Residual HIV-1 RNA in Blood Plasma of Patients Taking Suppressive Highly Active Antiretroviral Therapy

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The discovery of highly active antiretroviral therapy (HAART), which represents a combination of agents effective against human immunodeficiency virus 1 (HIV-1) usually including reverse transcriptase and protease inhibitors, has led to successful treatment of many patients in diverse cohorts in the developed world.1,2 Such patients have demonstrated “undetectable” levels of viral RNA in the peripheral blood plasma after treatment with HAART. Most importantly, correlations with these alterations of in vivo HIV-1 expression with HAART have included dramatic changes in mortality and morbidity.3 Thus, suppressive HAART has assisted in converting HIV-1 infection from a poorly treatable and fatal disease into one that can be, in many patients, chronic or at least subacute.

Recent studies have demonstrated that HIV-1 replicates at extremely high rates in untreated infected individuals.4,5 The turnover of virions in vivo yields a viral half-life in the range of a few hours to minutes.6 Productively infected CD4+ T lymphocytes account for more than 99% of infected cells in the peripheral blood and lymphoid tissue, and in treatment-naive patients, this robust viral production is dramatically affected by HAART. In cohorts of therapeutically naive patients, HAART may lead to viral loads in the peripheral blood plasma of fewer than 400 to 500 copies/mL in significant numbers of individuals. Fewer than 50 copies/mL of plasma HIV-1 RNA can be demonstrated, if assessed by clinical ultrasensitive techniques, in a somewhat smaller number but still a majority of patients.

Context Despite suppressive treatment with highly active antiretroviral therapy (HAART), replication-competent virus can still be isolated from peripheral blood mononuclear cells and genital cells of many individuals receiving suppressive HAART.

Objective To determine whether free virion RNA can be detected in the blood plasma and/or genital tract fluids from patients receiving suppressive HAART.

Design Prospective cohort study conducted from November 1998 to May 1999.

Setting Academic medical center.

Patients Human immunodeficiency virus 1–infected individuals (20 men and 2 women) shown in our laboratories to have fewer than 50 copies/mL of HIV-1 RNA in peripheral blood plasma while taking suppressive HAART.

Main Outcome Measures Free virion RNA levels in peripheral blood plasma and genital fluids, quantified using an ultrasensitive reverse transcriptase polymerase chain reaction able to quantify cell-free virion RNA to a lower limit of 5 copies/mL and qualitatively detect viral RNA below this level.

Results In all 22 patients, residual viral RNA could be detected in the peripheral blood plasma (mean level, 17 copies/mL). The presence of viral RNA suggests that ongoing viral replication is occurring, albeit at low levels, in each patient evaluated. Viral RNA levels were lower in most patients’ genital fluids compared with blood plasma and in 12 patients were undetectable.

Conclusions These data suggest that low-level replication of HIV-1 in patients taking suppressive HAART may be demonstrated not only in peripheral blood mononuclear cells but also in peripheral plasma as cell-free virion RNA. Complete ablation of viral replication may require intensification of antiretroviral therapies beyond standard suppressive HAART.

JAMA. 1999;282:1627-1632 www.jama.com

See also pp 1633 and 1668.
in several cohorts. This correlates with an inhibition of the vast majority of HIV-1 replication in certain infected hosts treated with HAART.

Nevertheless, several studies have now demonstrated that resting CD4+ T lymphocytes and cells in other body compartments, including the male genital tract, may maintain replication-competent proviral species in patients taking suppressive HAART with undetectable viral RNA in peripheral blood. These proviral species have been shown to generate replication-competent virus when cultured with CD8+ T lymphocyte–depleted peripheral blood mononuclear cells of uninfected individuals. In addition to these latently infected cells, there have also been studies that demonstrate that cells in the peripheral blood and lymphoid tissue may still generate low levels of viral replication, even under the setting of suppressive HAART.

From November 1998 to May 1999, we studied HIV-1–infected patients referred to our clinics and laboratories with fewer than 50 copies/mL of plasma viral RNA while taking HAART to determine whether blood plasma viral RNA, an indicator of ongoing viral replication, could be detected.

**METHODS**

A total of 22 HIV-1–infected men and women were drawn from a sample of more than 160 patients with known plasma HIV-1 RNA levels below 400 to 500 copies/mL while receiving HAART. Patients were followed up at hospitals within the Jefferson Health System and collaborating hospitals within the Delaware Valley.

All 22 individuals had consistently fewer than 400 to 500 copies/mL of viral RNA in peripheral blood plasma, as assayed by standard clinical reverse transcriptase polymerase chain reaction (RT-PCR) (Roche Inc, Indianapolis, Ind) or branched DNA analysis (Chiron Inc, Norwood, Mass). Each of the patients in this study was found to have peripheral plasma viral RNA levels of fewer than 50 copies/mL, using a laboratory-based quantitative RT-PCR method (quantitation to 5 copies/mL). Interassay variability (ie, reproducibility) was less than 10%. In 14 patients for whom clinical Roche ultrasensitive RT-PCR or Chiron ultrasensitive branched-chain DNA assays also were obtained, each demonstrated fewer than 50 copies/mL of HIV-1 RNA in blood plasma.

The 22 individuals were selected based on continuously undetectable plasma viral loads (fewer than 50 copies/mL at the time of the study), stable maintenance of virally suppressive HAART regimens for 5 to 55 months, and provision of genital fluid samples. All patients were immunologically (ie, CD4+ T-lymphocyte counts) and virologically stable during HAART. All patients referred initially for study who had fewer than 50 copies/mL of viral RNA in plasma were included in this analysis, except for 2 patients who did not provide genital fluid samples. Patients 8 through 14 had been reported on previously for proviral DNA and latent replication-competent virus in peripheral blood mononuclear cells and seminal cells. Screening of these patients for clinical study protocols, as well as for analysis of viral RNA in plasma and genital fluids, was approved by the Thomas Jefferson University Institutional Review Board and each patient signed an informed consent form.

**Plasma and Genital Fluid Samples**

Blood plasma samples were collected via peripheral phlebotomy. Fifty milliliters of peripheral blood was obtained and Ficoll-Hypaque gradient centrifugation was used to separate cells from plasma. The plasma was then passed through a 0.45-µm filter. Seminal samples were obtained from each man in the study by self-masturbation to ejaculation into a sterile container. Seminal cells were separated from seminal fluid by centrifugation at 500g for 10 minutes and subsequent passage through a 0.45-µm filter. Cervical/vaginal samples were obtained from HIV-1–infected women in this study by gently swabbing the cervical os and outer cervix with a cotton swab and via subsequent cervical vaginal collection with 2 mL of serum-free RPMI 1640 culture medium. All subjects in this study had abstained from sexual intercourse within 48 hours prior to time of sample collection. The women were not menstruating and no red blood cells were detected by microscopic analysis of the cervical/vaginal samples. The cervical/vaginal samples were centrifuged at 500g for 10 minutes with subsequent passage of the fluid through a 0.45-µm filter. Several HIV-1–seronegative individuals were used as controls in each batched group of PCR analyses.

**HIV-1 Virion RNA Isolation and Quantitative RT-PCR**

The blood plasma and genital fluids were concentrated via ultracentrifugation at 35,000 rpm for 1 hour. The supernatant was discarded and virion-associated genomic RNA was extracted from the subsequent pellet using a guanidinium thiocyanate method (Promega Inc, Madison, Wis). The isolated RNA was treated with 10 U of RQ1 DNase. The RNA was precipitated with transfer RNA (tRNA, Gibco Inc, Rockville, Md) as a carrier and resuspended in RNase-free water. Using this approach, reverse transcriptase–negative controls led to no detectable bands after PCR, which demonstrates the lack of viral DNA contamination.

Twelve microliters of each viral RNA sample (1 mL of plasma or genital fluid equivalent) was mixed with 1 µL of SK39 primer (50 µmol/L) followed by incubation at 55°C for 20 minutes. Then, 12 µL of a reverse transcriptase cocktail containing 5 µL of 5X reverse transcriptase buffer, 2.5 µL of 100-mmol/L dithiothreitol, 3.5 µL of 3-mmol/L deoxyribonucleoside triphosphates, and 1 µL of Moloney murine leukemia virus reverse transcriptase enzyme (Gibco Inc) was mixed with the RNA sample containing the primer. This mixture was then incubated at 37°C for 30 minutes. The reverse transcriptase was inactivated by boiling the samples for 10 minutes. The resultant complementary DNA was then analyzed by quantitative PCR with primers SK38 and SK39 to the gag region of the HIV-1 genome for 30 cycles, as described by Zhang et al. The amplified PCR prod-
ucts were hybridized with a probe labeled with phosphorus 32, SK19, and Southern blotting was then used to visualize the specific bands of the amplicons. A standard curve was developed using an in vitro transcribed gag RNA construct, as described previously. Comparison of the test samples with this serially diluted standard curve of the amplified in vitro transcribed standard was used to quantify viral unspliced RNA to 5 copies/mL, within the linear amplification range of this assay. Viral transcripts below 5 copies/mL also could be detected using this assay system. Quantitation of the viral transcripts was performed via analysis using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif).

**RESULTS**

The 22 patients represented an immunologically diverse group, with CD4+ T-lymphocyte counts between 0.10 and 1.27 × 10^9/L, and all 22 were treated with at least 3 antiretroviral agents (TABLE), including 2 reverse transcriptase inhibitors and at least 1 protease inhibitor or efavirenz (a nonnucleoside reverse transcriptase inhibitor), except for patient 4, whose therapy included 2 protease inhibitors and 1 reverse transcriptase inhibitor, and patient 15, who was treated with zidovudine and indinavir. The stability of the patients' HAART regimens was an important criterion of this cohort. Of note, patients 10 and 12 underwent a single switch from indinavir to nelfinavir during therapy, secondary to adverse effects (Table). In addition, several clinical complications occurred prior to initiation of HAART, aside from depression and renal stones (Table).

In all of the 22 patients evaluated with fewer than 50 copies/mL of virion RNA in the peripheral blood plasma, residual virion RNA was detectable at levels from fewer than 5 to 42 copies/mL (FIGURE and Table). The mean level of plasma viral RNA in these patients was approximately 17 copies/mL. In 3 patients (1, 10, and 11), viral RNA levels in peripheral blood plasma were reported, separated by at least 3 months between phlebotomies. Patients 1 and 11 had very similar levels of viral RNA in the periph-

### Table. Residual HIV-1 RNA in Blood Plasma and Genital Fluids of Patients Receiving Suppressive HAART

<table>
<thead>
<tr>
<th>Patients</th>
<th>Antiretroviral Therapy</th>
<th>CD4+ T Lymphocytes, ×10^9/L</th>
<th>Duration of Therapy, mo</th>
<th>Plasma (Mean, 17)</th>
<th>Seminal or Cervical Fluid</th>
<th>Clinical Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1†</td>
<td>Zidovudine/lamivudine/indinavir</td>
<td>0.11</td>
<td>7, 11</td>
<td>30, 12</td>
<td>&lt;5, Negative</td>
<td>Pneumocystis carinii pneumonia</td>
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<td>2</td>
<td>Zidovudine/lamivudine/nelfinavir</td>
<td>0.56</td>
<td>12</td>
<td>20</td>
<td>Negative</td>
<td>Depression</td>
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<tr>
<td>3‡</td>
<td>Zidovudine/lamivudine/indinavir</td>
<td>0.50</td>
<td>24</td>
<td>25</td>
<td>Negative</td>
<td>Depression</td>
</tr>
<tr>
<td>4</td>
<td>Delavirdine/nelfinavir/saquinavir</td>
<td>0.33</td>
<td>18</td>
<td>8</td>
<td>Negative</td>
<td>Herpes zoster, melanoma, cholecystitis, idiopathic thrombocytopenic purpura</td>
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<tr>
<td>5</td>
<td>Zidovudine/lamivudine/indinavir</td>
<td>1.05</td>
<td>12</td>
<td>10</td>
<td>Negative</td>
<td>Hypertension</td>
</tr>
<tr>
<td>6</td>
<td>Zidovudine/lamivudine/indinavir</td>
<td>0.34</td>
<td>42</td>
<td>40</td>
<td>&lt;5</td>
<td>None</td>
</tr>
<tr>
<td>7‡</td>
<td>Stavudine/lamivudine/nelfinavir</td>
<td>0.62</td>
<td>14</td>
<td>15</td>
<td>Negative</td>
<td>Hepatitis B</td>
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<tr>
<td>8</td>
<td>Zidovudine/lamivudine/indinavir</td>
<td>0.14</td>
<td>16</td>
<td>15</td>
<td>&lt;5</td>
<td>P carinii pneumonia, cryptococcosis</td>
</tr>
<tr>
<td>9</td>
<td>Stavudine/lamivudine/nelfinavir</td>
<td>0.28</td>
<td>5</td>
<td>8</td>
<td>&lt;5</td>
<td>Endocarditis</td>
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<tr>
<td>10†</td>
<td>Zidovudine/lamivudine/indinavir, nelfinavir§</td>
<td>0.65</td>
<td>18, 21</td>
<td>&lt;5, 15</td>
<td>&lt;5, Negative</td>
<td>Renal stones, eczema</td>
</tr>
<tr>
<td>11†</td>
<td>Zidovudine/lamivudine/indinavir</td>
<td>0.10</td>
<td>39, 55</td>
<td>15, 16</td>
<td>12, 8</td>
<td>None</td>
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<td>12</td>
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<td>18</td>
<td>10</td>
<td>&lt;5</td>
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<tr>
<td>13</td>
<td>Zidovudine/lamivudine/ritonavir/saquinavir</td>
<td>0.88</td>
<td>5</td>
<td>6</td>
<td>Negative</td>
<td>Thrush, herpes simplex</td>
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<tr>
<td>14</td>
<td>Didanosine/stavudine/indinavir</td>
<td>1.05</td>
<td>41</td>
<td>12</td>
<td>10</td>
<td>Renal stones, gonorhea, herpes simplex, amebiasis, rectal papilloma</td>
</tr>
<tr>
<td>15</td>
<td>Zidovudine/indinavir</td>
<td></td>
<td></td>
<td>0.78</td>
<td>36</td>
<td>7</td>
</tr>
<tr>
<td>16</td>
<td>Zidovudine/lamivudine/nelfinavir</td>
<td>0.37</td>
<td>19</td>
<td>32</td>
<td>&lt;5</td>
<td>Hepatitis B</td>
</tr>
<tr>
<td>17</td>
<td>Zidovudine/lamivudine/indinavir</td>
<td>0.49</td>
<td>24</td>
<td>12</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>18</td>
<td>Zidovudine/lamivudine/nelfinavir</td>
<td>0.55</td>
<td>30</td>
<td>21</td>
<td>Negative</td>
<td>Hypertension, mitral valve disease</td>
</tr>
<tr>
<td>19</td>
<td>Zidovudine/lamivudine/efavirenz</td>
<td>0.57</td>
<td>26</td>
<td>&lt;5</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>20</td>
<td>Stavudine/lamivudine/efavirenz</td>
<td>0.55</td>
<td>8</td>
<td>20</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>21</td>
<td>Zidovudine/lamivudine/efavirenz</td>
<td>1.27</td>
<td>13</td>
<td>42</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>22</td>
<td>Zidovudine/lamivudine/efavirenz</td>
<td>0.37</td>
<td>10</td>
<td>26</td>
<td>&lt;5</td>
<td>Syphilis, hermaphoria</td>
</tr>
</tbody>
</table>

*HIV-1 indicates human immunodeficiency virus 1; HAART, highly active antiretroviral therapy.
†Separate samples were obtained at least 3 months apart and analyzed on 2 different occasions.
‡Female.
§Indinavir was changed to nelfinavir during therapy.
Interleukin 2 therapy was received 17 months prior to analysis.
eral blood plasma on repetition, while there was a relatively small but somewhat higher level of HIV-1 RNA in the second sample obtained from patient 10 compared with the first. Of importance, this RT-PCR assay is able to quantify viral RNA to a level of 5 copies/mL but, nevertheless, could detect viral RNA at levels below 5 copies/mL. Human immunodeficiency virus 1-seronegative individuals, used as negative controls, never demonstrated specific bands at any magnitude in this assay system.

In addition, virion RNA levels were evaluated in the genital secretions from each of these patients. As has been demonstrated previously, the levels were lower than those found in peripheral blood plasma. Twelve patients had negative results for virion RNA levels in genital secretions, including both women analyzed in this study (patients 3 and 7). Eight patients demonstrated detectable viral RNA in seminal fluid but below the

**Figure.** Quantitative RT-PCR of HIV-1 RNA in Blood Plasma and Genital Secretions of Patients Receiving Suppressive HAART

RT-PCR indicates reverse transcriptase polymerase chain reaction; HIV-1, human immunodeficiency virus 1; and HAART, highly active antiretroviral therapy. Peripheral blood plasma and genital fluids from 22 patients with fewer than 50 copies/mL were further quantified using an ultrasensitive RT-PCR technique for HIV-1 virion RNA. In vitro transcribed RNA standards were used to quantify test samples to 5 copies/mL. Of note, this assay may also detect, albeit not quantify, viral copies below 5 copies/mL. Two HIV-1-seronegative patients were used as controls in each of the batched experimental runs. Specific primers and probes were used to detect the gag sequences in unspliced HIV-1 virion RNA. The Figure represents autoradiographs of Southern blotting analyses of RT-PCR–produced amplicons (see “Methods” section of text for details).
level of quantitation of 5 copies/mL. Only 2 patients demonstrated quantifiable virion RNA in seminal fluid (patients 11 and 14) (Figure and Table).

Thus, in all HIV-1–infected individuals taking suppressive HAART (fewer than 50 copies/mL of plasma viral RNA) in this study, residual HIV-1 RNA could be detected in each of the patient’s peripheral blood plasma.

COMMENT

The current study demonstrates that, in a cohort of patients with undetectable viral RNA for between 5 months and several years while taking HAART and with fewer than 50 copies/mL of viral RNA in peripheral blood plasma at the time of these analyses, all subjects had low but detectable levels of HIV-1 RNA in blood plasma. This was surprising in that these data demonstrated that viral expression could not only be shown by viral replication in selected cell types within patients taking suppressive HAART but by actual virion production within blood plasma. This correlates with data presented by several groups that showed low levels of ongoing viral replication in selected patients by analysis of peripheral blood mononuclear cells and lymphoid tissue.12-18 Our study expands and extends these data and demonstrates that some cell-free virion production may be quite common in patients taking suppressive HAART with fewer than 50 copies/mL of viral RNA in peripheral blood plasma. Thus, using specific techniques, the “cryptic” viral replication shown in certain cell types is also quantifiable using peripheral blood plasma analysis. Of importance, this viral replication may not only infect uninfected cells in the local microenvironment of viral-producing cells, but may infect cells at a distance within the body.

Only low levels of virion RNA were found in the genital secretions of these HIV-1–infected individuals. Fifty-five percent of these patients with fewer than 50 copies/mL of viral RNA in peripheral blood plasma demonstrated no detectable viral RNA in genital secretions, as shown by our assay system. Nonetheless, the viral levels in peripheral blood plasma do not take into account potential viral replication in other body fluids, including cerebrospinal fluid and the interstitial fluid between cells in certain solid tissues. In addition, the pathogenetic importance and potential for sexual transmission from highly virally suppressed individuals of very low-level cell-free virion RNA, found in genital secretions of 10 patients in this study, are unknown and require further study.

Plasma viral load suppression below 20 copies/mL has been demonstrated to yield a more long-term antiretroviral response in patients compared with those individuals who only obtain viral suppression below 500 copies/mL in plasma.24 Whether a clinical difference exists between suppression below 50 copies/mL and that below 20 copies/mL requires further study. A study of 6 patients also demonstrated the rate of HIV-1 rebound in peripheral blood plasma after discontinuation of HAART.25 The median time to obtain 500 copies/mL from a level of below 50 copies/mL was approximately 10 days after stopping therapy. Using regression analysis, it was suggested that approximately 10 copies/mL of viral RNA in peripheral blood plasma could have been the baseline level in these patients. This estimate is close to our mean viral load in peripheral blood plasma in the 22 patients studied in the current analyses (ie, 17 copies/mL). Because most25,26 but possibly not all27 patients rebound with high levels of plasma viral RNA when standard suppressive HAART is discontinued, even when fewer than 50 copies/mL have been demonstrated for significant periods in their peripheral blood plasma, this suggests ongoing viral replication and potentially infectious virions still present in peripheral blood plasma and possibly other body fluid compartments. If 3000 mL of blood plasma is considered an approximation of that found in most humans, this would suggest that, in the patients in our study, there was a mean level of 31 000 virion RNA copies in blood plasma during suppressive HAART. In another recent study, viral rebound occurred in 8 patients after discontinuation of HAART.28

The low-level viral replication in these patients may take place constantly or in “bursts.” Measuring viral RNA directly can facilitate study of mechanisms of viral persistence and initially hidden viral replication without the “noise” of active virally producing cells and high levels of cell-free virions, which occur in most HIV-1–infected patients not treated with HAART.29,30 Of note, viral reservoir decay characteristics suggest that in 1 analysis, 10 years,13 and in a second analysis, 60 years,31 will be necessary for patients treated with suppressive HAART to continue therapy for potential viral eradication. These are best-case scenarios, as they do not fully take into account low-level viral replication that occurs in most patients taking HAART, as demonstrated in the current study and by others.12-18

It is still unclear which cell types produce these residual virions. Sequencing of virion RNA at these low levels is difficult but may be approachable with new technologies being developed. It is not clear whether this very low-level viral replication leading to virion particles in the peripheral blood plasma in patients taking suppressive HAART comes from peripheral blood lymphocytes, tissue-bound macrophages,32 lymph nodes, or other compartments that may be relative “drug sanctuary” regions (eg, central nervous system, retina, and testes in men).11,33,34 Thus, further studies will be necessary to understand the in vivo molecular mechanisms behind “residual HIV-1 disease” in patients taking what is now clinically considered to be fully suppressive HAART.30 Of note, recent studies have shown that certain CD4+ T lymphocytes that are histocompatibility antigen–DR negative and, thus, not activated or stimulated, can be positive for low levels of viral RNA species in lymphoid tissue of patients taking combination antiretroviral chemotherapy.35 A spectrum of cells in various stages of activation may be involved in expressing HIV-1–specific RNA in patients taking suppressive HAART. These data also demonstrate the critical requirements for new and robust technologies to measure viral persistence in clinical microbiol-
ogy laboratories, rather than solely as research tools. In the era of HAART.

Plasma viral levels of patients treated during primary HIV-1 infection prior to seroconversion were not evaluated in the current study and may differ from patients already receiving long-term treatment. Nonetheless, patient 18 was treated with HAART within 2 months of HIV-1 seroconversion. Future studies should determine if true ablation of residual viral levels can be obtained.

In summary, the current data demonstrate that although HAART may lead to profound suppression of viral replication in many HIV-1–infected individuals, a low but detectable level of viral replication occurs in a significant majority of patients treated with these combinations of antiretroviral agents. Importantly, recent mathematical modeling of “Ongoing HIV dissemination During HAART” may be supported by the present data. This may have significant impact on clinical research and care because it suggests that most patients taking HAART, at least within the first few years of therapy, will have prompt viral rebound if HAART is discontinued, based on ongoing viral replication at low levels beyond the detection limit of most clinical assays of viral load. For the design of viral eradication studies of HIV-1, this suggests that there are important and possibly unrelated mechanisms of viral persistence during HAART. One mechanism may be true viral latency, in quiescent CD4+ T lymphocytes and other cell types. Additional data suggest that ongoing low-level viral replication must also be approached. Thus, although “stimulatory” therapy of latently infected cells may decrease the pool of persistently infected cells in patients taking suppressive HAART, “intensification” therapy to truly ablate viral replication maybe an important initial approach in any attempts at viral eradication, although weighing potential additional adverse effects from this approach must be considered.

Funding/Support: This work was supported in part by US Public Health Service grant AI38666 (Dr Pomerantz).

Acknowledgment: We thank Colleen Dascenzo for excellent assistance in obtaining the patient specimens, Rita M. Victor and Brenda O. Gordon for excellent secretarial assistance, and the Jefferson Health System, Pennsylvania Hospital, and MCP-Hahnemann University, Philadelphia, Pa, who volunteered for these studies.

REFERENCES


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