

## HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 IN THE SEMEN OF MEN RECEIVING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

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### ABSTRACT

**Background** Highly active antiretroviral therapy can effectively decrease the levels of human immunodeficiency virus type 1 (HIV-1) virions in peripheral plasma and seminal fluid of infected men. Whether the genital tract of HIV-1-infected men who are receiving highly active antiretroviral therapy and who have no detectable virus in the peripheral plasma harbors replication-competent virus is not known.

**Methods** We collected peripheral-blood and semen samples from seven men with HIV-1 infection who were receiving highly active antiretroviral therapy and who had no detectable viral RNA (fewer than 50 copies per milliliter) in plasma and analyzed the samples for cell-associated proviral DNA using a quantitative polymerase-chain-reaction assay. Replication-competent viruses were evaluated by cell-culture assays. Proviral DNA and replication-competent virus obtained from peripheral-blood and seminal cells were also analyzed by sequencing relevant viral genes.

**Results** Despite the long-term suppression of HIV-1 RNA in the plasma of the seven men, proviral DNA was detected in seminal cells in four. Replication-competent viruses were recovered from peripheral-blood cells in three men and from the seminal cells in two of these three men. The viruses recovered from the seminal cells had no genotypic mutations suggestive of resistance to antiretroviral drugs and were macrophage-tropic, a feature that is characteristic of HIV-1 strains that are capable of being sexually transmitted.

**Conclusions** In HIV-1-infected men who are receiving highly active antiretroviral therapy and who have no detectable levels of viral RNA in plasma, the virus may be present in seminal cells and therefore may be capable of being transmitted sexually. (N Engl J Med 1998;339:1803-9.)

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**S**EXUAL transmission has a major role in the spread of human immunodeficiency virus type 1 (HIV-1). The semen of infected men may contain high levels of HIV-1, and infectious viruses can be recovered from seminal cells or seminal fluid from these men.<sup>1-4</sup> Seminal cells are mixtures of spermatozoa, precursors of germ cells, T lymphocytes, macrophages, and epithelial cells, and HIV-1 proviral DNA has been detected in several types of these cells.<sup>4-8</sup> The level of HIV-1 RNA in seminal fluid can also be correlated with the level in plasma, and antiretroviral therapy decreases the

levels not only in plasma but also in seminal fluid.<sup>9,10</sup> There are often significant differences in the viral load and the viral sequences between semen and peripheral blood, suggesting that the replication of HIV-1 may be compartmentalized in vivo.<sup>11,12</sup>

Recently, therapy with combinations of antiretroviral drugs, referred to as highly active antiretroviral therapy, has resulted in the suppression of HIV-1 RNA in plasma to below the limits of detection of many assay systems and increased the survival of many HIV-1-infected patients.<sup>13,14</sup> In these patients, the viral load in lymphoid tissues is dramatically decreased,<sup>15,16</sup> but proviral DNA can still be detected in resting CD4 T lymphocytes in the peripheral blood, and replication-competent virus can be recovered from these cells, indicating that the cells could be a reservoir for viral replication.<sup>17-19</sup> HIV-1 can replicate in many types of cells and many locations in the body, but whether tissues and cells other than resting CD4 T lymphocytes in peripheral blood harbor replication-competent virus in patients who are receiving long-term highly active antiretroviral therapy is not known. We examined whether proviral DNA and replication-competent virus were present in the seminal cells of HIV-1-infected men who were receiving highly active antiretroviral therapy and in whom no viral RNA could be detected in plasma.

### METHODS

#### Study Subjects

We studied seven men with HIV-1 infection who had plasma levels of HIV-1 RNA below 400 copies per milliliter when measured by a reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay on three occasions at least one month apart. The men had pretreatment plasma HIV-1 RNA levels of at least 1000 copies per milliliter and had been taking highly active antiretroviral therapy for 5 to 41 months. They were identified from among a cohort of more than 400 men with HIV-1 infection who were treated in our clinics. Approximately four other men who met the eligibility criterion declined to participate in the study. On the days on which samples of peripheral blood and semen were obtained for the study, the plasma HIV-1 RNA levels in all seven men were below 50 copies per milliliter, as measured with a sensitive RT-PCR technique described previously.<sup>20-22</sup> The semen samples were collected by masturbation into clean containers. All samples were processed within two hours after collection. The

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study protocol was approved by the university's institutional review board, and all the men gave written informed consent.

### Quantitative HIV-1 DNA and RNA Analyses

Peripheral-blood cells were separated from plasma by discontinuous Ficoll centrifugation at  $1000\times g$  for 20 minutes at  $4^{\circ}\text{C}$ . Seminal cells were separated from seminal fluid by centrifugation at  $500\times g$  for 10 minutes at  $4^{\circ}\text{C}$ . The cell-associated HIV-1 proviral DNA levels and virion-associated RNA levels were measured as described previously.<sup>20-22</sup> To separate the virions from plasma and seminal fluid, the samples were centrifuged at  $150,000\times g$  for one hour. The RNA was isolated from the pellets by acid guanidinium thiocyanate-phenol-chloroform extraction.<sup>23</sup> DNA was extracted from peripheral-blood mononuclear cells and seminal cells with use of a standard method.<sup>20</sup> The HIV-1 *gag* DNA and RNA sequences were detected with the primer-probe set SK38, SK39, and SK19.<sup>20-22</sup> The  $\beta$ -globin gene was quantitated in the DNA from peripheral-blood mononuclear cells and seminal cells by a PCR assay with PCO3 and PCO4 as the primer pair, as described previously,<sup>20-22</sup> as a loading control, to confirm that each sample contained similar quantities of DNA before the initiation of PCR. The phosphorus-32-labeled Southern blots of these PCR mixtures were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The results of Southern blotting of the patients' samples were compared with those for extracts of ACH-2 cells, a T-cell line that contains one integrated HIV-1 genome per cell for the DNA PCR assay,<sup>24</sup> for the quantitation of proviral DNA and with an HIV-1 *gag* RNA construct transcribed in vitro for the RT-PCR assay<sup>22</sup> for the quantitation of RNA virions. Two healthy men were studied as negative controls to rule out cross-contamination of the samples.

### Coculture Assays

CD8 T lymphocytes were depleted from the isolated peripheral-blood mononuclear cells by binding to magnetic beads conjugated with anti-CD8 antibody (Biosource, Camarillo, Calif.). This process decreases the fraction of CD8 T lymphocytes in the peripheral-blood mononuclear cells from approximately 20 to 30 percent to 3 to 5 percent, as analyzed by flow cytometry. Depletion of CD8 T lymphocytes significantly increases in vitro outgrowth of HIV-1 from the peripheral-blood mononuclear cells<sup>17-19</sup> because CD8 cells secrete chemokines and other factors that inhibit the replication of the virus.<sup>25</sup> Macrophages and their precursors were depleted from peripheral-blood mononuclear cells by incubating the samples overnight to allow these cells to attach to the plastic plates. The remaining peripheral-blood lymphocytes were then stimulated with  $5\ \mu\text{g}$  of phytohemagglutinin per milliliter (Sigma, St. Louis) and 50 U of interleukin-2 per milliliter (GIBCO-BRL, Grand Island, N.Y.). Peripheral-blood lymphocytes were isolated from blood samples obtained from normal subjects with the same procedure. The peripheral-blood lymphocytes from the patients were then mixed in a 1-to-1 ratio with those from normal subjects (2 million cells each) and cultured in RPMI-1640 medium with 10 percent fetal-calf serum and penicillin plus streptomycin at  $37^{\circ}\text{C}$  for six weeks. Twice a week, half the medium was replaced with fresh medium, and once a week, half the cells were replaced with 2 million fresh peripheral-blood lymphocytes from normal subjects after stimulation with phytohemagglutinin and interleukin 2 and depletion of CD8 T lymphocytes.

The seminal-cell pellet was washed twice with cold phosphate-buffered saline, and 3 million cells were mixed with 2 million peripheral-blood lymphocytes from normal subjects after the depletion of CD8 T lymphocytes and stimulation with phytohemagglutinin and interleukin-2. After 24 hours, the cells were washed three times with phosphate-buffered saline, and the cultures were maintained in the presence of interleukin-2 (10 U per milliliter) for six weeks. Twice a week, half of the medium was replaced with fresh medium, and once a week, the cells were replen-

ished with 2 million fresh, treated peripheral-blood lymphocytes from normal subjects. HIV-1 p24 antigen was measured in the supernatants by an enzyme-linked immunosorbent assay (ELISA) (Dupont, Wilmington, Del.). All procedures were performed under level P3 biosafety conditions to minimize the possibility of cross-contamination.

### DNA-Sequence Analyses

The sequences of the V3 loop of the gp120 region of the viral envelope (*env*), protease, and *RT* genes of HIV-1 were determined by a nested PCR assay of both proviral DNA (directly from peripheral-blood and seminal-cell samples) and virion-encapsidated RNA (from replicating virus in the coculture). The viral RNA was reverse-transcribed with an antisense external primer, and the complementary DNA (cDNA) was amplified with the PCR. A second PCR was performed with a primer pair internal to the primer pair used in the first PCR, and the amplified DNA was isolated from agarose gels and analyzed by sequencing with an automated sequencer (Prism model 377, with XL upgrade, Perkin-Elmer Applied Biosystems, Foster City, Calif.). If there were variations in the sequences, the PCR fragments were cloned into the pGEM-T vector (Promega, Madison, Wis.). Multiple clones were selected from DNA of seminal cells and peripheral-blood mononuclear cells as well as from cDNA from replicating viral RNA, and the sequences were analyzed. The outer primer pair for the V3 loop of the gp120 region of the viral envelope was KK30 and KK40, and the inner primer pair was KK10 and KK20, as described previously.<sup>26</sup> (The sequences have been deposited in the GenBank data base under accession numbers AF098718 through AF098734.)

### Determinations of Viral Phenotype

To determine the viral phenotypes, viral isolates from the cocultures were cultured on MT-2 T lymphocytes and human macrophages (containing 250  $\mu\text{g}$  of HIV-1 p24 antigen equivalents per milliliter). The macrophages were isolated from peripheral-blood mononuclear cells from normal subjects, as described previously.<sup>22</sup> Viral growth in the MT-2 cells was determined on the basis of the formation of syncytium and the production of p24 antigen in culture. The growth of HIV-1 in macrophages was determined on the basis of the detection of p24 antigen in the culture supernatants by ELISA (Dupont).

## RESULTS

### Detection of Cell-Associated HIV-1 Proviral DNA

The characteristics of the seven men are shown in Table 1. The levels of HIV-1 RNA in the plasma and seminal fluid of these men were below 50 copies per milliliter and thus were much lower than the levels in men with untreated HIV-1 infections who were evaluated by the same assay (1500 to 75,000 copies per milliliter of seminal fluid).<sup>22</sup> These results suggest that in our subjects, highly active antiretroviral therapy inhibited viral replication not only in the bloodstream but also in the genital tract. However, cell-associated viral DNA was detected in peripheral-blood mononuclear cells from all the men (Fig. 1). The number of copies of HIV-1 *gag* DNA ranged from 5 to 40 per million peripheral-blood mononuclear cells.

Cell-associated proviral DNA was also detected in the seminal cells from four men (Fig. 1 and Table 1). The number of copies of HIV-1 *gag* DNA ranged from less than 5 to 90 per million seminal cells. The sequence analyses of the V3-loop region of gp120

TABLE 1. CLINICAL AND VIROLOGIC CHARACTERISTICS OF SEVEN MEN WITH HIV-1 INFECTION.\*

PATIENT NO.	ANTIRETROVIRAL THERAPY	DURATION OF THERAPY	CD4 T-LYMPHOCYTE COUNT	HIV-1 RNA		HIV-1 DNA		CULTURE RESULTS	
				PLASMA	SEMINAL FLUID	PBMCs	SEMINAL CELLS	PBMCs	SEMINAL CELLS
		mo	cells/mm <sup>3</sup>	copies/ml		copies/10 <sup>6</sup> cells			
1	Zidovudine, lamivudine, indinavir	16	140	<50	<50	8	<5	Negative	Negative
2	Stavudine, lamivudine, nelfinavir	5	282	<50	<50	18	8	Negative	Negative
3	Zidovudine, lamivudine, indinavir, nelfinavir†‡	18	649	<50	<50	7, 15§	<5, <5§	Positive	Negative
4	Zidovudine, lamivudine, indinavir	39	100	<50	<50	25, 30§	90, 20§	Positive	Positive
5	Zidovudine, lamivudine, indinavir, nelfinavir†	18	840	<50	<50	5	<5	Negative	Negative
6	Zidovudine, lamivudine, ritonavir, saquinavir	5	882	<50	<50	35	5	Positive	Positive
7	Didanosine, stavudine, indinavir¶	41	1050	<50	<50	40	5	Negative	Negative

\*PBMCs denotes peripheral-blood mononuclear cells.

†The initial protease inhibitor was changed to nelfinavir.

‡The patient was also treated with low-dose interleukin-2.

§Separate samples were obtained at least two months apart and analyzed on two different days.

¶The patient was also treated with acyclovir.

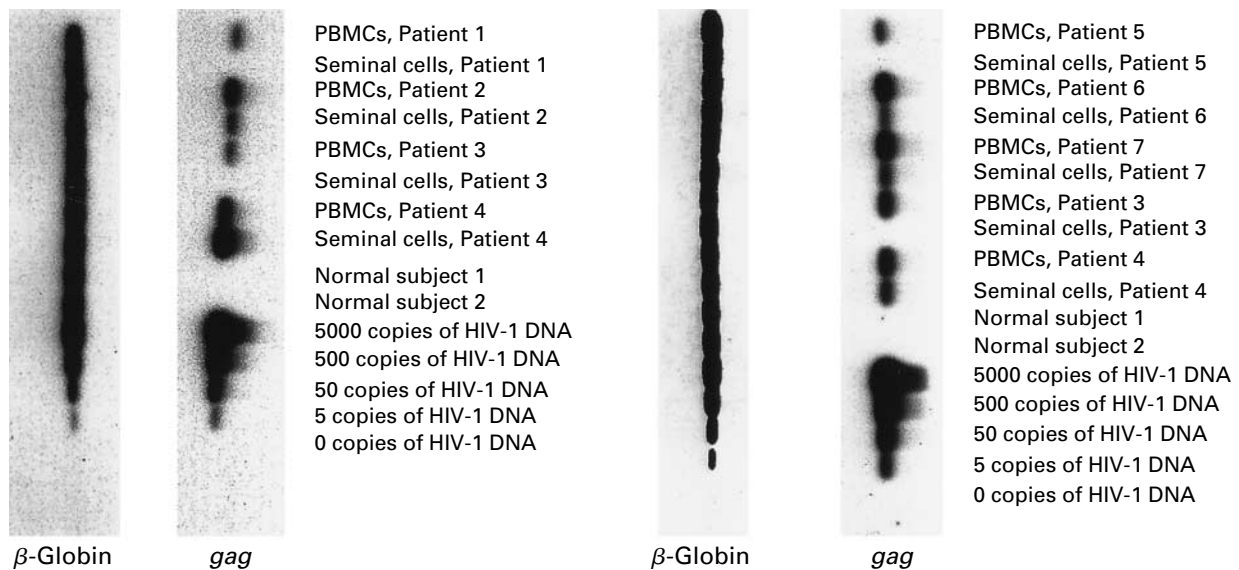


Figure 1. Detection of Proviral DNA in Seminal Cells and Peripheral-Blood Mononuclear Cells from Seven Men with HIV-1 Infection. Cellular DNA was extracted from seminal cells and peripheral-blood mononuclear cells (PBMCs), and amplified by the PCR, with gag SK38 and SK39 as the primer pair. As a means of confirming that each sample contained similar quantities of DNA before PCR,  $\beta$ -globin DNA was also amplified in each sample, with PCO3 and PCO4 as the primer pair.<sup>20-22</sup> The HIV-1 DNA standards were prepared from ACH-2 cells (a T-cell line with one integrated HIV-1 genome per cell)<sup>24</sup> and amplified by the PCR at the same time as were the samples from the patients; values are given as the number of copies per million cells. Blood and semen samples from two healthy men served as negative controls. A second set of samples was obtained from Patients 3 and 4 two to three months after the first set.

**TABLE 2.** GENOTYPES AND PHENOTYPES OF HIV-1 FROM PERIPHERAL-BLOOD MONONUCLEAR CELLS AND SEMINAL CELLS.\*

SAMPLES	AMINO ACID SEQUENCE OF V3-LOOP REGION	NO. OF CLONES	POSITIVE CHARGE IN V3-LOOP REGION†	VIRAL GROWTH IN CULTURE‡	
				MACROPHAGE	MT-2
<b>Consensus sequence§</b>	CTRPNNNTRKSIHI--GPGRAFYTTGEIIGDI RQAHC				
Bal	.....L.....		3		
NL4-3	.....R-QR.....V.I.K.....NM.....		8		
<b>Patient 4</b>					
PBMCs					
DNA					
1	.....S.....R.....--.....K.F.....D.....	1	3		
2	.....V.S.....P.....--.....DVM.....	1	3		
3	.....V.S.....P.....--.....DV.....	6	3		
Culture	.....V.S.....P.....--.....DV.....	2	3	Positive	Negative
Seminal cells					
DNA					
1	.....S.....P.....--.....DV.....G.....	1	3		
2	.....S.T.RR.P.....--.....DV.....	1	4		
3	.....G.....--.....F.S.L.....K.....	1	4		
Culture	.....N.....--.....F.SL.....K.....	2	5	Positive	Positive
<b>Patient 6</b>					
PBMCs					
DNA					
1	.....R.....--.....D.....R.....	2	5		
2	.....G.....--.....ST.AQ.....N.....	1	3		
3	.....G.....--.....ST.AQ.....K.....	1	3		
4	.....NV.L--.....F.....L.....R.K.....R.....	1	7		
Culture	.....GV.....--.....K.....	2	3	Positive	Negative
Seminal cells					
DNA					
1	.....--.....A.....	1	3		
2	.....P.....--.....A.K.....P.....	1	5		
3	.....G.....--.....ST.AQ.....N.....	3	3		
Culture	.....--.....A.....	2	3	Positive	Negative

\*PBMCs denotes peripheral-blood mononuclear cells. The DNA numbers denote the proviral sequences obtained.

†A charge of 4 or less indicates a macrophage-tropic virus, and a charge of 5 or more usually indicates a T-cell-line-tropic virus.

‡Viral isolates were cultured on human macrophages and MT-2 lymphocytes.

§NL4-3 is a T-cell-line-tropic laboratory strain of HIV-1 that uses CXC chemokine receptor 4, and Bal is a macrophage-tropic laboratory strain of HIV-1 that uses CC chemokine receptor 5.

indicated that the proviral DNA was not the result of contamination with common laboratory strains, as analyzed by a search of the GenBank data base (Table 2) (and data not shown). In two men (Patients 3 and 4), the levels of proviral DNA in seminal cells and peripheral-blood mononuclear cells were examined again two to three months after the first analysis. There was a small decrease in the level of proviral DNA in seminal cells in Patient 4. Patient 3 had no detectable proviral DNA in seminal cells on either occasion.

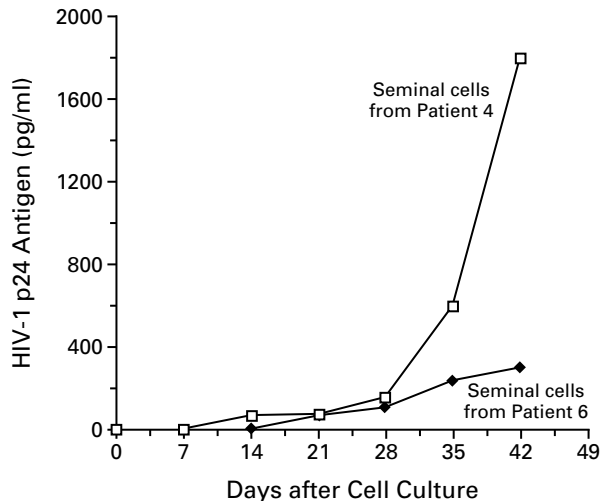
**Recovery of Replication-Competent HIV-1**

Replication-competent HIV-1 was recovered from peripheral-blood lymphocytes from three men and seminal cells from two of the three (Table 1 and Fig. 2). In Patient 4, replication-competent HIV-1 was recovered from both peripheral-blood lymphocytes and seminal cells on two occasions. The analyses of

the sequences in the V3 loop of the gp120 region of the viral envelope with the use of the GenBank data base indicated that these viruses were primary isolates and not laboratory strains causing contamination (Table 2) (and data not shown). The viral sequences differed among these three men, thus demonstrating that there was no cross-contamination of the samples. When CD8 T lymphocytes were not depleted from the peripheral-blood cells used in the cocultures, we could not isolate viruses from either peripheral-blood lymphocytes or seminal cells from any of the men (data not shown).

**Genotypic and Phenotypic Characterization of Replication-Competent HIV-1**

To investigate whether the replication-competent viruses recovered from seminal cells could be sexually transmitted, the sequences in the V3-loop region and the phenotype of the viruses were exam-



**Figure 2.** Growth Kinetics of Replication-Competent HIV-1 from Seminal Cells.

Approximately 3 million fresh seminal cells from HIV-1-infected men were incubated for 24 hours with 2 million peripheral-blood lymphocytes from normal subjects after the depletion of CD8 T lymphocytes and stimulation with phytohemagglutinin and interleukin-2. The cell mixtures were then washed and cultured in the presence of interleukin-2 (10 U per milliliter) for six weeks. Every week, half the cells were replaced with 2 million treated peripheral-blood lymphocytes from normal subjects, and the supernatants were analyzed for HIV-1 p24 antigen by ELISA. Two samples from each culture were analyzed.

ined (Table 2). According to the net amino acid charges in the V3 loop (a charge of +4 or less indicates a macrophage-tropic virus, and a charge of +5 or more usually indicates a T-cell-line-tropic virus) and the growth patterns in cell culture,<sup>27,28</sup> the virus isolated from the seminal cells of Patient 6 was a typical macrophage-tropic strain, whereas the virus isolated from the seminal cells of Patient 4 had dual tropism. Most HIV-1 strains transmitted sexually are macrophage-tropic or have dual tropism<sup>11,29,30</sup>; therefore, the replication-competent viruses isolated from the seminal cells of these men are potentially capable of initiating a primary infection in a sexual partner, even though these men were receiving highly active antiretroviral therapy and had undetectable levels of viral RNA in plasma.

The differences in the sequences of the V3-loop region between the viruses recovered from peripheral-blood lymphocytes and those recovered from seminal cells from Patients 4 and 6 suggest that at least some viral replication is compartmentalized within the male genital tract and the peripheral blood, as described previously.<sup>11</sup> The sequences also indicate that the recoverable, replication-competent viruses from seminal cells and peripheral-blood lymphocytes are probably derived from the proviral DNA in these cells (Table 2). In Patient 6, the replication-competent

virus from seminal cells and the DNA of a provirus from seminal cells had the same V3-loop sequence, whereas in Patient 4, the sequences differed slightly. In Patient 6, however, the V3 sequence of replication-competent virus from the peripheral-blood lymphocytes differed substantially from that of the proviral DNA from peripheral-blood mononuclear cells.

Strains of HIV-1 that are resistant to RT inhibitors as well as protease inhibitors can be transmitted sexually.<sup>31</sup> To determine the drug sensitivity of the replication-competent HIV-1 and proviral DNA isolated from seminal cells and peripheral-blood mononuclear cells of our patients, we sequenced the RT and protease regions.<sup>32</sup> We found a single mutation coding for drug resistance in the protease gene (isoleucine was substituted for leucine at position 10) of the proviral DNA from peripheral-blood mononuclear cells from Patient 4. No drug-resistance mutations were detected in either the replication-competent virus recovered from the seminal cells or the seminal-cell-associated proviral DNA from Patient 4 or Patient 6. Similarly, no drug-resistance mutations were detected in seminal-cell-associated proviral DNA from Patients 2 and 7. Finally, as previously reported,<sup>18</sup> no other drug-resistance mutations were detected in the proviral DNA from peripheral-blood mononuclear cells from Patient 2, Patient 6, or Patient 7 (data not shown).

## DISCUSSION

We isolated replication-competent virus from the seminal cells of HIV-1-infected men who were receiving highly active antiretroviral therapy and who had no detectable levels of viral RNA in plasma. This finding suggests that the genital tract can be a reservoir for HIV-1 replication in men. This phenomenon could be due to a very slow turnover of some cells harboring proviral DNA. Theoretically, if no drug-resistant mutants developed during highly active antiretroviral therapy, there would be no reinfection of cells in microenvironments in which there were inhibitory concentrations of the drugs. However, the blood-testes barrier may prevent antiviral drugs from entering testicular tissue in high concentrations, therefore creating a viral sanctuary. Other sites of tissue-blood endothelial barriers, including the brain and the retina,<sup>33,34</sup> have also been shown to harbor HIV-1-infected cells. Although levels of HIV-1 RNA were below the level of detection in the seminal fluid of the men in the present study, there could still be covert viral replication in the genital tract, as may occur in lymphoid tissue.<sup>19</sup>

We did not identify the types of cells in the genital tract that contained replication-competent HIV-1. In untreated HIV-1-infected men, the virus is found in macrophages and CD4 T lymphocytes in the semen<sup>18</sup>; germ cells such as spermatogonia and their

progeny may also contain provirus, although the sequences in germ cells may be defective or incomplete.<sup>6,7,35</sup> Germ cells do not have CD4 molecules on their surfaces,<sup>36</sup> and therefore, HIV-1 should not be able to enter them, unless there is a CD4-independent mechanism of entry.

Reinfection of peripheral-blood mononuclear cells and seminal cells should not occur in patients who are receiving highly active antiretroviral therapy and who have undetectable levels of HIV-1 RNA in plasma and seminal fluid. Resting CD4 T lymphocytes from local lymphoid tissue, which may have a relatively long life,<sup>37</sup> could be reservoirs for the virus. The sequences of HIV-1 isolated from the seminal cells differed from those obtained from peripheral-blood lymphocytes in some men; therefore, some of the infected seminal cells may not have come directly from the peripheral blood. These T lymphocytes or macrophages could have been infected by blood-borne viruses before therapy was initiated, but they would have had to survive for very long periods. In preliminary studies of seminal cells separated by magnetic beads conjugated with anti-CD3 antibody, proviral DNA was detected in both the T-lymphocyte-replete (CD3 antigen-positive) and T-lymphocyte-depleted cellular fractions (data not shown).

Seminal cells harboring proviral DNA could be vehicles for the sexual transmission of HIV-1. These infected seminal cells could come in direct contact with the target cells (i.e., CD4 T lymphocytes, macrophages, and dendritic cells) in the mucosa of the sexual partners, resulting in the transmission of the virus. The virions produced from seminal cells after transfer from a man who is receiving highly active antiretroviral therapy to a sexual partner should be infectious because the concentrations of antiviral drugs in the semen would be diluted to low levels. Our genotypic and phenotypic analyses indicate that the replication-competent viruses recovered from the seminal cells of our study subjects are macrophage-tropic or have dual tropism, suggesting that they have the potential to initiate and establish a primary infection in the sexual partners of these men.<sup>10,29,30</sup> Our studies also demonstrate that replication-competent viruses from the seminal cells remain sensitive to antiretroviral drugs. This finding further suggests that proviral DNA in seminal cells, from which these viruses were most likely derived, may represent archival or fossil viral sequences derived by replication soon after primary infection.

In summary, replication-competent viruses can be recovered from seminal cells of HIV-1-infected men who are receiving highly active antiretroviral therapy and who have undetectable levels of viral RNA in plasma, suggesting that sexual transmission of HIV-1 is possible despite the use of seemingly effective therapy.

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