(Fig. 5A) rescued the fusion defect (Fig. 5B). Thus, inhibition of myotube formation by MKP-1 overexpression is reversible. The formation of multinucleated myotubes upon readdition of tetracycline to differentiated C2MKP-1 myocytes was not accompanied by changes in the amounts of MHC (Fig. 5C), indicating that the formation of multinucleated myotubes upon readdition of tetracycline stemmed from the preexisting population of differentiated myocytes.

These observations indicate that down-regulation of MKP-1 late during myogenesis is functionally required for the process of myotube formation. Thus, expression of the muscle-specific genes is necessary but not sufficient to commit terminally differentiated myocytes irrevocably to the formation of multinucleated myotubes. The uncoupling of muscle-specific gene activation from myotube formation suggests that a distinct pathway involving a member or members of the MAPK family regulates the formation of multinucleated myotubes.

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9. C2C12 myoblasts were washed in ice-cold phosphate-buffered saline and lysed in 1% NP-40, 50 mM Hepes (pH 7.4), and 150 mM NaCl in the presence of 1 mM sodium orthovanadate, 5 mM NaF, 5 mM phenylmethylsulfonyl fluoride, aprotonin (5 μg/ml), leupeptin (5 μg/ml), 1 mM benzamidine, and pepstatin (0.1 μg/ml). Cell lysates were clarified at 20,000*g* for 20 min, and protein concentration was assessed by Bradford assay. Following immunoprecipitation of p42Erk2 (Santa Cruz, Santa Cruz, CA; SC-154) from 250 μg of total cell lysate, p42Erk2 activity was assessed by immunocomplex kinase assay with myelin basic protein as substrate (0.25 mg/ml) with 50 μM adenosine triphosphate (ATP) and 5 μCi of [γ-^{32}P]ATP for 15 min at 30°C. Immunocomplex kinase reactions were stopped by the addition of 0.5 M EDTA, and 20-μl aliquots of the supernatant were spotted onto p81 phosphocellulose paper, washed extensively, and counted by liquid scintillation. JNK activity was assessed by incubating a glutathione-S-transferase (GST) fusion protein of c-Jun (GST–c-Jun, containing amino acids 5 through 89 of c-Jun) with 250 μg of total cell lysate. After washing the beads, the kinase reactions were initiated by the addition of 25 μM ATP and 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C. Reactions were terminated by the addition of sample buffer, and GST–c-Jun was resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE), excised, and counted by liquid scintillation.
10. Approximately 50 to 100 μg of total cell lysate prepared as described was resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). Membranes were blocked in nonfat dry milk (5%) and immunoblotted with either anti-MyoD (5.8A), anti-myogenin (F5D), anti-myosin heavy chain (MF20), anti-p21, anti-cyclin D1 (Transduction Laboratories, 1 μg/ml), or anti-p27 (Transduction Laboratories, 1 μg/ml) antibodies. Primary antibodies in nonfat dry milk (5%) were incubated with PVDF membranes for 2 to 4 hours at room temperature or overnight at 4°C. Proteins were visualized with either sheep antibody to mouse immunoglobulin G (IgG) or donkey antibody to rabbit IgG peroxidase-linked secondary antibodies (Amersham) at a dilution of 1:4000 and were developed with Enhanced Chemiluminescence (Amersham).
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21. NIH 3T3 cells were transfected with the human cyclin D1 promoter fused to the luciferase gene (1 μg) and β-galactosidase (1 μg) with either wild-type [pEXV3.MEK(WT)] (4 μg) or constitutively active MEK [pEXV3.MEK(EE)] (4 μg) and pSG5.MKP-1–Myc (4 μg). We prepared lysates 2 days after transfection, and luciferase activity was measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) and β-galactosidase activity was assayed according to the manufacturer's protocol. Results are expressed as relative luciferase units normalized to β-galactosidase activity.
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23. NIH 3T3 cells were cotransfected with plasmids encoding MyoD (0.5 μg), rabbit muscle creatine kinase (MCK) promoter/enhancer luciferase reporter gene (1 μg), and β-galactosidase (1 μg), with either vector control, MEK(WT), or MEK(EE) (3 μg). We harvested cells 2 days after transfection and measured the luciferase and β-galactosidase activities. Expression of MEK(EE) inhibited MyoD transcriptional activity from the MCK luciferase reporter gene by ~50% relative to MEK(WT).

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Recovery of Replication-Competent HIV Despite Prolonged Suppression of Plasma Viremia

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In evaluating current combination drug regimens for treatment of human immunodeficiency virus (HIV) disease, it is important to determine the existence of viral reservoirs. After depletion of CD8 cells from the peripheral blood mononuclear cells (PBMCs) of both patients and normal donors, activation of patient CD4 lymphocytes with immobilized antibodies to CD3 and CD28 enabled the isolation of virus from PBMCs of six patients despite the suppression of their plasma HIV RNA to fewer than 50 copies per milliliter for up to 2 years. Partial sequencing of HIV pol revealed no new drug resistance mutations or discernible evolution, providing evidence for viral latency rather than drug failure.

Treatment with potent antiretroviral regimens can produce sustained suppression of HIV-1 replication, with reduction of HIV RNA in infected individuals to below the limits of detection in blood for 2 years or more (*1*). Although viral DNA remains detectable in PBMCs as well as in lymphoid tissues, several groups have reported that virus cannot

be recovered from PBMCs or lymphoid tissue samples from these patients by standard coculture techniques (*2*, *3*). The inability to isolate infectious HIV from these patients has led to speculation that the residual proviral DNA represents defective viral genomes incapable of replication and that HIV may be at the point of eradication (*3*–*5*). This possibility is supported by studies of patients treated early after infection who showed reductions in virus-specific antibody titers after prolonged therapy with potent antiretroviral regimens, suggesting elimination of viral antigen production (*3*, *6*). Moreover, defective genomes are found in a high proportion of proviral DNA from PBMCs of chronically infected patients (without viral suppression) (*7*).

Estimates of 2 to 3 years have been made for a theoretical time to eradication of HIV infection (*4*), but Perelson *et al.* stated that such projections assumed no third phase of decay or long-lived, latently infected cell population (*4*). Recently, Chun *et al.* have shown, in treated patients with incomplete viral suppression, that integrated proviral DNA resides in resting memory CD4 cells ($CD45RO^+$, HLA DR^-) and that such cells are capable of producing replication-competent HIV upon appropriate activation (*8*).

Here, we assessed the replication capacity of HIV-1 provirus persisting in the PBMCs of patients with complete and sustained suppression of plasma viremia by attempting recovery of virus with both standard and enhanced coculture conditions. The six patients examined were enrolled in the San Diego cohort of the Merck 035 study (*1*). All patients received the triple drug regimen of zidovudine (AZT), lamivudine (3TC), and indinavir (IDV) and were selected for this study on the basis of reductions of HIV RNA in blood to <50 copies/ml for 52 to 92 weeks (mean, 74 weeks) (*9*). At the time of blood collection for our experiments, plasma samples were analyzed with the Roche Ultrasensitive HIV RNA assay (*10*). In one case, the HIV RNA was at the limit of detection of 50 copies/ml despite having been undetectable during the preceding 12 months, whereas in all other cases it was undetectable (arbitrary sensitivity of 50 copies/ml) (Table 1). Residual viral RNA and proviral DNA load in lymph node and blood had been assessed in patients A, B, and C after 1 year of treatment (*2*). In each case, lymph node viral RNA concentrations had been 4 log copies per gram of lymph node tissue lower than in treated patients with detectable plasma virus. However, proviral DNA had remained detectable in all PBMC and lymph node samples, even though virus isolation had been unsuccessful by standard coculture protocols at the 1-year time point. Furthermore, sequencing of the protease gene and the first 242 codons of the reverse transcrip-

Table 1. HIV RNA concentrations in plasma from study patients. Samples were first assayed with the Amplicor assay (Roche). For samples with values of <400 copies/ml, the Ultrasensitive assay (Roche) was used with a limit of detection of 50 copies/ml. Assay results corresponding to virus isolation time points are shown in bold (n.d., not done).

Time on therapy (weeks)	Patient A (copies/ml)	Patient B (copies/ml)	Patient C (copies/ml)	Patient K (copies/ml)	Patient L (copies/ml)	Patient M (copies/ml)
0	62,680	19,200	51,704	39,322	13,644	50,030
4	1,195	651	2,223	199	104	2,267
8	<50	144	414	<50	44	797
12	<50	<50	184	<50	<50	483
16	<50	<50	78	<50	<50	349
20	<50	<50	<50	<50	<50	167
24	<50	<50	228	<50	<50	167
28	<50	<50	<50	71	<50	65
32	<50	<50	<50	<50	<50	<50
36	<50	<50	<50	<50	<50	86
40	<50	<50	<50*	<50	<50	88
44	<50	<50	<50	<50	<50	301
48	<50	<50	<50	<50	<50	<50
52	<50	<50	<50	<50	60	<50
56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
60	<50	<50	<50	<50	<50	<50
64	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
68	<50	<50	<50	<50	<50	<50
72	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
76	<50	<50	<50	<50	<50	<50
80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
84	<50	<50	<50	<50	<50	<50
88	<50	n.d.	n.d.	n.d.	<50	n.d.
92	<50	**<50**	<50	<50	<50	<50
96	n.d.	n.d.	**<50**	n.d.	**<50**	n.d.
100	<50	**<50**	<50	**<50**	<50	**50**
104	**<50**	n.d.		<50		<50
108	<50	<50				

*A separate assay performed at the same time point yielded a value of 119.

Table 2. Result of cultures by standard techniques or with the use of enhanced conditions, as described in the text. Positive cultures all had rising HIV RNA concentrations and positive p24 concentrations. Serial HIV RNA concentrations in culture supernatants are from a single representative condition out of the six enhanced conditions used. HIV RNA on supernatants from the standard cultures were all negative, with the exception of patient M (*18*). For all patients: PHA stimulated CD8-depleted HC and anti-CD3/anti-CD28–stimulated CD8-depleted patient cells. Patient A, + TNF; patient B_1, + macrophages; patient B_2, + macrophages and TNF; patient C, no addition; patient K, + macrophages; patient L, + macrophages and TNF; patient M, + macrophages. Passage codes: +, viral cultures have been successfully passaged to obtain high-titer stocks; *, experiments are still ongoing. B_1 and B_2 are the first and second culture from patient B's cells.

Patient	Culture		HIV RNA supernatants [log(RNA copies/ml)]				Passaged
	Standard	Enhanced	Day 1	Day 7	Day 14	Day 21	
A	–	+	<1.7	2.8	5.8	8.7	+
B_1	–	+	<1.7	3.1	4.4	5.6	+
B_2	–	+	<1.7	3.8	4.6†	4.5	+
C	–	+	<1.7	4.3	4.8	5.4	*
K	–	+	3.1	4	4.9	5.1	*
L	–	+	<1.7	3.8	6	6.6	+
M	+	+	3.3	5.7	6.1	7.5	+

†RNA quantitated from day 18 of culture.

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tase (RT) from both sources of residual RNA and DNA at the 1-year time point had demonstrated little or no change in viral sequences from study entry, which suggested that replication in vivo was suppressed below the extent necessary for discernible evolutionary changes (*11*).

In our study, the majority of CD8 T cells were removed from both the patient and healthy donor cell preparations before culture (*12*) to avoid the induction of soluble factors and chemokines suppressive to viral replication and alloreactive cytolytic T lymphocytes targeting dividing CD4 cells (*13*). The chosen activation conditions were based on observations by Spina *et al.* that immobilized antibody to CD3 and CD28 strongly activated CD4 lymphocytes, permitting more extensive HIV replication in CD45RO$^+$ memory cells than did the standard activation stimuli of phytohemagglutinin (PHA) and recombinant interleukin-2 (rIL-2) (*14*). The patient PBMCs were so activated; however, other investigators have reported selective downmodulation of the CCR5 viral coreceptor on lymphocytes after stimulation with anti-CD3 and anti-CD28, but not with PHA mitogen (*15*). To provide permissive CD4 cells for spread and growth of both syncytium-inducing and non–syncytium-inducing HIV isolates generated from the activated patient cells, we prestimulated the healthy donor cells with PHA for 1 to 3 days before their addition to the coculture. Recombinant IL-2 was then added to the coculture and replenished at 3- to 4-day intervals to maintain optimal lymphocyte proliferation. In some replicate cultures, monocytes removed by fibronectin adhesion before CD8 cell depletion were added back to patient CD4 cells, or patient CD4 cells were pretreated with recombinant tumor necrosis factor–α (rTNF-α), to overcome potential barriers to viral transcription that might be attributable to abnormalities in the TAT-Tar axis (*16*).

Virus was recovered from all six patients by means of these enhanced coculture conditions, whereas standard coculturing techniques (*17*) (in which coculturing is done with PHA- and IL-2–stimulated donor PBMCs) resulted in virus isolation from only one patient (patient M, Table 2). In one case (patient B) virus was recovered on two separate occasions 2 months apart, with the enhanced conditions only. Viral RNA concentrations in culture supernatants during the first 21 days of coculture from patient B were

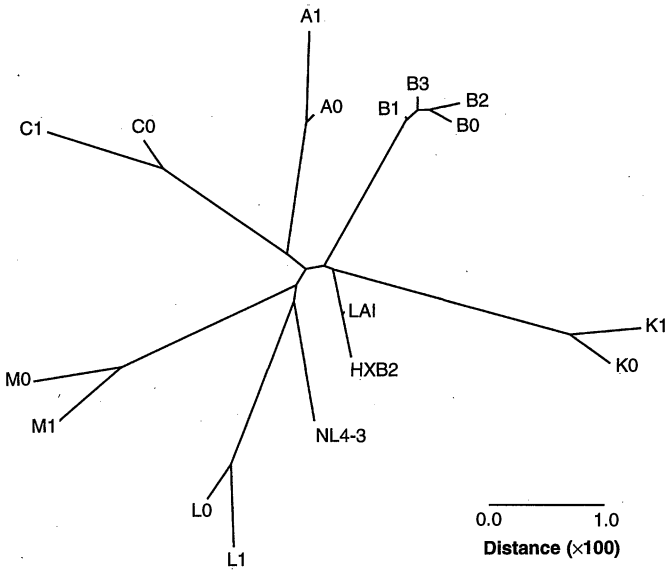

Fig. 1. Inferred phylogenetic tree demonstrating the relation of sequences at baseline, of post-treatment virus isolates, and of laboratory strains of HIV-1. Letter designations correspond to sequences from patients A to C and K to M. Suffix codes: 0, sequences obtained directly from baseline plasma; 1, sequences from the first coculture supernatant; 2, sequences from the second coculture; and 3, sequences from the passaged, high-titer virus stock. LAI, HXB2, and NL4-3 refer to sequences of the respective laboratory strains of HIV-1. The tree was inferred by a maximum likelihood method (*20*). Branch lengths are scaled to genetic distance, with a distance (×100) of 1.0 corresponding approximately to a 1% difference in nucleotide sequences (with weighting of substitutions at individual positions and a transition/transversion ratio of 1.4). These laboratory reference strains were chosen to rule out contamination as an explanation for the virus isolations. When trees were inferred by use of maximum parsimony analysis (with bootstrap analysis of 100 data sets), the results showed nearly identical branch orders and bootstrap values of 99 and 100 at internal nodes separating sequences of each patient from all others (*18*).

Table 3. Lack of emergence of drug resistance mutations in six patients treated with up to 2 years of triple therapy and undetectable plasma RNA. In five of six patients, substitutions in position 63 of the protease corresponding to known natural polymorphisms (*24*) were seen at baseline and in virus isolates. In patient K, a natural polymorphism was found in position 71 at baseline and after 2 years. Box denotes data indicating a residue where the sequence after 2 years of treatment was wild type in one culture despite a resistance mutation at baseline. (–) indicates identity with consensus clade B sequence. Abbreviations for amino acids: A, Ala; D, Asp; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; T, Thr; V, Val; and Y, Tyr.

Patient	Weeks on treatment	Sample	Amino acid residue associated with resistance and Clade B reference sequence																	
			Protease gene											Reverse transcriptase gene						
			10 L	20 K	24 L	32 V	46 M	54 I	63 L	71 A	82 V	84 I	90 L	41 M	67 D	70 K	184 M	215 T	219 K
A	0	Plasma	–	–	–	–	–	–	P	–	–	–	–	–	–	R	–	–	–
	104	Culture	–	–	–	–	–	–	P	–	–	–	–	–	–	R	–	–	–
B	0	Plasma	–	–	–	–	–	–	P	–	–	–	–	–	–	R	–	–	–
	92	Culture 1	–	–	–	–	–	–	P	–	–	–	–	–	–	–	–	–	–
	100	Culture 2	–	–	–	–	–	–	P	–	–	–	–	–	–	R	–	–	–
	92	Passage 1	–	–	–	–	–	–	P	–	–	–	–	–	–	–	–	–	–
C	0	Plasma	–	–	–	–	–		P	–	–	–	–	–	N	R	–	–	Q
	96	Culture	–	–	–	–	–		P	–	–	–	–	–	N	R	–	–	Q
K	0	Plasma	–	–	–	–	–	–	T	V	–	–	–	L	–	–	–	Y	–
	100	Culture	–	–	–	–	–	–	T	V	–	–	–	L	–	–	–	Y	–
L	0	Plasma	–	–	–	–	–	–	P	–	–	–	–	–	N	–	–	–	Q
	96	Culture	–	–	–	–	–	–	P	–	–	–	–	–	N	–	–	–	Q
M	0	Plasma	–	–	–	–	–	–	–	–	–	–	–	L	N	R	–	Y	–
	100	Culture	–	–	–	–	–	–	–	–	–	–	–	L	N	R	–	Y	–

measured after varying several components of the isolation conditions. CD8 depletion from both the patient and healthy control donor PBMCs was critical for successful virus isolation from these patients (*18*), whereas the addition of soluble TNF-α (R&D Systems, Minneapolis) or autologous macrophages may have accelerated virus recovery from some of the cultures (*19*). In those experiments where HIV RNA concentrations rose in culture supernatants (typically by day 7 to 14), HIV p24 antigen became detectable between 11 and 21 days (range of 36 to 10,630 pg/ml). With the exception of patient M, p24 antigen was not detectable in the standard cocultures carried out to 28 days. In patients A, B, L, and M, culture supernatants were passaged in donor PBMCs stimulated with PHA and IL-2 to produce high-titer viral stocks (p24 of 240,000 to 530,000 pg/ml). Similar passaging and expansion of viral isolates from the other two patients is in progress.

Partial sequencing of HIV pol (*20*) was performed on virus from culture supernatants collected before peak p24 concentrations from all six patients, as well as in one patient after in vitro passage of the virus. Sequences obtained directly from baseline patient plasma collected 2 years earlier showed high sequence homology to the virus recovered in coculture (Fig. 1) (*20*). The differences (mean 9/1000 base positions) between sequences from study baseline and after 2 years of treatment are minimal, in contrast to mean differences of 17/1000 base positions found in incompletely suppressed patients on triple therapy participating in the same study after only 8 to 12 months (*11*). For each patient, the sequences from the two time points resembled each other and did not resemble any laboratory strain; this finding excluded laboratory contamination to explain virus recovery, and relatively little viral evolution over the 2-year period was evident from analysis of the sequence data.

No new substitutions associated with drug resistance (*21*) were identified in any of the virus recovered after 2 years (Table 3). In patient B, the $Lys^{70} \rightarrow Arg$ mutation, associated with AZT resistance, was absent in virus recovered from the first coculture experiment but was present in virus from a second isolation; this finding suggested the existence of mixtures of genotypes in the PBMCs of this patient, with stochastic events in coculture favoring the outgrowth of one or the other genotype. Plasma HIV RNA concentrations have remained undetectable (<50 copies/ml for all patients) 1 to 4 months after the recovery of virus (Table 1). In aggregate, these observations demonstrate that the recovery of virus in these patients was not attributable to drug failure, but rather to the continued persistence of long-lived and latently infected T cell populations.

Patient M had low but measurable viral RNA in plasma 1 year earlier (301 copies/ml) and at the time of these experiments (50 copies/ml). There was no recognized nonadherence to medications. In this case alone, virus was also recovered with standard coculture conditions. This sporadic appearance of small but detectable amounts of HIV RNA in plasma may indicate a less complete suppression of virus replication than was the case for the other five patients. However, even in this patient, the lack of acquisition of resistance mutations and the modest plasma viremia argue against the development of drug resistance as a reason for these differences (Table 3).

Whether the in vitro conditions used to recover virus in these studies translates to a comparable capacity for reactivation of viral infection (in the absence of suppressive antiviral therapy) in the complexity of the in vivo environment is not known. Nevertheless, these data suggest that it would be premature to discontinue suppressive therapy in such patients within this time frame. At the same time, the absence of discernible viral evolution, including the lack of emergence of new drug resistance substitutions, argues for the durability of the antiviral drug regimen used and provides encouragement that antiretroviral suppression can be sustained by quenching the replication of the viral progeny of these latently infected CD4 lymphocytes should they become activated in vivo.

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9. Study patients had been treated with the three-drug regimen of AZT, 3TC, and IDV for a period of about 2 years (*1*) and were chosen for the study because, with the exception of sporadic detection of HIV RNA in the first year, they had plasma and serum HIV RNA concentrations at or below 50 copies/ml shortly after initiation of therapy, which persisted through the time point when the coculture was performed. All patients had been treated with AZT in the past, before enrollment in the Merck 035 study. All patients gave written informed consent, and the conduct of this study conforms to and was approved by the local institutional review board.
10. HIV RNA quantitation was performed with the Amplicor assay (Roche Molecular Systems) on cell-free virus supernatants from cocultures. Plasma and serum RNA concentrations were determined using the Ultrasensitive assay (Roche), a modification of the Amplicor assay (*22*). Briefly, this assay incorporates a high-speed spin (23,500*g* for 1 hour at 4°C) and resuspension of the RNA pellet in one-fourth of the typical diluent volume before the reverse transcription polymerase chain reaction (RT-PCR) and improves the sensitivity of detection from 400 copies/ml to 50 copies/ml. Repeat assays were performed on appropriate dilutions of supernatants when results from standard Amplicor assays exceeded the upper limit of reliability for the assay. HIV p24 antigen was measured by enzyme-linked immunosorbent assay (ELISA; Coulter, Hialeah, FL).
11. H. Günthard, J. K. Wong, C. Ignacio, D. Havlir, D. D. Richman, in (*5*), p. 43.
12. Patient PBMCs were prepared from acid-citrate-dextrose (ACD) anticoagulated peripheral blood (60 cc on each occasion) by ficoll-Hypaque density gradient centrifugation, and plasma was separated and stored at −80°C in aliquots of 1 ml. Monocytes were removed by fibronectin adhesion (*23*). CD8 cells were depleted by treatment with CD8 monoclonal antibody (mAb) OKT-8 (Johnson and Johnson, Ortho Diagnostics, Raritan, NJ) and panning on tissue culture plates coated with goat antibody to mouse immunoglobulin (Biosource, Camarillo, CA). Treatment resulted in reduction of CD8 lymphocytes to ~4% and enrichment of CD4 lymphocytes to 67% of the cell preparations from healthy control (HC) donors and enrichment of CD4 lymphocytes to 26 to 37% of preparations from patients. For each culture condition, 2×10^6 CD8-depleted lymphocytes were activated with immobilized CD3 mAb (Leu-4, Becton-Dickinson Immunocytometry Systems, San Jose, CA) and CD28 mAb (clone 9.3), bound to culture wells (six-well non–tissue culture treated plates; Costar, Cambridge, MA) (*14*). Isolated macrophages from the patients were added back to some culture wells at numbers equaling 10% of the lymphocyte population. For each patient, replicate cultures were also initiated by pretreatment of patient CD8-depleted lymphocytes with rTNF-α (R&D Systems, Minneapolis, MN) at a final concentration of 100 U/ml 24 hours before addition of donor cells. After 24 to 72 hours of stimulation with PHA (3 μg/ml, Sigma), either 5×10^6 total PBMCs or 5×10^6 CD8-depleted PBMCs from a healthy donor were added to each well containing patient cells (day 1 of culture). Cultures were maintained in the presence of rIL-2 (final concentration, 10 U/ml; DuPont–New England Nuclear Research Products, Boston, MA). For patient B, the first coculture experiments (B_1) were performed with the following enhanced conditions: (i) total HC PBMCs + total patient PBMCs; (ii) total HC PBMCs + CD8-depleted patient cells; (iii) total HC PBMCs + CD8-depleted patient cells with autologous macrophages; (iv) CD8-depleted HC cells + total patient PBMCs; (v) CD8-depleted HC cells + CD8-depleted patient cells; and (vi) CD8-depleted HC cells + CD8-depleted patient cells with autologous macrophages. For all other patients and for patient B in the second coculture experiment (B_2), four enhanced conditions were used: conditions v and vi from the first set of coculture experiments, and conditions v and vi with pretreatment of patient cells with TNF-α. For these experiments, PBMCs from four different HC donors were used. At day 1 of culture, 1 ml of medium was collected from each well without disturbing the cell layer and stored at −80°C for baseline HIV p24 antigen and RNA determinations. At days 4, 11, 18, and 25, half of the culture medium was replaced with fresh medium containing rIL-2 without disturbing the cell layers. At days 7, 14, 21, and 28, cells were resuspended, viability counts were performed, and approximately half of the cell suspensions were split and fed with fresh medium with rIL-2 (final concentration, 10 U/ml) to maintain a viable cell concentration of ~10^6 cells/ml. Cell-free supernatant was divided into aliquots and stored at

−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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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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