Evaluation of Distinct Blood Lymphocyte Populations in Human Immunodeficiency Virus Type 1–Infected Subjects in the Absence or Presence of Effective Therapy

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Virus reservoirs can persist in human immunodeficiency virus type 1 (HIV-1)–infected subjects despite effective plasma virus suppression. To compare viral dynamics in the absence and presence of antiretroviral therapy, blood mononuclear cells from 19 subjects with high plasma RNA levels and 18 subjects following prolonged virus suppression were examined, by use of in situ hybridization, to detect virus RNA expression before and after in vitro T cell activation. This approach reveals circulating lymphocytes expressing HIV-1 RNA before activation and an increase in cells with detectable HIV-1 RNA transcription after in vitro activation. The frequencies of these 2 cell populations are strongly correlated with plasma virus load and appear to be stable once a new steady state is established during therapy. The frequency of viral RNA–positive cells is equivalent to the frequency of cells that produce infectious virus. Thus, in HIV-1–infected subjects there are distinct virus reservoirs comprising both latent and replication-active cells.

The natural history of human immunodeficiency virus type 1 (HIV-1) infection, including the prolonged clinically stable period following initial exposure, is characterized by persistently high levels of viral replication in lymphoid tissue [1–7]. The extent of viral replication is reflected in the amount of viral RNA (vRNA) in the plasma [8–10], which is closely correlated with the risk of disease progression [11]. Administration of highly active antiretroviral therapy (HAART) inhibits de novo infection and rapidly decreases plasma vRNA to undetectable levels in many patients, leading to dramatic clinical improvement [9, 10, 12, 13]. The rapid decay rate of plasma vRNA is thought to reflect the rapid death of replication-active cells in vivo, which suggests that blocking de novo infection could lead to complete clearance of infected cells over time. However, an important obstacle to complete eradication of HIV-1 infection is a population of latently infected CD4 T cells that can produce infectious virus following in vitro stimulation, and that population may persist despite effective, prolonged plasma virus suppression [14–16]. In addition to the latent reservoir, indirect evidence suggests ongoing, low-level viral replication may occur despite suppression of plasma HIV RNA levels to undetectable levels [16, 17].

The analytical challenge of measuring the extremely low frequency of latently infected cells in subjects on effective HAART makes the design of therapeutic trials that specifically target these cells for elimination difficult. We have developed a novel strategy to determine the frequency of latently infected cells in HIV-1–infected subjects. This approach distinguishes between latently infected T cells and those engaged in viral transcription by determining RNA expression before and after in vitro activation of T cells. A resting CD4 T cell that contains integrated, replication-competent proviral DNA initiates viral transcription by activation of cellular transcription factors, similar to the induction of cytokine gene transcription [18–27]. This initial level of transcription is then stabilized and amplified by the viral regulatory genes tat and rev to achieve a fully replication-active state [26, 28, 29]. Rather than evaluating replication-competent cells by their ability to propagate additional rounds of infection in coculture, we directly measured the frequency of vRNA-positive (vRNA⁺) cells by in situ hybridization (ISH) analysis before and after in vitro T cell activation with phytohemagglutinin (PHA).

Our hypothesis, which is based in part on our studies of viral dynamics in biopsy specimens of HIV-1–infected tissues [30], was that both latently infected and replication-active cells in

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Informed consent for collection of blood was obtained from all subjects. The institutional review board of all institutions involved reviewed and approved the study.

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blood contribute to the residual low-level viremia following prolonged potent suppression of plasma virus load. In this report, we evaluate the relative frequencies of these two distinct virus reservoirs in blood obtained from individuals with different levels of immunosuppression and a range of plasma virus load values in the presence or absence of effective HAART.

Understanding the relative sizes of the latently infected cell pool and cells that actively express HIV-1 RNA, compared with the results of conventional coculture assays for cells producing infectious virus, might provide important insights into the nature of the total reservoir of viral genetic material in HIV-1 infection. For example, if a large proportion of the proviral DNA detected within peripheral blood mononuclear cells (PBMC) from an individual were genetically defective, then the number of cells capable of producing infectious virus should be significantly less than the number of cells capable of initiating HIV-1 RNA expression. The alterations in these cellular HIV-1 reservoirs when plasma viremia is suppressed by HAART, as assessed via cross-sectional analysis of subjects receiving or not receiving effective therapy, may also guide the design of future therapeutic strategies.

Materials and Methods

Study population. Blood was obtained from HIV-1-infected subjects during routine clinic visits. The participating clinical sites were the University of Alabama at Birmingham 1917 Clinic and the Duke University Infectious Disease Clinic. Thirty-seven HIV-1-infected subjects were analyzed. Eighteen subjects had maintained plasma virus loads at undetectable levels on HAART for 1–24 months, as evidenced by repeated virus load measurements of <50 or 200 copies/mL. Nineteen subjects were receiving no or suboptimal antiretroviral therapy and had detectable plasma virus loads. Subjects represented a wide range of clinical histories (mean CD4 cell count, 364/µL; range, 21–1186). Plasma virus loads ranged from <50 to >8,000,000 copies/mL.

ISH. ISH was performed as described elsewhere [31, 32]. Cytosmears (smears of PBMC suspensions) on baked glass slides were acetylated in 0.1 M triethanolamine, pH 8.0, with 0.25% acetic anhydride. PBMC were prehybridized for 1 h at 50°C or at room temperature in 50% formamide, 2× standard saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate), 1× Denhardt’s solution, 500 µg/mL herring sperm DNA, and 250 µg/mL yeast transfer RNA. A digoxigenin-UTP-labeled antisense HIV-1 RNA probe (including gag, pol, vpu, env, nef, U3, and R sequences) was denatured and applied to the PBMC in prehybridization buffer at a final concentration of 100 fmol/mL. PBMC were hybridized with the probe at 50°C overnight.

After hybridization, the PBMC were treated with RNase A (50 µg/mL in STE buffer [sodium chloride, Tris, EDTA]) for 1 h at 37°C. After being rinsed with STE buffer, the PBMC were washed with decreasing salt buffers: 2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC at room temperature. The anti-HIV RNA probe was detected by use of a sheep anti-digoxigenin F(ab) antibody (Boehringer Mannheim, Indianapolis) conjugated with alkaline phosphatase at room temperature in Tris-buffered saline with 1% bovine serum albumin. After excess antibody was washed off, nitro blue tetrazolium (NBT; Sigma, St. Louis) substrate was added, and the PBMC were incubated overnight in a dark, humidified chamber. The reaction was stopped in Tris-EDTA buffer, and the slides were washed in Tris-EDTA buffer, soaked, and coverslipped with Aquamount (Fisher, Atlanta).

Cytosmears were prepared by directly micropipetting ~10³ cells onto a circular area on an RNase-free double-plus coated glass specimen slide (Fisher). After being stained with NBT, cells expressing HIV-1 RNA (vRNA⁺) appear bluish-purple under the microscope (figure 1) and can be counted directly to determine the frequency of vRNA⁺ cells present in a population of PBMC. vRNA⁺ cells were counted by scanning each cytosmear at high power (200×) and recording grid positions of the positive cells when rare (<1/10³ cells). vRNA⁻ cells were scored by the presence of NBT substrate, the cell’s morphology, the absence of background, and whether the candidate cell was contained in the correct focal plane. Next, vRNA⁺ cells were recounted or confirmed by grid position by an independent observer blinded to the first analysis.

The total number of cells for each sample was determined by counting the density of cells in representative areas of the cytosmears, by use of a calibrated ocular grid, together with direct measurement of the total area of the cytosmear, to express the result as the frequency per 10³ PBMC examined. Negative controls for ISH consisted of PBMC from HIV-seronegative donors. No positive cells were detected in these cytosmears (data not shown). For each new preparation of HIV RNA ISH probe, ISH was performed on normal donor PHA blasts, which were infected with characterized primary isolates of HIV-1 or a chronically infected cell line that constitutively expresses HIV-1 RNA. The sensitivity of this ISH detection methodology and validation of frequency counts has been analyzed extensively in lymph node tissue specimens of HIV-infected individuals [30].

Calculation of the inducible vRNA⁺ cell frequency. ISH for HIV-1 RNA was done on 2 different cell populations from the same preparation of PBMC directly after isolation by ficoll density gradient centrifugation and after 24 h of activation in vitro with PHA (Sigma) (figure 2). The frequency of cells that were vRNA⁺ after in vitro activation (activated vRNA⁺) represent the combination of cells that were already vRNA⁺ when plated (circulating vRNA⁺) as well as those cells that were induced to express vRNA by activating the host cell (inducible vRNA⁺). Therefore, the frequency of “inducible cells” is equal to the frequency of “activated vRNA⁺ cells” minus the frequency of “circulating vRNA⁺ cells.” All frequencies are expressed as vRNA⁺ cells per 10³ PBMC.

Synthesis of probes for ISH. Probes for HIV-1 RNA in both sense and antisense orientations were produced by in vitro transcription using SP6 or T7 RNA polymerase from 3 plasmids (HIV-1 nucleotide sequences 225–3378; 5546–7308, and 7308–9107 as in GenBank accession L02317), which were linearized by convenient restriction enzyme digestion. During in vitro transcription, digoxigenin-UTP was incorporated into the RNA transcripts for immunologic detection of the RNA duplex. The 3 separate RNA probes were transcribed, quantified by trace incorporation of [³H]-UTP during transcription, and then hydrolyzed for 10 min in 20 mM NaHCO₃, at pH 10.2 to yield smaller fragments of ~300 bp. The 3 RNA probes were mixed together at equimolar amounts so
that each probe was present at a final concentration of 100 fmol/mL for ISH.

**Preparation of PBMC from whole blood.** Blood was obtained from HIV-1–infected subjects during routine clinic visits. Blood was collected into 8.5-mL acid citrate/dextrose vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and processed immediately or after overnight shipping. PBMC were isolated by Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. Some patient PBMC were counted and assayed immediately by ISH, in vitro activation, and coculture. Other patient PBMC were counted and viably frozen at 5 × 10⁶ cells/mL in 90% fetal bovine serum (FBS; Hyclone, Logan, UT) and 10% DMSO (Sigma). Typically, 1 × 10⁶ to 10 × 10⁶ PBMC isolated from patient blood were placed directly onto slides for ISH to determine the frequency of vRNA⁺ cells circulating in patient blood (circulating vRNA⁺).

**Preparation of viably frozen patient PBMC.** PBMC were frozen at a controlled rate of 1°C per minute to −70°C in an isopropanol-insulated container or a controlled-rate freezer for 12–24 h and then transferred to vapor-phase liquid nitrogen for long-term storage. Viably frozen PBMC were rescued from liquid nitrogen storage by thawing in a 37°C water bath for 2–3 min. The cells were then added dropwise to 10 mL of complete RPMI 1640 containing 10% FBS, centrifuged at 300 g for 10 min, and resuspended in culture medium. Cells were then counted and checked for viability by trypan blue dye exclusion. In general, 90% of the viably frozen cells were recovered during the rescue process.

**In vitro activation of T cells in PBMC.** Patient PBMC were cultured with 5 μg/mL PHA in RPMI supplemented with 10% FBS, 20 U/mL recombinant human interleukin-2 (IL-2) (Roche, Indianapolis), and 2 mM L-glutamine for 20–24 h in a humidified CO₂ incubator at 37°C. In vitro activations were performed in the presence of 1.2 μM nevirapine to suppress de novo infection. To prepare cytosmears for ISH, cells were resuspended vigorously in each well and transferred to a microcentrifuge tube. The wells were washed with PBS containing 1% bovine serum albumin (Sigma), and the wash suspension was added to the tube. Cells were pelleted at 1000 g for 5 min in a microcentrifuge and then resuspended in Hanks’ balanced salt solution. The cell suspension was then micropipetted directly onto a double-plus coated specimen slide, air dried for 10 min, and fixed in 3% paraformaldehyde in diethylpyrocarbonate (DEPC)–treated 1× PBS for 1–3 h. The fixed cytosmears were then rinsed with DEPC-treated 2× SSC and allowed to air dry completely before ISH was performed. Some slides were stored at −70°C for several days before ISH was done.

**Limiting-dilution coculture of patient PBMC.** Freshly isolated or viably frozen PBMC from HIV-1–infected subjects were activated with PHA for 20–24 h and then cocultured with allogeneic 1- to 3-day-old PHA blasts from seronegative donors [33, 34]. Some
of the PHA-activated PBMC were also placed on a slide at the time of coculture to determine the frequency of vRNA+ cells by ISH. Patient PBMC were cultured in complete RPMI supplemented with 10% FBS, 2 mM L-glutamine, and 20 U/mL IL-2 at several different concentrations with 1 × 10^6 donor blasts, with replicate wells at each dilution to allow frequency calculations by limiting-dilution analysis. For all cocultures, half of the culture medium (1 mL) was removed from each well at day 7 and replaced with 5 × 10^3 fresh donor PHA blasts per well in 1 mL of fresh medium plus IL-2. At day 14, coculture supernatants from each well were assayed by ELISA (Coulter, Miami) for p24 capsid production. The frequency of cells producing infectious virus (infectious cells/10^6 PBMC) was estimated for each patient from the relationship of the log of the fraction of p24-negative wells versus the input number of patient cells, by use of Poisson statistics.

Quantitative measurement of HIV-1 DNA in PBMC. HIV-1 DNA was quantitated by a modification of a previously described procedure [35]. The synthetic competitor for HIV-1 DNA analysis was constructed by ligation of the 5' and 3' respective oligonucleotide templates into the general cloning vector pQPCR1, as described elsewhere [35]. This pQPCR.HRV2 competitor contains several primer sets, one of which was a site in the HIV-1 pol gene designed to amplify a 415-bp fragment extending from position 2713 to 3127 in the HIV genome (based on the position number in GenBank account K03455). All competitors were linearized with XhoI to generate linear DNA competitor molecules, which were gel-purified and quantitated by optical density measurements at 260 nm. DNA was prepared by solubilizing suspensions of PBMC from HIV-1 subject patients in 2,3,4,6-tetra-O-acetyl-beta-D-guanidinium isothiocyanate at 1 or 10 × 10^6 cells/mL. DNA was isolated from 10^6 cells on QIAamp columns (QIAamp Blood and Tissue kit; QIAGEN, Valencia, CA) according to the manufacturer's instructions.

DNA eluates from the QIAamp columns were resuspended in 100-mL elution buffer AE (QIAGEN), and 10 mL was used in a 50-mL polymerase chain reaction (PCR; 100,000 cell equivalents). The single-tube quantitative PCR technique, a modification of the multitube quantitative PCR technique, was performed by adding an unknown DNA to a competitor cocktail containing 5 different synthetic HIV competitors at a series of concentrations differing by 0.4 log_10 multiples. For example, competitor cocktail 1 consists of competitor A at log_10 3 (10 copies), competitor B at log_10 1.4 (25 copies), and so on. Each competitor differed from the others by an internal 25-bp segment of DNA, which is the basis for differential hybridization and detection.

After PCR, the products were detected by use of an EIA as described elsewhere [35], except that each PCR product was plated into 12 microtiter wells instead of 4. Two wells each were probed with detection oligonucleotides for the HIV-1 gene and stuffer detection oligos specific for competitors A–E. Calculation of endpoints was performed as described elsewhere [35]. The pol gene primers used were upstream oligo 5'-CATACAATACTCCAGTTTGGCCA and downstream oligo 5'-AAGTCAGATCCATACAAAATCA. PCR conditions for pol amplification were as follows: 0.8 μM primers, 2.6 mM Mg^2+, 50°C annealing for 35 cycles. The detection oligonucleotides for hybridization of the internal HIV sequence was 5'-TGGATGTTGGTGATGCATTATTTTGAGTC, whereas each different competitor species was detected with a distinct 25-mer sequence, by use of standard hybridization conditions.
Results

Detection of circulating and inducible vRNA\(^+\) cells. To determine whether individual vRNA\(^+\) cells could be detected in patient PBMC preparations, ISH analysis was performed before and after in vitro activation with PHA as outlined in figure 2. Although the frequencies of vRNA\(^+\) cells were quite low, individual positive cells could be clearly detected and enumerated by direct microscopic observation. Since immunohistochemical detection involves an enzymatic product, the signal was localized in the cellular focal plane inside individual lymphocytes (figure 1). Thus, the detection and counting of individual positive cells could be performed accurately by distinguishing bits of debris from positive cells.

To determine whether the detection of vRNA\(^+\) cells by ISH was reproducible, independent determinations were made by use of multiple aliquots of viably frozen PBMC specimens from 12 patients with various clinical histories (table 1). Determination of the frequency of positive cells was reproducible (mean intrapatient coefficient of variation, 17%). To confirm that all the cells with detectable vRNA expression after 1 day of activation represented cells actually infected in vivo, we performed the in vitro T cell activation in the absence or presence of nevirapine (1.2 \(\mu M\)), using PBMC from 5 subjects. Equivalent frequencies of vRNA\(^+\) cells were detected (data not shown), confirming that de novo infection was not occurring during in vitro activation.

Equivalence of vRNA transcription and production of infectious virus. To determine the clinical and biologic relevance of the assay results, we compared the frequency of vRNA\(^+\) cells by ISH with the frequency of cells capable of producing infectious virus (measured by production of p24 capsid protein) in coculture (figure 3). The frequency of vRNA\(^+\) cells detected by ISH was equivalent to the frequency of infectious cells determined by coculture analysis, for 11 subjects. The Pearson correlation coefficient for this comparison was .97 and was highly significant (P < .00001). Furthermore, the regression analysis shows an intercept at the origin and a slope of 1.0, indicating that these assays measure equivalent entities.

This analysis included PBMC from subjects who were on

<table>
<thead>
<tr>
<th>ID</th>
<th>No. vRNA(^+)/10^6 PBMC in replicate analyses(^a)</th>
<th>Intrapatient analyses Mean (± SD)</th>
<th>Intrapatient analyses % CVb</th>
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<tr>
<td>1</td>
<td>1.56 25.7 16.2 50.3 41.3</td>
<td>29.8 (±15.5)</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>9.1 9.3 20.0 23.1</td>
<td>15.4 (±7.2)</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>220 145 207 190</td>
<td>191 (±32.7)</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>74.8 70.7 76.4 47.4</td>
<td>67.3 (±13.5)</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>6.2 7.6 9.3</td>
<td>7.7 (±1.6)</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>7.4 9.4 7.9</td>
<td>8.2 (±1.0)</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>3.1 3.2</td>
<td>3.2 (±0.1)</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>1.5 1.5</td>
<td>1.5 (±0.0)</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>1.7 2.2</td>
<td>2.0 (±0.4)</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>1.8 2.0</td>
<td>1.9 (±0.1)</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>2.0 1.7</td>
<td>1.9 (±0.2)</td>
<td>11</td>
</tr>
<tr>
<td>12</td>
<td>0.9 0.9</td>
<td>0.9 (±0.0)</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE. ID, patient identification no.

\(^a\) 2-5 independent ISH analyses were performed. No. of replicate analyses was determined by no. of cells available from each subject. ISH was performed following 24 h of activation with phytohemagglutinin, except that PBMC from subjects 6-8 were analyzed directly after isolation without culture. PBMC from subjects 10 and 11 were analyzed as fresh (first) and viably frozen (second) samples.

\(^b\) Overall mean intrapatient coefficient of variation (% CV) was 17% (range, 0-52).

Determination of plasma virus load. Plasma virus load determinations were made by use of the Roche Monitor assay [36] and the Roche Ultrasensitive assay following the manufacturer’s instructions. Plasma virus loads are expressed as \(\log_{10}\) RNA copies or RNA copies per milliliter.

Figure 3. Correlation of frequencies of activated viral RNA-positive (vRNA\(^+\)) cells measured by in situ hybridization and infectious cells measured by coculture. Frequencies of vRNA\(^+\) cells/10^6 peripheral blood mononuclear cells (PBMC; horizontal axis) were plotted on \(\log_{10}\) scale against frequency of infectious cells/10^6 PBMC (vertical axis) for 14 human immunodeficiency virus type 1-infected subjects. Each point on graph represents analysis of individual subject. ●, Subjects with active viral replication; ◇, subjects who had maintained undetectable plasma virus loads while receiving highly active antiretroviral therapy (HAART); ▼, subjects who had maintained undetectable plasma virus loads while receiving HAART and for whom vRNA\(^+\) cells were detected but infectious cells were not. These subjects were plotted on vertical axis at upper limit estimated by limiting dilution coculture. Plasma virus loads of patients analyzed ranged from 6.6 \(\log_{10}\) copies/mL plasma to undetectable (<1.7 \(\log_{10}\) or 50 copies/mL plasma). Both vRNA\(^+\) cells and infectious cells were measured over 4-\(\log_{10}\) range and were highly correlated and directly proportional. Pearson product moment correlation coefficient for 2 variables was .97 (P < .00001), and slope of regression line was 1.0. Spearman rank correlation for 2 variables was .98 (P < .0001).

Table 1. Reproducibility of viral RNA-positive (vRNA\(^+\)) cell frequency determination by independent in situ hybridization (ISH) analyses of multiple aliquots of viably frozen peripheral blood mononuclear cells (PBMC) from 12 human immunodeficiency virus type 1-infected patients with various clinical histories.
inadequate or no therapy and 3 subjects who had maintained undetectable plasma virus loads for 3, 12, and 24 months at the time PBMC were obtained (figure 3). In PBMC from all 3 subjects, high-titer infectious virus stocks were recovered from the p24-positive limiting-dilution coculture wells, confirming the production of replication-competent virus (data not shown). This observation suggests that most of the cells from these subjects that are capable of vRNA transcription can also produce infectious virus in vitro. For 3 other subjects who had also maintained undetectable plasma virus loads (figure 3), vRNA+ cells were detected by ISH at very low frequencies, but infectious cells were not detected in coculture. However, the number of cells required to perform an authentic limiting-dilution analysis exceeded the number of cells actually plated for these subjects.

In addition, total copies of proviral DNA were measured in PBMC from the 11 subjects for whom infectious cells were detected (table 2). Consistent with previous reports [6, 17, 37–39], a 1- to 3-log10 excess of proviral copies over infectious cells was detected in PBMC from these subjects. Thus, assuming one copy of proviral DNA per cell at the low frequency infection present in vivo, cells capable of vRNA expression or production of infectious virus represent only a small fraction of those with detectable proviral DNA.

Viral dynamics in the blood of HIV-1–infected subjects. After confirmation of the reproducibility of the assay, we determined the frequencies of circulating vRNA+ cells and those capable of inducible vRNA expression in PBMC specimens obtained from HIV-1–infected subjects. These specimens were obtained from 19 subjects with active viral replication and 18 subjects receiving HAART who had maintained undetectable plasma vRNA for 1–24 months (table 3). The frequencies of both the circulating vRNA+ cells in the blood (present prior to in vitro activation) and the inducible vRNA+ cells after in vitro activation (figure 4) were higher in subjects not receiving antiviral drug treatment. The frequency of circulating vRNA+ cells was positively correlated with plasma virus load (Pearson correlation coefficient, .73; P < .00001). The mean frequency of circulating vRNA+ cells was only −0.4 log10 higher in subjects without virus suppression (mean, 3.3 vs. 0.6/106 PBMC; P > .05), even though the plasma virus loads varied over 5 logs. In those subjects receiving effective HAART with undetectable plasma vRNA, the frequency of circulating vRNA+ cells was extremely low but still detectable in 16 of the 18 subjects examined (table 3).

The frequency of inducible vRNA+ cells was also positively correlated with plasma virus load (Pearson correlation coefficient, .79; P < .00001), and the range of vRNA+ cell frequencies was larger than the range of circulating vRNA+ cells (figure 4). The mean frequency of inducible vRNA+ cells in those patients with detectable active viral replication was significantly higher than that in subjects receiving HAART (mean, 35 vs. 0.8/106 PBMC; P < .02). The higher frequency of inducible cells in the presence of active viral replication is consistent with the detection of recently infected cells that contain preintegration complex vDNA. These cells can be induced to transcribe vRNA after in vitro activation [37, 39–41]. In those subjects on HAART for whom the plasma virus load was undetectable, the frequency of inducible vRNA+ cells was similar to that previously reported for the resting T cells with stable integrated vDNA [15].

New steady state in subjects receiving HAART. Because previous studies reported that the frequency of replication-competent, latently infected, resting CD4 T cells does not decrease with time on HAART [15], we compared the frequencies of both circulating vRNA+ cells and cells capable of inducible vRNA expression with the duration of HAART (figure 5). This comparison demonstrated that the frequencies of both the circulating and inducible vRNA+ cells fall quickly with induction of therapy, similar to the amount of vRNA in plasma. However, once undetectable virus load is achieved, the frequency of both subsets of cells is stable. No significant difference in frequencies was observed between those subjects receiving HAART for a few months versus those treated for >1 year. The decrease in the frequency of circulating vRNA+ cells during the induction of therapy is consistent with effective suppression of de novo infection. The substantial decrease in the frequency of inducible vRNA+ cells observed after initiation of HAART may be due to the decay of labile preintegration complex vDNA when de novo replication is suppressed and plasma virus load falls [9, 10]. The stability of inducible cells in subjects receiving HAART

### Table 2. Viral RNA (vRNA) expression, infectivity, and total proviral DNA copies in peripheral blood mononuclear cells (PBMC) from 11 human immunodeficiency virus type 1–infected subjects for whom infectious cells were detected.

<table>
<thead>
<tr>
<th>Frequency/10^6 PBMC</th>
<th>vDNA: infectious cells</th>
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<tr>
<td>ID</td>
<td>vRNA+ cells</td>
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<tr>
<td>1</td>
<td>151</td>
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<tr>
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<tr>
<td>11</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Mean 50 51 1445 239 2.4

**NOTE.** ID, patient identification no.

* Frequency of vRNA-positive (vRNA+) cells was detected in PBMC by in situ hybridization analysis.

* Frequency of infectious cells was determined in PBMC by p24 capsid protein production in limiting-dilution coculture analysis.

* No. of copies of proviral DNA (vDNA) was determined by quantitative competitive polymerase chain reaction.

* Ratio of copies of vDNA to infectious cells.

* log10 of ratio of vDNA to infectious cells.
Table 3. Clinical histories for 37 human immunodeficiency virus type 1 (HIV-1)-infected study subjects.

<table>
<thead>
<tr>
<th>ID</th>
<th>Plasma virus loadb</th>
<th>No. of CD4 cells/μLc</th>
<th>Duration of HAART, monthsd</th>
<th>Frequency of vRNA+ cells/106 PBMCe</th>
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NOTE. vRNA+, viral RNA positive; PBMC, peripheral blood mononuclear cells; ID, patient identification no.; HAART, highly active antiretroviral therapy; ND, not done; NC, not calculated.

a Detected by in situ hybridization for HIV-1 RNA. Circulating indicates that cells were examined directly without activation, activated indicates that cells were cultured in presence of phytohemagglutinin for 24 h before examination, and inducible is calculated difference between other 2 frequencies.

b Cell-free plasma was isolated from patient blood within 3 h of collection and stored at −70°C for measurement of plasma virus load, which is expressed as RNA copies per milliliter of plasma, as determined by Roche (Indianapolis) Monitor or Ultrasensitive assay. Plasma and PBMC used for analysis were isolated from the same blood sample.

c Absolute CD4 cell counts were measured by flow cytometry.

d Each subject had repeated plasma virus load measurements below detectable limits (<50 or <200 copies/mL) for indicated time.

Discussion

Understanding the status of HIV-1 infection in subjects who have sustained suppression of detectable plasma vRNA while receiving combination antiretroviral therapy is a critical research goal. Although few controlled studies have been reported, anecdotal reports suggest that withdrawal of antiretroviral therapy in subjects with established HIV-1 infection usually results in rapid rebound of active viral replication [6], but the biologic nature of the persistent infection is not clear.

To address this issue, we developed a novel analytical approach to investigate latent infection. The approach was based on the ability to induce active vRNA transcription after T cell activation that is detected by ISH analysis. Using this procedure, we report two new observations that have important implications for understanding HIV-1 pathogenesis. First, cells capable of transcriptional activation as detected by ISH are long term suggests the presence of a stable form of latent cell containing integrated proviral DNA.
der vRNA^+^ cells in large populations of cells is critical to this analysis. A key element of this technique is the discrimination of cells with positive ISH signal from cellular debris that nonspecifically binds the labeled riboprobe. The immunoenzymatic detection of an ISH signal using digoxigenin-labeled riboprobes is as sensitive as techniques involving radiolabeled probes [31]. Furthermore, since the signal is discretely localized at the site of hybridization, bits of debris can be morphologically distinguished from authentic signal.

Although direct counting of cells is time consuming, it is reproducible (table 1) and correlates with other independent measures of frequency of virus infection, such as coculture analysis by limiting dilution (figure 3). In fact, even though the interpatient variation in frequency of vRNA^+^ cells shown in figure 3 was >200-fold, the intrapatient variation was <3-fold, even after 5 independent measurements. In addition, a limited analysis of patient PBMC samples was done to compare the frequency of vRNA^+^ cells by ISH to an externally standardized quantitative competitive reverse transcription-PCR analysis [35]. There was a close correlation between the amount of vRNA detected in cell extracts and the frequency of positive cells by ISH (R.D.H., unpublished data). Unfortunately, sufficient cells were not available to perform both of these assays in the current study.

Furthermore, we have recently developed micromanipulation methodology to physically isolate individual ISH-positive cells, separate vRNA after denaturation of the RNA duplex, and perform sequence analysis after reverse transcription and nested PCR amplification. Analysis of individual positive cells from the specimens described in this report yielded unique HIV-1 sequences (R.P.B., unpublished data). Since individual positive cells contain unique sequences, even when present on the same slide, contamination with free virions is unlikely. Thus, the reproducibility of the ISH detection and the strong correlation of the frequencies detected with coculture analysis indicate that this method accurately measures the frequency of replication-active HIV-1-infected cells.

The novel observation that the frequency of vRNA^+^ cells is equivalent to the frequency of infectious cells determined by limiting-dilution coculture analysis has important implications. The large excess of proviral DNA compared with the frequency of infectious cells reported in this study (table 2) and by other investigators [6, 17, 37–39] is unlikely to represent abundant defective genomes, since only a few mutations would be expected to prevent transcriptional activation, whereas many could affect virus infectivity.

An alternative explanation for the presence of abundant mutations within proviral genomes that is consistent with the equivalence of transcriptional competence compared with infectivity reported here is the preferential accumulation of tran-
Figure 5. Cross-sectional analysis of frequencies of circulating and inducible viral RNA-positive (vRNA+) cells vs. no. of months patients received highly active antiretroviral therapy (HAART). Human immunodeficiency virus type 1-infected subjects with detectable plasma virus loads who were not receiving effective antiretroviral therapy at time of study were plotted at 0 months. ●, Subjects with active viral replication; ○, subjects who had maintained undetectable plasma virus loads while receiving HAART (<50 or <200 copies/mL for 1–24 months). Each point on graph represents analysis of individual subject. Top, Frequencies of circulating vRNA+ cells/10^6 peripheral blood mononuclear cells (PBMC; vertical axis) were plotted on log10 scale against no. of months patients received effective HAART (horizontal axis). No significant correlation between frequency of circulating vRNA+ cells and time receiving HAART therapy was observed. Bottom, Frequencies of inducible vRNA+ cells/10^6 PBMC (vertical axis) were plotted on log10 scale against no. of months receiving effective HAART (horizontal axis) for subjects for whom inducible vRNA+ cells were calculated. No significant correlation between frequency of inducible (latent) vRNA+ cells and duration of HAART was observed.

scriptionally silent defective proviral genomes over the course of chronic infection. Although this mechanism may operate to some extent, we speculate that this mechanism would result in substantial accumulation of total proviral DNA over the course of infection. Since the mechanism of elimination of infected cells involves active transcription of the proviral genome (for either viral cytopathic effects or immune clearance), no selective pressure would exist against cells that contained transcriptionally silent defective genomes. Many CD4 T cells become infected and die each day throughout the course of infection [9, 10]. If a significant fraction of the newly integrated proviral genomes was defective for transcription, these cells would not be eliminated, and the total amount of proviral DNA would accumulate in the population. However, no significant accumulation of proviral DNA over the course of infection has been observed [6, 17, 37–39], and the large discordance between pro-

viral DNA and infectious cells is established during very early infection [17], prior to the development of selective pressures favoring transcriptionally silent genomes. Additional factors that may contribute to the excess of proviral DNA include positional effects of the proviral integration site and heterogeneity in T cell activation.

First, it is likely that some sites of proviral integration are silenced in a lineage-specific manner when an infected T cell reverts to a resting state. Altered patterns of transcriptional activation related to the integration site in chromatin have been demonstrated in transfected cell lines [42], in transgene constructs from different founder lines [43–45] and in other retroviral systems [46]. This phenomenon has been suggested, on the basis of analysis of some chronically infected tumor cell lines, to occur in HIV-1 infection as well [47–49]. Thus, it seems reasonable to suggest that transcription factors produced in activated T cells would fail to stimulate proviral long terminal repeats (LTRs) present in inaccessible heterochromatin. These proviruses would be nonfunctional but not because of lethal mutations in the nucleotide sequence. This integration-site mechanism is distinct from the viral transcription mutation mechanism discussed above, since transcription could be initiated immediately after integration (when the chromatin is open) but would become defective for transcriptional induction only when the T cell returns to a resting phenotype.

A second mechanism that could result in an excess of proviral DNA compared with the frequency of cells able to induce transcription in vitro (or ignite a new infection at limiting dilution) is heterogeneity of T cell activation. Proviral transcription is initiated from the 5 LTR of the viral genome, which shares many sequence motifs with the IL-2 promoter and other cytokine genes [18–27]. Signal transduction through the T cell receptor is not simply an “on/off” switch: It involves multiple levels of signals that can be qualitatively different in their functional outcome [50–55]. These distinct levels of stimulation can translate subtle differences in avidity of the T cell receptor–major histocompatibility complex–peptide complex into a wide range of signals mediated by several independent pathways [56–58]. One consequence of the complexity of T cell activation is heterogeneity in the specific pattern of responses, even within clonal populations, of different promoter sequences [59–61]. Such heterogeneity may also be involved in the initial activation of transcription from the HIV-1 LTR in resting, latently infected cells. Any cellular heterogeneity during in vitro T cell activation would contribute to the apparent excess of total proviral DNA over cells with biologically competent provirus.

Previously reported results from several groups have documented that cells with recoverable infectious virus are present despite prolonged effective HAART [6, 15, 16] and that the frequency of these cells is independent of the length of time on effective therapy [15, 17]. Although coculture analysis of blood samples obtained from patients with prolonged plasma virus suppression has been assumed to measure only latently infected
cells, this result has not been explicitly validated. The data presented here suggest that the viral replication detected under in vitro activation conditions actually is the net result of cells with preexisting active replication (circulating cells) and latently infected cells that express vRNA only after activation signals (inducible cells).

Conventional coculture methods involve activation conditions (PHA, IL-2, or both) to stimulate cell cultures, such that ongoing active and latent HIV RNA expression cannot be distinguished. Although other investigators have purified resting CD4 T cells from blood of subjects on HAART and demonstrated a subset of stable latently infected cells [15, 17, 39, 40], these data do not rule out the coexistence of a low frequency of replication-active cells. The observation that coculture results correlate closely with the frequency of activated vRNA\(^+\) cells confirms the concept that coculture methods alone do not segregate replication-active cells from latently infected cells. The presence of persistent replication-active cells would suggest that current PCR technology fails to detect plasma vRNA at steady state on HAART, given the extremely low frequency of infected cells and the rapid clearance of free virions in vivo. Within the limits of the cohort available for investigation, the frequencies of both of these cells appear fairly stable after the first month of HAART.

We found that the frequencies of both the circulating and the inducible vRNA\(^+\) cells were higher in subjects not receiving effective therapy, compared with those receiving HAART (figure 4). Although there was an \(\approx100,000\)-fold range of plasma virus loads among untreated and treated subjects, the difference in mean frequency of circulating vRNA\(^+\) cells between individuals receiving or not receiving HAART was <10-fold. This observation suggests that most of the vRNA\(^+\) cells in subjects with active viral replication and high plasma virus load are sequestered in tissue sites, as opposed to circulating freely in blood. This implication has been directly confirmed by analysis of lymph node biopsy specimens by the same ISH methodology, and a direct correlation was found between the frequency of lymph node vRNA\(^+\) cells and plasma virus load [30].

The differing degree of decline in plasma virions versus vRNA\(^+\) cell frequency in blood may be a reflection of the cellular redistribution that occurs after HAART induction. Antiretroviral drugs cause a large reduction in replication-active cells, which results in decreased antigen-driven immune activation in lymphoid tissue. The decreased amount of viral replication in tissue allows resolution of immune activation, consistent with the dramatic decrease in expression of inflammatory adhesion molecules observed in lymphoid tissue in untreated versus treated subjects [62]. The extremely low frequency of residual replication-active cells is more evenly distributed between blood and tissue compartments in the absence of intense immune activation. The net result of these 2 effects is a modest fall in the frequency of circulating vRNA\(^+\) cells after HAART.

What is the relationship between circulating vRNA\(^+\) cells and resting latently infected (inducible vRNA\(^+\)) cells in vivo? One possibility is that latently infected and replication-active cells derive from different mechanisms: Circulating vRNA\(^+\) cells represent ongoing rounds of de novo infection at very low levels despite substantial inhibition of viral replication by HAART, whereas the latent cell pool is sessile. It is possible that nonadherence to drug regimens or reduced drug potency accounts for the apparently consistent level of de novo infection. An alternative explanation for the coexistence of these 2 populations of cells is that most of the circulating vRNA\(^+\) cells derive from in vivo activation of latently infected cells. These two possible mechanisms are not mutually exclusive, since activation of latent cells may occur during limited rounds of de novo infection that result from suboptimal drug concentration. This scenario implies that in vivo activation of latent cells is reasonably frequent but does not lead to net clearance of this population with time on HAART.

As discussed in detail elsewhere [63], the dominant clearance mechanism of vRNA\(^+\) cells in vivo is most likely an antigen-driven effector cytotoxic T lymphocyte response. Multiple lines of evidence indicate that virus load is directly controlled by an active CD8 T cell–mediated immune response, including the recent demonstration that depletion of CD8 T cells in simian immunodeficiency virus–infected rhesus macaques results in rapid increase in viremia from the normal “set point” level [64, 65]. Since the induction of HAART significantly decreases the amount of viral antigen available to continuously activate effector CD8 T cells, the clearance rate of vRNA\(^+\) cells in subjects receiving HAART may be reduced, resulting in a longer life span for these cells. If this is the case, some latently infected cells that become activated and express vRNA may revert to a latent state before elimination by active cytotoxic T lymphocyte or viral cytopathic effects, resulting in the stability of the latent population. Such a mechanism accounts for the rapid accumulation of latently infected cells during primary infection before an immune response has been induced [17].

The reduction of viral antigen in subjects receiving HAART may extend the life time of infected cells, leading to establishment of a new steady state [65]. The concept that latently infected cells can generate a low frequency of replication-active cells but that these cells rarely infect other cells, because of effective HAART, may account for the slow emergence of drug-resistant mutants and the rapid rebound of viremia if therapy is discontinued. The hypothesis that biologically competent HIV-1 infection persists in the presence of HAART because of reduction in an immune clearance mechanism has substantial clinical implications. If interventions, such as therapeutic vaccination, could augment this clearance mechanism, it is conceivable that infection could be cleared or at least controlled by a boosted immune response, even if viral drug-resistance mutations ultimately arise. The analytical approach of measuring inducible vRNA in blood lymphocytes may provide a
useful assay to assess the effectiveness of candidate immune adjuvants to HAART.

Acknowledgments

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References

26. Lu X, Welsh TM, Peterlin BM. The human immunodeficiency virus type 1 long terminal repeat specifies two different transcription complexes, only one of which is regulated by Tat. J Virol 1993; 67:1752-60.