0	8.3	96.6	<40	+	0.5
18	AZT, d4T, 3TC, IDV	10.5	9.8	98.1	ND	+	3.2
19	AZT, 3TC, RTV	22.0	8.1	97.2	+	+	2.8
20	AZT, 3TC, RTV	17.0	6.4	97.5	+	+	0.5
21	AZT, 3TC, IDV	17.0	11.7	93.1	ND	+	16.2
22	AZT, 3TC, IDV	15.5	6.6	98.4	<40	+	1.6

*Patients were adult volunteers who gave informed consent before giving blood for this study. †Drugs used in HAART regimens included the nucleoside analogs AZT, stavudine (d4T), and 3TC; the nonnucleoside reverse transcriptase inhibitor nevirapine (NVP); and the protease inhibitors RTV, SQV, IDV, and nelfinavir (NFV). In most cases, treatment with three drugs for which the patient had no prior experience was started simultaneously. In two cases, one protease inhibitor was substituted for another (that is, RTV→SQV) because of toxicity, but in all cases, treatment with three or four drugs was maintained. Several of the patients had had previous monotherapy, generally with nucleoside analogs (*27*). ‡Determined by flow cytometry as the percentage of $CD4^+$ $HLA\text{-}DR^+$ cells among the total $CD4^+$ T cell population. §Resting $CD4^+$ T cells were purified by magnetic bead depletion and flow cytometry as previously described (*10*, *11*). The purity was determined by reanalysis after sorting as the percentage of $CD4^+$ $HLA\text{-}DR^-$ cells among the total sorted population. ‖Determined by semiquantitative PCR with gag primers as previously described (*11*). The sensitivity of the assay, as assessed with an HIV-1 plasmid diluted into genomic DNA from an uninfected donor, was about approximately 40 copies in 10^6 cells. Failure to detect HIV-1 DNA on repeated analysis is indicated as <40 copies per 10^6 cells. ND, not done. ¶Virus isolation was negative (−) only in the case of four patients from whom insufficient cells could be recovered to carry out a full duplicate limiting dilution series starting with 5×10^6 cells per well (+ indicates that virus isolation was positive). For patients 2, 4, and 5, the CD4 counts were low, making isolation of resting $CD4^+$ T cells difficult. #Calculated from the limiting dilution culture data with the maximum likelihood method of Myers *et al.* (*28*). The 95% confidence intervals for individual determinations spanned about 1.4 logarithms. The goodness of fit for almost all measurements was 1.0. In cases in which insufficient cells were obtained to carry out a full dilution series and all tested wells were negative, an upper bound on the frequency of cells with replication-competent provirus was estimated by assuming that the next highest cell concentration would have been positive.

although proviral DNA is readily detectable in most patients on HAART (Table 1) (*3*, *7*, *8*, *14*, *15*), the critical issue is whether virus persists in a replication-competent form. After 2 to 12 months on HAART, standard culture assays for replication-competent virus in unfractionated PBMCs are typically negative (*7*, *14*). We reasoned that detection of latent virus in resting $CD4^+$ T cells might require enrichment for the relevant cell population, afforded by purification of $CD4^+$ $HLA\text{-}DR^-$ cells, and a high-efficiency activation step (*16*) in which all of the resting cells are induced to undergo blast transformation and become permissive for viral replication. Therefore, dilutions of purified resting $CD4^+$ T cells were activated in the presence of phytohemagglutinin (PHA) and irradiated PBMCs from an HIV-1–negative donor. These conditions allow activation of virtually 100% of resting T cells (*17*). The day after activation and again on day 7, CD8-depleted PHA blasts from an HIV-1–negative donor were added to amplify infectious virus.

In each of 18 cases in which a sufficient number of resting $CD4^+$ T cells were obtained for analysis, replicating virus was isolated by this culture system (Table 1). One of these patients had been on HAART for 30 months with no detectable plasma virus. Two other patients had been on HAART for over 20 months. Data were quantified by limiting-dilution assay. In wells seeded with the highest numbers of purified resting $CD4^+$ T cells (5×10^6 or 1×10^6), large amounts of p24 were present in the supernatants on day 14. Wells containing less than 2×10^5 $CD4^+$ $HLA\text{-}DR^-$ cells were usually negative. Control wells lacking patient cells were invariably negative for p24. Virus isolation failed only in the case of four patients from whom an insufficient number of resting $CD4^+$ T cells were isolated to set up replicate wells at 5×10^6 or 1×10^6 cells per well.

In principle, activation of resting $CD4^+$ T cells carrying defective viral genomes could result in the production and release of noninfectious viral particles. However, if all of the viral particles released after activation of resting $CD4^+$ T cells were noninfectious, amounts of p24 in the supernatant should decrease over time as the cultures are fed and split. This was not the case, as levels of p24 in our culture system were generally undetectable on the first day after stimulation and increased markedly between days 7 and 14, indicative of a spreading infection (*18*). To confirm that resting $CD4^+$ T cells from patients on long-term HAART harbor infectious virus, we further passaged p24 positive supernatants onto $CD4^+$ T lymphoblasts from HIV-1–negative donors, and increasing supernatant p24 val-

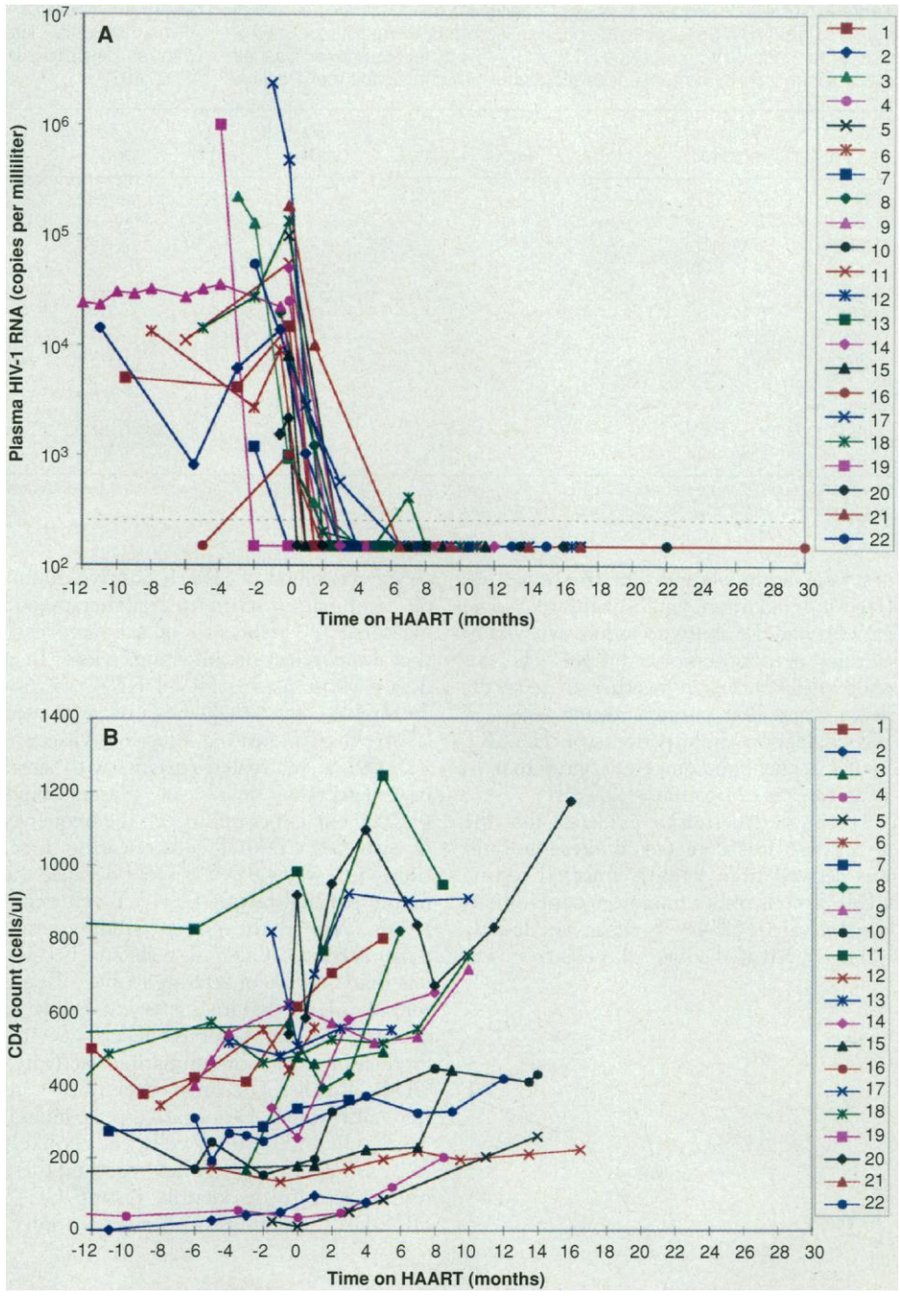

Fig. 1. Effectiveness of HAART in suppressing viremia and reversing CD4 depletion. (**A**) Time course for changes in plasma HIV-1 RNA after initiation of HAART. The zero time point is taken as the day on which the patient began a completely suppressive combination therapy regimen, typically a three- or four-drug regimen with at least one protease inhibitor (Table 1). In patient 19, monotherapy had produced a decline in plasma viremia to undetectable levels even before the initiation of combination therapy. Nevertheless, the zero time point was considered to be the day combination therapy was started. The dashed line indicates the limit of detection of the assay (200 copies per milliliter). Assays in which HIV-1 RNA was undetectable are arbitrarily plotted as 150 copies per milliliter. In patient 17, an acute seroconvertor, plasma HIV-1 RNA was still detectable at 3 months (570 copies per milliliter) and subsequently declined to undetectable levels. In this case, the longer interval to undetectable plasma HIV-1 RNA probably reflects the extremely high levels present before treatment (2.36×10^6 copies per milliliter). For patient 21, at the earliest visit after treatment at 1.5 months, plasma HIV-1 RNA was undetectable by the bDNA assay (limit of detection, 10,000 copies per milliliter). On the basis of the subsequent responses, it is likely that HAART therapy produced more rapid decline than that shown. After viral RNA became undetectable, plasma HIV-1 RNA levels remained undetectable in all patients, with the exception of a single reading of 415 copies per milliliter on one occasion for patient 17. (**B**) Time course for changes in CD4 count after initiation of HAART.

Table 2. Nucleotide sequences of eight isolates from resting CD4+ T cells of patients on HAART. Sequences at codons associated with resistance to RTV or SQV for patients who received RTV or SQV (or both) are shown. Dashes indicate identity with the consensus sequence shown in the top row. Positive culture supernatants were amplified by RT-PCR and sequenced with the Affymetrix (Santa Clara, California) high-density oligonucleotide array (GeneChip) as described (*14, 21*) (GenBank accession numbers AF027391 to AF027402).

Patient (isolate)	Protease inhibitor	10 ctc/L	20 aag/K	33 tta/L	36 atg/M	46 atg/M	48 ggg/G	54 atc/I	71 gct/A	82 gtc/V	84 ata/I	90 ttg/L
1	RTV, SQV	---	---	---	---	---	---	---	---	---	---	---
9* (5M1)	RTV, SQV	---	---	---	---	---	---	g--/V†	---	-c-/A†	---	---
9* (5M2)	RTV, SQV	---	---	---	---	---	---	---	---	---	---	---
11	RTV, SQV	---	---	---	---	---	---	c--/L†	---	---	---	---
13	RTV	---	---	---	---	---	---	---	---	---	---	---
16‡	RTV	---	---	---	--a/I†	---	---	---	a--/T	---	---	---
19	RTV	---	---	---	---	---	---	---	---	---	---	---
20	RTV	---	---	---	---	---	---	---	---	---	---	---
Known resistance mutations for → RTV			R	F	I	I		L, V	V	A, T, F, S	V	M
→ SQV		I					V	V			V	M

*Patient 9 was on RTV monotherapy for 15 months before starting HAART (AZT, 3TC, RTV, and SQV) 9.5 months before analysis. †Resistance mutations. ‡Patient 16 was on monotherapy for 7 months before beginning a combination regime.

ues were again observed in these cultures (Fig. 2). In addition, viral isolates from some patients had the ability to induce syncytia in cultures of nontransformed CD4+ T lymphoblasts (*19*). Taken together, these results demonstrate that purified resting CD4+ T cell populations from patients on HAART harbor replication-competent virus that in some cases is cytopathic.

To provide definitive evidence that the infectious virus detected in these cultures was derived from latently infected resting CD4+ T cells rather than from contaminating activated CD4+ T cells, we directly isolated activated CD4+ T cells from the peripheral blood of HIV-1–positive donors on combination antiretroviral therapy and measured the frequency of activated cells that were releasing infectious virus. In a donor whose plasma HIV-1 RNA was undetectable, the frequency of activated CD4+ T cells releasing infectious virus was <0.0002%. In treated patients with small but detectable levels of plasma virus (~1000 copies per milliliter), the frequency of activated CD4+ T cells releasing infectious virus was <0.002% (*18*). Because the highly purified resting CD4+ T cell populations used in the present study contain <1% activated CD4+ T cells and because less than 0.002% of activated CD4+ T cells release infectious virus, it is impossible to account for the observed levels of virus production by contaminating activated CD4+ T cells. The only other trace contaminants were CD4-negative cells, including B cells and CD8+ T cells, cells unlikely to be sources of virus. Taken together, these results definitively identify resting CD4+ HLA-DR− T cells as a reservoir for infectious HIV-1 in infected individuals on long-term HAART.

The replication-competent virus present in these cells is likely to be in an integrated form. When purified resting CD4+ T cells are infected in vitro and then incubated for 6 days, it becomes difficult to recover virus by activation of the cells (*11*). Thus, neither virions that remain bound to the cell surface nor unintegrated HIV-1 DNA molecules formed in the cells were capable of giving rise to a productive infection after this incubation. When resting cells from patients on HAART were preincubated before activation, the measured frequencies were very similar to those obtained by immediate activation, indicating that the recovered virus was derived from cells carrying stable integrated forms (*18*).

To determine the frequency of cells carrying replication-competent provirus among resting CD4+ T cells, we analyzed the limiting dilution virus culture data by a maximum likelihood method (Table 1 and Fig. 3). Overall, the measured frequencies

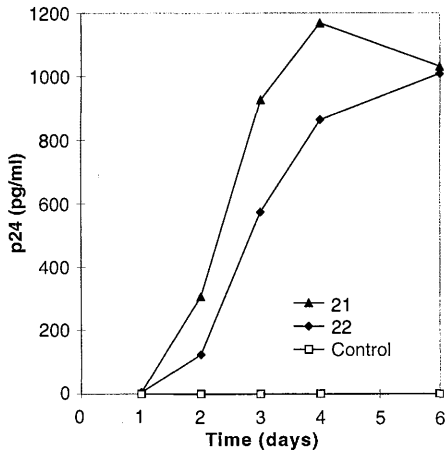

Fig. 2. Replication competence of viruses derived from latently infected resting CD4+ T cells. Supernatants from positive limiting dilution cultures were used to infect CD4+ T lymphoblasts from an HIV-1–negative donor. Amounts of p24 antigen in a 1/250 dilution of culture supernatant were measured by ELISA. Increasing p24 amounts in the supernatants of these cultures demonstrate the successful passage of infectious virus. Control supernatants were from wells containing mock-infected PHA blasts.

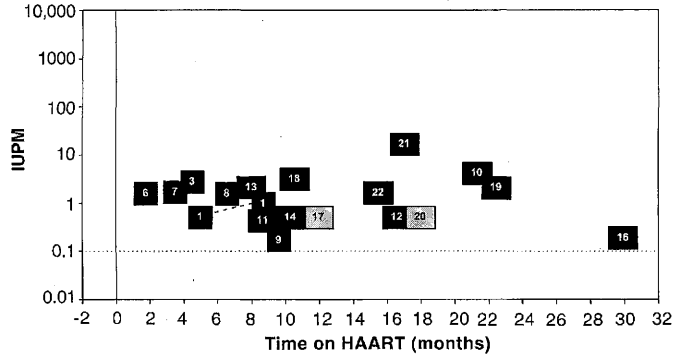

Fig. 3. Relation between the frequency of latently infected CD4+ T cells and the time on HAART. Frequencies are expressed in terms of infectious units per 10^6 resting CD4+ T cells (IUPM). Each square indicates the isolation of virus from a patient on HAART for the indicated number of months. The patient number is indicated inside the square. Black squares indicate long-term infected patients. Shaded squares (patients 17 and 20) are acute seroconvertors treated within 10 weeks of presentation. The dashed line indicates that assays done on patient 1 after 5 and 8.5 months of HAART showed about the same amount of latently infected cells. The dotted line indicates the normal limit of the detection of the assay.

Table 3. Nucleotide sequences of four isolates from resting CD4+ T cells of patients on HAART. Sequences at codons associated with resistance to IDV for patients who received IDV are shown. Dashes indicate identity with the consensus sequence shown in the top row. Procedures used are described in the legend to Table 2.

Patient	Protease inhibitor	10 ctc/L	20 aag/K	24 tta/L	46 atg/M	54 atc/I	71 gct/A	82 gtc/V	84 ata/I	90 ttg/L
14	IDV	---	---	---	---	---	---	---	---	---
18	IDV	---	---	c--/Q	---	---	---	---	---	---
21	IDV	--t/L	---	---	---	---	---	---	---	---
22	IDV	---	---	---	---	---	---	---	---	---
Known resistance mutations		I, R, V	M, R	I	I, L	V	T, V	A, F, T, V	V	M

ranged from 0.2 to 16.2 per 10^6 resting CD4+ T cells. These frequencies are lower than the frequencies of cells carrying persistent replication-competent forms of HIV-1 DNA previously measured in a different group of patients who were not on HAART [1 to 81 per 10^6 resting CD4+ T cells, mean 7 per 10^6 (*11*)]. In a cross-sectional analysis, frequencies of latently infected CD4+ T cells did not decrease appreciably with increasing time on HAART (Fig. 3). These results suggest a slow decay rate for this compartment, consistent with the long-term survival of memory CD4+ T cells in uninfected individuals (*12*). These cross-sectional data cannot be used to define the actual decay rate of this compartment because of a potential problem that can occur in cross-sectional analysis under specific conditions (*20*). Precise determination of the decay rate of latently infected CD4+ T cells will require prospective sequential quantitation of such cells in individual patients over a period of years. In a single patient studied on two occasions (after 5 months and 8.5 months of HAART), no decrease in the frequency of latently infected cells was noted (Fig. 3).

Of the 18 patients from whom virus was isolated, two were newly infected patients treated within 10 weeks of the onset of symptomatic primary infection (patients 17 and 20). The isolation of virus from these individuals suggests that the latently infected CD4+ T cell compartment can become established early in infection.

To determine whether the viruses that persisted in latently infected resting CD4+ T cells had developed resistance to the relevant antiretroviral drugs, we determined nucleotide sequences of the HIV-1 *pol* gene for 12 viral isolates (Tables 2, 3, and 4). Protease inhibitor monotherapy results in rapid selection of drug-resistant variants with characteristic mutations at particular codons in the protease gene (*21*, *22*). For patients treated with ritonavir (RTV), multiple mutations arise in a defined sequence beginning at residue V82 (*21*, *23*). Analysis of eight sequences from seven different RTV-treated patients showed little evidence for the evolution of resistance (Table 2). Only one isolate from one patient (9) had a V82 mutation. This isolate also had a characteristic $Ile^{154} \rightarrow Val$ (I54V) mutation and may have arisen during the 15 months of RTV monotherapy that the patient received before starting a four-drug regimen of zidovudine (AZT), lamivudine (3TC), RTV, and saquinavir (SQV). Another isolate from the same patient did not show any mutations associated with resistance to RTV. Neither isolate had the L10, G48, I84, or L90 mutations associated with resistance to SQV. The isolate from patient 16 had M36I and A71T mutations that may reflect the RTV monotherapy the patient received before starting combination therapy or may represent preexisting polymorphism at these positions (*24*). For this patient, comparison with available baseline plasma RNA and proviral DNA sequences revealed no changes in the reverse transcriptase or protease coding regions after 30 months of combination therapy. One additional RTV-treated patient (patient 11) had a single I54L mutation. None of the isolates from RTV-treated patients showed characteristic changes at K20, L33, M46, I84, or L90. Isolates from four other patients treated with RTV combination therapy for between 5 and 22 months showed no resistance-associated mutations. None of the four patients treated with indinavir (IDV) had any of the characteristic resistance mutations seen at L10, K20, L24, M46, I54, A71, V82, I84, or L90 (Table 3). Thus, viruses grown from resting CD4+ T cells of patients on HAART did not show the pattern of multiple mutations characteristic of high-level resistance. Each of the 1.1-kb sequences was distinct from others in the data set, from any published laboratory strain, and from any of the >200 sequences from other clinical isolates analyzed previously in the laboratory. This finding is illustrated by the analysis of positions where polymorphisms not associated with drug resistance occur (Table 4). In the case in which two biological clones were obtained from the same patient (that is, patient 9), the sequences were clearly related to each other. In addition, the appearance of characteristic resistance-associated mutations in isolates from patients who had received prior monotherapy (that is, patient 9) provides strong evidence linking the sequences to the histories of individual patients. Taken together, these results indicate that in the patients studied, combination therapy was effective in suppressing viral replication and the attendant selection of resistant variants.

The isolation of viruses with drug-sensitive genotypes from the resting CD4+ T cells of patients successfully treated for long periods with HAART suggests that these viruses are derived from long-lived

Table 4. Nucleotide sequences of 12 isolates from resting CD4+ T cells of patients on HAART. Sequences at other codons where there are polymorphisms not associated with protease inhibitor resistance are shown. Dashes indicate identity with the consensus sequence shown in the top row. Procedures used are described in the legend to Table 2.

Patient (isolate)	19 cta/L	37 aat/N	41 aga/R	62 ata/I	77 gta/V	93 att/I
1	---	---	---	g--/V	a--/I	---
9 (5M1)	---	-g-/S	-a-/K	---	a--/I	---
9 (5M2)	---	-g-/S	-a-/K	---	---	---
11*	---	---	---	---	---	---
13*	---	---	---	---	---	---
14	g--/V	---	---	---	a--/I	c--/L
16*	---	---	---	---	---	---
18	---	---	-a-/K	---	a--/I	c--/L
19	---	---	---	---	---	---
20	---	-g-/S	-a-/K	---	---	c--/L
21*	---	---	---	---	---	---
22	---	-g-/S	-a-/K	g--/V	---	---
Known polymorphisms	I, Q, V, T, F	S	K, E	V	I	L, V

*These sequences are distinguishable on the basis of mutations at other codons, including substitutions at the third position of several codons.

cells that were initially infected before the initiation of therapy. The ability of replication-competent viruses to persist in a latent form in resting $CD4^+$ T cells is also consistent with the finding that frequencies of latently infected $CD4^+$ T cells did not decrease appreciably with increasing time on HAART. The isolation of replication-competent virus from patients who have responded well to long-term HAART has also been achieved by Wong *et al.* (*25*) and Chun *et al.* (*26*). Whether latently infected $CD4^+$ T cells can be efficiently reactivated to produce virus in vivo is unknown; however, the existence of a small but relatively stable compartment of latently infected cells should be considered in deciding whether treatment should be stopped in patients with no other evidence of residual virus.

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16. The frequency of resting $CD4^+$ T cells carrying replication-competent HIV-1 was determined as described (*11*). Briefly, highly purified resting cells were plated in duplicate fivefold serial dilutions beginning at 5×10^6 cells per well in culture medium consisting of RPMI with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and 4 mM L-glutamine supplemented with recombinant interleukin-2 (100 U/ml). Resting $CD4^+$ T cells were activated by the addition to each well of highly purified PHA (0.25 μg/ml; Wellcome Diagnostics, Research Triangle Park, NC) and a >10-fold excess of freshly isolated PBMCs from one or more HIV-1 seronegative donors. These PBMCs were inactivated by γ irradiation with 5000 roentgens in a cesium source irradiator and then washed twice. The next day, PHA was removed, and CD8-depleted PBMCs, which had been stimulated with PHA for 3 days, from an HIV-1–negative donor were added to each well. Cultures were fed and split as needed, and freshly prepared CD8-depleted PHA blasts were added on days 7 to 9. Supernatants collected on day 14 were analyzed for p24 antigen by enzyme-linked immunosorbent assay (ELISA). Preculturing purified resting $CD4^+$ T cells before activation did not consistently decrease the infected cell frequencies, indicating that stable, peristent forms of latent virus predominate in these patients.
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20. If the initial levels of latently infected cells before treatment are positively correlated with the time on therapy at which a given patient is studied, then the actual decay rate may be more rapid than the rate suggested by a cross-sectional plot of IUPM versus time. However, there is no reason to believe that the initial levels are correlated with time on therapy. The initial levels of latently infected cells before treatment in these patients are unknown. In a previous study (*11*) of untreated patients and patients on nucleoside analog therapy, the frequency of cells carrying persistent, replication-competent forms of virus ranged from 1 to 81 per 10^6 resting $CD4^+$ T cells. The frequency of cells carrying any replication-competent form of virus ranged from 1 to 420 per 10^6 resting $CD4^+$ T cells in the peripheral blood. These values for the frequencies of latently infected cells were not correlated with CD4 count or viral load. Therefore, there is no reason to expect a correlation between the pretreament level of latently infected T cells and the time at which a given patient was evaluated in our study.
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Amidation of Bioactive Peptides: The Structure of Peptidylglycine α-Hydroxylating Monooxygenase

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Many neuropeptides and peptide hormones require amidation at the carboxyl terminus for activity. Peptidylglycine α-amidating monooxygenase (PAM) catalyzes the amidation of these diverse physiological regulators. The amino-terminal domain of the bifunctional PAM protein is a peptidylglycine α-hydroxylating monooxygenase (PHM) with two coppers that cycle through cupric and cuprous oxidation states. The anomalous signal of the endogenous coppers was used to determine the structure of the catalytic core of oxidized rat PHM with and without bound peptide substrate. These structures strongly suggest that the PHM reaction proceeds via activation of substrate by a copper-bound oxygen species. The mechanistic and structural insight gained from the PHM structures can be directly extended to dopamine β-monooxygenase.

Many mammalian bioactive peptide hormones, neurotransmitters, and growth factors have a COOH-terminal carboxamide (*1–4*). Surprisingly, the amide is not generated by an NH_2 transfer, but rather by N-oxidative cleavage of a glycine-extended prohormone (*2*, *3*). One enzyme, peptidylglycine α-amidating monooxygenase (PAM) (E.C. 1.14.17.3), catalyzes the α-amidation of these diverse physiological regulators, many of which are much less active with free carboxylates. Bioactive peptides produced by PAM are distributed widely across vertebrate and invertebrate species (*5*). The critical role of PAM is highlighted by data showing that a null mutation of the PAM gene in *Drosophila* is larval lethal (*6*). In humans, PAM is the target of drug design for diseases ranging from rheumatoid arthritis to cancer.

PAM is a bifunctional enzyme encoded by a complex single-copy gene that is subject to tissue-specific and developmentally regulated alternative splicing (*1-4*, *7–9*). Two independent enzymatic domains—a monooxygenase domain and a lyase domain—cat-

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