The Effect of Commencing Combination Antiretroviral Therapy Soon after Human Immunodeficiency Virus Type 1 Infection on Viral Replication and Antiviral Immune Responses

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Twelve subjects were treated with zidovudine, lamivudine, and ritonavir within 90 days of onset of symptoms of acute infection to determine whether human immunodeficiency virus type 1 (HIV-1) infection could be eradicated from an infected host. In adherent subjects, with or without modifications due to intolerance, viral replication was suppressed during the 24-month treatment period. Durable suppression reduced levels of HIV-1–specific antibodies and cytotoxic T lymphocyte responses in selected subjects. Proviral DNA in mononuclear cells uniformly persisted. The persistence of HIV-1 RNA expression in lymphoid tissues and peripheral blood mononuclear cells suggests that elimination of this residual pool of virus should be achieved before considering adjustments in antiretroviral therapeutic regimens. In addition, given the reduction in levels of virus-specific immune responses, it would seem prudent to consider enhancing these responses using vaccine strategies prior to the withdrawal of antiviral therapy.

Acute infection with human immunodeficiency virus type 1 (HIV-1) is characterized by high levels of viral replication [1, 2], often associated with a clinical syndrome that includes fever, rash, lymphadenopathy, headache, myalgia, and gastrointestinal and neurologic symptoms [3–5]. Probably because of a genetic bottleneck during or soon after transmission, the initial expansion of HIV-1 in the new host typically involves a relatively clonal virus population [6, 7]. With the onset of a cellular immune response, principally cytotoxic T lymphocytes (CTL), there is a partial, and at times marked, reduction in viremia [8, 9]. Characteristically, a quasi-steady-state (set point) is established by 9–12 months after the initial infection, when HIV-1 production and clearance are in approximate balance [10–12]. In characterizing 74 persons newly infected with HIV-1, Schucker et al. [13] showed that the inflection point in the reduction in plasma HIV-1 RNA levels occurs, on average, as early as 117 days after infection. Despite the presence of strong cellular and humoral immune responses during this period of chronic infection, viral replication remains high and drives a gradual destruction of the immune system [14, 15], dominated by an inexorable loss of CD4 T lymphocytes. Severe immunodeficiency and death ultimately ensue in nearly all untreated infected persons.

The level of HIV-1 in plasma at the set point is highly predictive of the subsequent clinical course [10–13, 16]. Between 93% and 99% of plasma virus is produced by rapid rounds of infection and destruction of activated CD4 T cells, with the rest produced by long-lived chronically infected cells, such as tissue macrophages and dendritic cells, latently infected lymphocytes, and release of trapped virions from lymphoid tissue [17]. Effective antiviral therapy rapidly interrupts the replication of HIV-1 in CD4 T cells and prevents the spread of infectious virus from the longer-lived cells; the half-lives of HIV-1 production in these two compartments are ~1 day and ~2 weeks, respectively [17]. Although quantitatively minor, a third viral compartment consisting of resting memory CD4 T cells harboring integrated, replication-competent HIV-1 has been identified and could serve as a persistent reservoir of latent virus despite prolonged antiviral therapy [18].

At the time this clinical trial was designed, having observed
a 99% reduction in plasma HIV-1 RNA in subjects treated with protease inhibitor monotherapy [19, 20], we asked whether HIV-1 infection could be eradicated with a combination of the most active therapies available when applied in the best of clinical situations: a newly infected host with a drug-susceptible virus population and a minimally affected immune system.

We selected the combination of ritonavir, a potent protease inhibitor, with the reverse transcriptase (RT) inhibitors zidovudine and lamivudine. Used alone, ritonavir can cause ~2 log₁₀ reductions in plasma viremia [19, 20], and the combination of zidovudine with lamivudine also has a powerful antiviral effect [21]. We therefore anticipated that the triple combination would be highly effective at reducing HIV-1 replication and restoring CD4 T cells in vivo. Triple therapy with ritonavir (1200 mg/day), zidovudine (600 mg/day), and lamivudine (300 mg/day) was begun in July 1995 in 12 subjects within 90 days of symptoms of acute HIV-1 infection. Patient acceptance and tolerance of the regimen was poor, and subsequent experience with ritonavir clearly showed that the drug in the 600 mg twice-a-day dosage can be difficult to tolerate, particularly in combination with zidovudine (unpublished data). Despite these inherent limitations, we report here a detailed analysis of the virologic effects of this regimen in both peripheral blood and lymphoid tissue and the immunologic consequences of this regimen over the first 2 years of treatment.

Our more recent research and that of others indicate that eradication of HIV-1 from the infected host may require treatment regimens that are more sophisticated and of longer duration. This proof-of-concept study remains valuable nevertheless for assessing the virologic and immunologic effect of early intervention with highly active antiretroviral therapy. In addition, data generated from this and other studies [22, 23] using highly effective combination therapy have strongly influenced recent guidelines on treating HIV infection [24].

Methods

Study design. Twelve subjects newly infected with HIV-1 were recruited for this open-label, noncontrolled pilot study between July 1995 and February 1996. Entry criteria included plasma viremia detectable by the branched DNA (bDNA) assay [2.0, Chiron Diagnostics, Emeryville, CA], with a detection limit of 500 HIV-1 RNA copies/mL plasma, and either an initial negative test for HIV-1 antibodies followed by the appearance of ≥2 new bands on a Western blot or a convincing clinical history for acute HIV-1 infection within the prior 90 days plus a documented negative antibody test within 120 days of screening. Subjects were treated with escalating doses of ritonavir over 7–10 days to achieve a maximum final dose of 1200 mg/day. Zidovudine and lamivudine were used from the outset at dosages of 600 mg/day and 300 mg/day, respectively. Therapy was initiated on an inpatient basis but continued on an outpatient basis. After the initial hospitalization of ~7 days, subjects were seen weekly to week 4, biweekly to week 12, and then monthly, at which time blood was collected and plasma and cells separated and cryopreserved for the virologic and immunologic studies detailed below. Adherence to therapy was determined by drug reconciliation and patient history. Semen was collected from all subjects after 1 year of therapy. Biopsy of gastrointestinal tissue by flexible sigmoidoscopy to obtain gastrointestinal or gut-associated lymphoid tissue (GALT) was performed in all subjects after a minimum of 1 year of therapy. After 15 months of therapy, 1 patient (subject 9) consented to a repeat gut biopsy, simultaneous biopsies of cervical lymph node and tonsil, and a lumbar puncture. Additional subjects consented to biopsies of either cervical or inguinal node or tonsil after ~24 months of therapy. Tissues were snap-frozen and stored at −70°C for subsequent polymerase chain reaction (PCR) studies, and some were formalin-fixed and paraffin-embedded for in situ examination.

Virologic measurements. Plasma HIV-1 RNA was quantified longitudinally using both the bDNA signal amplification assay [25] and the ultrasensitive RT-PCR assay [26] (Roche Diagnostics, Alameda, CA), which have detection limits of 500 and 50 RNA copies/mL, respectively. Infectious HIV-1 titers in peripheral blood and lymphoid tissue were determined by limiting-dilution cocultures and expressed as TCID₅₀/10⁶ mononuclear cells [27]. The coculture procedure of patient peripheral blood mononuclear cells (PBMC) was modified when infectious titers were persistently <0.1 TCID₅₀/10⁶ cells by directly stimulating donor and patient PBMC with phytohemagglutinin (PHA) and depleting them of CD8 T cells [28–30]. Semen was collected and processed within 6 h. After a 1:4 dilution with RPMI, cells were separated from seminal plasma by centrifugation at 800 g. Cells were stored at −70°C for subsequent analysis. Levels of multiply spliced (MS) and unspliced (US) HIV-1-specific mRNA and proviral DNA in mononuclear cells from blood, genital secretions, and tissue were determined by PCR as described previously [31, 32]. In situ hybridization on formalin-fixed, paraffin-embedded lymphoid tissue was performed using HIV-1–specific antisense probes corresponding to ≥90% of the viral genome [33, 34]. GALT was histologically graded 1–4 as follows: 1, scattered lymphoid cells; 2, small lymphoid aggregate; 3, large well-defined aggregate; and 4, germinal center present.

Humoral and cellular immunology. CD4 and CD8 T cell counts were assessed longitudinally using standard techniques. Routine screening ELISA and Western blots were performed. Plasma antibody titers to HIV-1 gp120 and p24 proteins were measured as described elsewhere [35]. HIV-1–specific CTL precursor frequency (CTLp) was quantitated using autologous B lymphoblastoid cell targets infected with recombinant vaccinia viruses expressing HIV-1–specific antigens (gag, pol, env, or nef). Direct CTL effector activity was measured using freshly isolated PBMC, per published methods [8].

Results

Study design, compliance, and adherence. All study subjects were homosexual men with symptomatic acute HIV infection whose treatment commenced, on average, 65 days (range, 45–90) from onset of symptoms. For subjects able to define when they became infected, their symptoms commenced a mean of 15 days (range, 6–20) after the reported high-risk sexual exposure. Seven of the original 12 participants have now remained on either the original regimen or a modified highly
active regimen for up to 24 months (table 1). Gastrointestinal adverse events led 2 subjects (1 and 12) to discontinue drugs and withdraw early from the study at weeks 4 and 52. Subjects 4 and 10 voluntarily withdrew from the trial after 24 and 8 weeks, respectively, because of the rigorous protocol schedule. Participants were questioned at each clinic visit to detail the number of missed doses, if any, of the 3 study medications, to monitor for adherence to the prescribed drug regimen. At the month 20 visit, when results of plasma RNA measurements using a new ultrasensitive assay with a lower detection limit of 50 RNA copies/mL plasma were made available, subject 11 described reducing ritonavir doses by 50% after only 2 months of therapy and not taking any of the prescribed drugs about 25% of the time. These 5 subjects (1, 4, 10, 11, and 12) were formally removed from the trial, although some findings for subject 11 are presented below. Subject 8 briefly interrupted therapy after 19 months because of emotional distress unrelated to therapy and was promptly restarted on the identical regimen after a rapid virologic rebound was documented (see below).

In addition, at the month 21 visit, when a clear rebound in HIV-1 RNA was noted, subject 6 described altering his treatment regimen. He reduced his dose of ritonavir after ~1 year to 50%, and ceased all 3 drugs 2 months thereafter (table 1). After recommencing therapy with the original regimen, he remained in the trial, and findings are presented below. All 5 remaining subjects (2, 3, 5, 7, and 9) were >95% adherent to the prescribed regimen, judged both by clinical history and drug reconciliation. However, 2 participants required replacements for ritonavir; 1 by indinavir at month 8 because of allergy (subject 3), the other by nevirapine at month 12 because of complications with chronic hepatitis C infection (subject 5). Subject 2 had been maintained on a reduced (400 mg/day) dose of zidovudine because of grade 4 elevations in serum creatine phosphokinase levels beginning at week 12. Subject 9 received a slightly reduced (1000 mg/day) dose of ritonavir beginning at week 6 because of early grade 4, drug-related elevations in serum levels of transaminases on the original dose. We do not believe that the use of replacement therapies or dose alterations in these 4 subjects compromises the analyses, since HIV-1 replication in these subjects was successfully suppressed, consistent with the original intent of the clinical experiment.

Effects of triple-drug therapy on plasma viremia. Longitudinal plasma viremia (HIV-1 RNA levels) measured by both signal amplification (bDNA) assays with a detection limit of 500 copies/mL and PCR-based assays with a detection limit of 50 copies/mL are shown in figure 1A for the adherent subjects (2, 3, 5, 7, and 9) and in figure 1B for the nonadherent subjects (6, 8, and 11).

All 12 participants initially responded to triple-drug therapy. An undetectable level of HIV RNA in plasma, as determined by bDNA (<500 RNA copies/mL), was reached after a mean of 28.8 days and a median of 9.5 days (range, 4–97). The mean maximum reduction in plasma RNA was 2.2 log_{10} (data not shown). In 7 subjects with baseline plasma RNA values >10,000, a typical biphasic pattern of HIV-1 RNA decay was observed. Mean and median half-lives for the initial rapid phase were 2.4 and 1.9 days, respectively (range, 1.2–5.3). In the 6 patients in whom a second slower phase was measurable, the mean and median half-lives were 22.4 and 19.2 days, respectively (range, 11.2–46.2). At the time they discontinued therapy, subjects 1 and 10 both had undetectable plasma viremia by the bDNA assay. However, viremia in subjects 4 and 12 was rebounding at discontinuation, presumably reflecting the difficulties they had in following the therapeutic protocol (data not shown).

Plasma viremia remained persistently below the detection threshold of 50 copies/mL in compliant subjects 2, 3, 7, and 9 (figure 1A). Sporadic increases in plasma viremia to levels between 50 and 500 copies/mL were detected in subject 5 around month 12, when ritonavir was replaced by nevirapine because of complications with chronic hepatitis C infection (figure 1A). An unexplained further sporadic increase in plasma viremia was noted near month 18, but no sustained viral replication has since been documented in this person. Plasma viremia in

Table 1. Baseline characteristics of the study subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Primary syndrome</th>
<th>Duration of primary infection $^b$</th>
<th>Time (days) from symptoms to treatment</th>
<th>Duration (months)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adherent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>Moderate</td>
<td>A</td>
<td>60</td>
<td>26</td>
<td>Zidovudine, 400 mg/day</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>Mild</td>
<td>A</td>
<td>75</td>
<td>25</td>
<td>Ritonavir replaced by indinavir</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>Severe</td>
<td>A</td>
<td>60</td>
<td>25</td>
<td>Ritonavir replaced by nevirapine</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
<td>Moderate</td>
<td>B</td>
<td>45</td>
<td>23</td>
<td>Ritonavir, 1000 mg/day</td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>Mild</td>
<td>A</td>
<td>75</td>
<td>20</td>
<td>Ritonavir, 600 mg/day at 12 months; stopped all drugs at month 14</td>
</tr>
<tr>
<td>Nonadherent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>Moderate</td>
<td>A</td>
<td>45</td>
<td>24</td>
<td>Ritonavir, 600 mg/day at 12 months; stopped all drugs at month 14</td>
</tr>
<tr>
<td>8</td>
<td>38</td>
<td>Moderate</td>
<td>A</td>
<td>45</td>
<td>21</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>22</td>
<td>Severe</td>
<td>B</td>
<td>90</td>
<td>20</td>
<td>Frequent drug interruptions</td>
</tr>
</tbody>
</table>

$^a$ All subjects are homosexual men.

$^b$ A, evolving antibody reactivity by Western blots; B, history consistent with primary infection together with negative HIV-1 antibody test within prior 120 days.
Figure 1. Longitudinal plasma HIV-1 RNA copies and CD4 cells for 8 subjects on therapy >12 months. Open red circles, HIV-1 RNA by branched DNA (lower limit, 500 copies/mL); + and −, detectable and undetectable (<50 copies/mL) plasma HIV-1 RNA by ultrasensitive polymerase chain reaction, respectively; green dots, absolute CD4 cell count.

nonadherent subject 11 was never suppressed to <50 copies/mL but still remained below 500 copies/mL despite his less-than-optimal therapy. Furthermore, despite complete discontinuation of all medications at month 18, the level of HIV RNA detectable in plasma was never above 500 copies/mL for 6 months but was detectable by the ultrasensitive RNA-PCR assay (figure 1B). Although subject 6 completely discontinued therapy at month 14 after 2 months of ritonavir at a reduced dose, his plasma viremia remained low (50–500 copies/mL) for 4 months before rebounding to >500 copies/mL (figure 1B). Conversely, subject 8 chose to discontinue therapy shortly after his month 19 visit, and his plasma viremia level rebounded rapidly (figure 1B).

Virus isolation and detection of HIV-1 in tissues. The results of cross-sectional analyses of HIV-1 infectivity and viral mRNA in PBMC and GALT of 5 adherent and 3 nonadherent subjects are presented in table 2, as are tonsil and lymph node tissue findings of subjects 2, 3, and 9. To assess whether infectious HIV-1 could be isolated from PBMC, qualitative cocultures were performed on CD8-depleted, PHA-stimulated cells after therapy for 19–24 months. No infectious HIV-1 was detected (<0.1 TCID50/10⁶ CD4 T cells) in subjects 2, 3, and 7, consistent with their undetectable plasma viremia. No infectious HIV-1 was detected in subject 8 at a time that he was both adherent and aviremic. However, unexpectedly, the coculture was positive for subject 9, whose plasma viral RNA was consistently <50 copies/mL. In keeping with their low but persistent plasma viremia, the cocultures for subjects 5, 6, and 11 were positive.

We next used a PCR assay to measure MS and US HIV-1 mRNA in subjects’ PBMC. The presence of MS mRNA is
indicative of ongoing viral replication, whereas the detection of US mRNA could be indicative of the presence of virions regardless of their infectivity [31, 32]. As shown in table 2, both MS and US mRNA species were detected in the PBMC of subject 11. For the other subjects, no MS mRNA was found in PBMC, although US mRNA was detectable in some subjects (2, 3, 5, and 6). No HIV-1 mRNA of either form was detectable in PBMC from subjects 7, 8 (with a brief exception), and 9, 3 of the subjects who were most able to adhere to the original treatment protocol up to the time these analyses were done. HIV-1 DNA was uniformly detectable in PBMC of every subject, regardless of the degree of suppression of plasma viremia (table 2). PBMC isolated from semen after 12 months of therapy in subjects 2, 3, 5, 6, 7, 8, 9, and 11 were all positive for proviral DNA and negative for both MS and US mRNA (data not shown). HIV RNA was undetectable by the ultrasensitive RNA-PCR method in the cerebrospinal fluid obtained by lumbar puncture from subject 9 after 15 months of therapy (data not shown).

Results of analyses of the HIV-1 content of GALT samples during months 12–17 are shown in table 2. Mononuclear cells were isolated from biopsy specimens by tissue homogenization followed by ficoll-hypaque density gradient before placement in quantitative cocultures to detect infectious HIV-1. In each case, GALT obtained during months 12–17 of treatment were negative for infectious virus (i.e., <0.1–1.0 TCID₅₀/10⁶ mononuclear cells). Adequate numbers of CD4+ T cells could not be isolated from GALT, precluding more sensitive qualitative coculture, as described for PBMC.

Messenger RNA was extracted from the total GALT RNA pool for detection of HIV-1 MS and US mRNA. As summarized in table 2, no MS mRNA was detected in any case, whereas US mRNA was detectable in nearly all subjects. Proviral DNA was routinely detected in the mononuclear cells

Table 2. Cross-sectional analyses of HIV-1 infectivity and viral mRNA in peripheral blood mononuclear cells (PBMC) and gut-associated lymphoid tissue (GALT) of adherent and nonadherent study subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time of sampling (months)</th>
<th>Qualitative culture</th>
<th>mRNA (copies/µg)</th>
<th>DNA (copies/10⁶ cells)</th>
<th>Time of sampling (months)</th>
<th>GALT tissues</th>
<th>Culture (TCID₅₀/10⁶MC)</th>
<th>mRNA (copies/µg)</th>
<th>DNA (copies/10⁶MC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>NA</td>
<td>(+)</td>
<td>7489</td>
<td>5290</td>
<td>1496</td>
<td>NA</td>
<td>Desc. colon</td>
<td>1.0</td>
<td>439</td>
</tr>
<tr>
<td>Adherent</td>
<td>2</td>
<td>24</td>
<td>(−)</td>
<td>&lt;50</td>
<td>372</td>
<td>353</td>
<td>16</td>
<td>Desc. colon</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>(−)</td>
<td>&lt;50</td>
<td>459</td>
<td>503</td>
<td>17</td>
<td>Desc. colon</td>
<td>&lt;0.1</td>
<td>351</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>(+)</td>
<td>&lt;50</td>
<td>1921</td>
<td>528</td>
<td>16</td>
<td>Desc. colon</td>
<td>&lt;1.0</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>(−)</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>167</td>
<td>15</td>
<td>Desc. colon</td>
<td>&lt;0.1</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>(+)</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>112</td>
<td>12</td>
<td>Desc. colon</td>
<td>&lt;0.1</td>
<td>50</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>6</td>
<td>20</td>
<td>(+)</td>
<td>&lt;50</td>
<td>295</td>
<td>254</td>
<td>13</td>
<td>Desc. colon</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>11</td>
<td>18</td>
<td>(+)</td>
<td>312</td>
<td>1521</td>
<td>1753</td>
<td>13</td>
<td>Desc. colon</td>
<td>&lt;0.1</td>
<td>50</td>
</tr>
</tbody>
</table>

NOTE. MC, mononuclear cells; NA, not available; Desc., descending; (+), positive; (−), negative; ND, not done.

a 1–2 × 10⁵ CD4/CD8 cells depleted and phytohemagglutinin-stimulated.
b At time of study, patient completely adherent to regimen.
derived from GALT of all subjects. Tonsil and cervical lymph node biopsies were performed on subject 9 after 15 months of therapy. Analyses of these tissues yielded results similar to those derived from GALT: no culturable virus and undetectable MS mRNA, but detectable US mRNA and proviral DNA. At 24 months, patients 2 and 3 underwent tonsillar biopsy and inguinal lymph node biopsies, respectively. These tissues were negative in viral coculture and contained no detectable US or MS mRNA.

In situ hybridization was also performed to detect HIV-1 RNA expression in lymphoid tissues (figure 2). An optimal biopsy from an HIV-1–infected untreated control with a large well-defined lymphoid nodule is shown in figure 2A. The large amount of trapped viral RNA and the intense signal from some productively infected cells from the untreated control are evident in panels 2B and 2C, respectively. Gastrointestinal biopsy samples from 7 subjects (2, 3, 5–8, and 11) who remained on therapy ≥12 months are shown in panels 2D–2K. Although only a limited number of biopsies could be performed on each subject, HIV-1 RNA-expressing cells could not be detected in samples from any of these subjects. In 3 GALT biopsy sections from subjects 2, 6, and 7 in which lymphoid follicles were observed, no trapped HIV-1 was detected. A more extensive series of biopsies was performed on subject 9 (figure 2L–2Q); HIV-1–specific RNA was rarely detected in individual cells in rectal, tonsil, and cervical node tissue (figure 2N, 2P, 2Q). Germinal centers in tonsil and lymph node were free of trapped virus (figure 2L, 2M), and the occasional RNA-positive cell had relatively few grains (7–37) compared with untreated controls (figure 2C), in which there were too many grains to count. In situ hybridization of the tonsil and inguinal node tissue from subjects 2 and 3 obtained after 24 months of therapy were negative for trapped virus in the germinal centers, and no RNA-expressing cells were seen in >30 sections examined (data not shown).

Cellular and humoral immunology. Lymphocyte subset analyses are presented longitudinally in figure 1 for 8 subjects. At 18 months, mean CD4 cells had increased significantly by 290 cells/mm³ (P < .001), whereas mean CD8 cells fell by 129 cells/mm³ (P = .3).

The binding antibody responses (midpoint titers) to the HIV-1 gp120 and p24 proteins are presented for subjects 2, 3, 5–9, and 11 in figure 3. In general, antibody titers remained substantially lower than the titers of ≥10⁴ typically found in chronically infected persons [35]. In addition, antibody titers to gp120 were 1–2 logs higher than those to p24, which were often undetectable. Among subjects with consistently undetectable levels of plasma HIV-1 RNA, only subject 9 had a rising anti-gp120 titer, and the increase was modest. In subject 6, there was a rapid increase in antibody titers about the time he discontinued therapy; of note, the increase in his anti-gp120 titer preceded the rise in plasma viremia (figure 1B). Subject 11 had the highest antibody titers to both gp120 and p24, probably because of the relatively incomplete suppression of HIV-1 replication in this noncompliant subject.

“Fresh” (unstimulated) CTL responses to env, gag, pol, and nef antigens were very low (<10% specific lysis) at all times in each trial participant and did not change in response to reductions in plasma viremia (data not shown). CTLp analyses revealed three different patterns of response in the 7 persons studied (figure 4). No CTLp were detectable in subject 5 at baseline, on day 28, or at month 20. Subjects 9 and 11 had either a broadening or an increase in CTLp levels over time, suggesting that HIV-1 replication was still occurring in some tissue compartment. Of note was the late broadening in CTLp frequencies in subject 9, about the time his anti-gp120 titers were gradually rising, despite the absence of detectable plasma viremia and his sustained compliance with the therapeutic protocol (figures 3, 4). However, subjects 3, 6, 7, and 8 all had undetectable or significantly reduced CTLp levels at the end of the analytical period.

Discussion

The original goal of this clinical experiment was to completely suppress viral replication in a cohort of newly infected subjects and thereby attempt virus eradication. This represents a major scientific challenge. The initial triple combination regimen was difficult to maintain; 4 subjects withdrew, dosage adjustments were made in 2 others, and poor compliance was noted in an additional 3 subjects. We believe this was due to the use of ritonavir in daily doses of 1200 mg. Subsequent studies by our group using alternative protease inhibitor–containing regimens (e.g., indinavir, 2400 mg/day, or ritonavir, 800–1200 mg/day, in combination with saquinavir, 800–1200 mg/day) with double-nucleoside RT inhibitor therapy have resulted in improved patient acceptance and adherence while achieving virologic results similar to those documented above [36, 37]. This supports the feasibility of the use of combination therapy in the acute and early stages of HIV-1 infection.

Because the use of replacement therapies or dose alterations did not compromise suppression of HIV-1 replication in the study subjects, the virologic and immunologic data collected reflect the original intent of the clinical experiment. The initial virologic response to the triple-drug regimen was robust, and the dynamics of the response comparable to our previous reports [15, 17]. Comprehensive virologic studies of blood, tissue, and genital secretions for up to 24 months of therapy suggest complete suppression of ongoing productive HIV replication in subjects 2, 3, 7, and possibly 9. A tonsillar biopsy on patient 2 and an inguinal node biopsy on patient 3 after 24 months of therapy support these conclusions; there was an absence of follicular dendritic cell (FDC)–associated HIV RNA in multiple germinal centers, and HIV RNA-expressing cells were absent from up to 30 sections of tonsillar or lymphoid tissue examined by in situ hybridization (data not shown). The demonstration
Figure 2. Lymphoid tissue histology and in situ hybridization (ISH). A, B. Rectal biopsy in untreated control with submucosal lymphoid nodule (hematoxylin-eosin stain, ×25). ISH with large amounts of HIV-1 RNA (blue-green). C. Sigmoid biopsy in positive control with multiple HIV-1 RNA cells (×50). D. Subject 2, grade 4 gut-associated lymphoid tissue (GALT) germinal center (×100), ISH-negative for HIV RNA in cells and follicular dendritic cell (FDC) network. E. Subject 3, grade 2 GALT, no HIV RNA by ISH (×50). F. Subject 5, grade 3 GALT, no HIV RNA by ISH (×50). G and H. Subjects 6 and 7, respectively, grade 4 GALT, no HIV RNA by ISH (×50). J and K. Subjects 8 and 11, respectively, grade 2 GALT, no HIV RNA by ISH (×50). L. Subject 9, germinal centers in tonsil and lymph node, no FDC-associated HIV RNA (L, ×100; M, ×157.5). N, P, Q. Subject 9, rectum, lymph node, and tonsil tissue with rare HIV RNA by ISH (N, P, ×100; Q, ×157.5). (D–Q, Giemsa stain.)
Figure 3. Longitudinal midpoint antibody titers to env (gp120) (red dots) and gag (p24) (green triangles) in 8 subjects on triple therapy >12 months.

of plasma HIV RNA levels below detection by an ultrasensitive assay (50 RNA copies/mL) and the absence of MS mRNA expression in blood and tissue, combined with low to undetectable levels of HIV-1-specific immune responses, supports this conclusion.

The virologic data were somewhat equivocal in subject 5, with two isolated instances of measurable levels of HIV RNA, albeit at very low levels. In addition, infectious virus could be isolated from $1.5 \times 10^7$ CD8-depleted, PHA-stimulated CD4 cells. It is unclear whether this reflects drug failure due to emerging resistance or the occasionally visible expression of virus from slowly decaying infected cellular compartments in blood and tissue. Our genotypic analysis of the culturable virus showed no evidence of drug-resistant mutations (data not shown), a finding consistent with the lack of persistent measurable plasma viremia in this patient. Despite undetectable plasma HIV-1 RNA levels, subject 9 had both rising antibody titers to env and broadening of the CTL response, both unexpected findings. Virus-positive, CD8-depleted, PHA-stimulated CD4 cell cocultures and persistent HIV-1 RNA expression in biopsied tissue may in this case represent early escape from the therapeutic regimen. However, genotypic analysis of the cultured virus revealed wild type sequences that were more consistent with the virus emerging from cells latently infected prior to the initiation of therapy. Moreover, results from in situ hybridization studies of a tonsillar biopsy done 4 months after the observed increase in both antibody levels and the broadening of the CTL response revealed neither RNA expression nor FDC-trapped virus (data not shown). Levels of MS and US mRNA species as determined by PCR from this tonsil biopsy were below the level of detection (table 2), and the plasma HIV-1 RNA level also remained undetectable. Clearly, it is possible that a viral compartment persists somewhere in this patient, allowing sufficient HIV-1 replication to stimulate specific immune responses, but insufficient in quantity to allow detection by current methodologies. Alternatively, since subject 9 appeared to be as completely suppressed as subjects 2, 3, and 7 (described above), the immune phenomena observed may reflect viral persistence and expression without ongoing viral replication.

Cross-sectional measurements revealing low levels of US mRNA expression in the blood of apparently well-suppressed persons suggests the possibility of very low level ongoing viral replication. Alternatively, US mRNA may arise from activation of latently infected cells resulting in RNA expression without de novo infection of the susceptible cell population. In lymphoid tissue, detectable US mRNA may similarly arise from chronically infected long-lived cells or may represent low-level residual virions trapped on follicular dendritic cells. Given the presence of detectable proviral DNA in blood and tissue, it is likely that detectable US mRNA species arise in part from a pool of latently infected resting CD4 lymphocytes stochastically activated to produce virus, a portion of which may be infectious.

The size of the persistent latent pool harboring infectious provirus is indeed critical. Our inability to culture virus from $1.5 \times 10^7$ circulating CD8-depleted PHA-stimulated CD4 T cells suggests that the pool size of these latently infected resting
cells harboring infectious provirus may be $\approx 3 \times 10^4$. Veseran et al. [38] measured levels of proviral DNA from this cohort and estimated the average half-life of this pool as $\approx 3$ months (decay constant of $\approx 0.007$). Thus, on average, $\approx 210$ cells would be activated to produce infectious virus each day. Whether this low level of potential viral replication in vivo can be controlled in the absence of antiviral drugs by either HIV-specific immunologic factors (CTL or antibody) or nonspecific antiviral factors (chemokines, interleukins, soluble factors, or cytokines) is unknown. However, the persistence of proviral DNA in genital secretions and the unanswered question of viral persistence in the central nervous system, both immunologically privileged sites, raises additional concerns of the feasibility of immunologic control of viral replication in the absence of antiviral therapy.

The virologic and immunologic profiles observed in subject 6 after drug discontinuation are of particular interest. That there was a 4-month lag between drug cessation and clear viral rebound with an apparently drug-sensitive virus population is a provocative finding. Following drug discontinuation, a new equilibrium was established. After a lag period, the HIV RNA level climbed precipitously, only to drop again with reinstitution of the original regimen. Perhaps the early phase of limited viral replication reflects host factors, presumably immunologic, that initially substantially restrict viral replication. The rebound of a drug-sensitive virus population could have resulted from de novo mutation resulting in immunologic escape. Alternatively, the rebound viremia might represent the activation of latent virus from a cell infected before the initiation of therapy, producing a drug-sensitive yet antigenically distinct virus population.

The rapid rebound after the temporary discontinuation of antiviral therapy in subject 8 highlights the importance of the persistence of proviral DNA despite successful suppression of viral replication in peripheral blood and tissue (GALT) for $\approx 19$ months. Although control of viral replication was easily regained with the reintroduction of the same drug regimen, the rapidity of the rebound suggests that the multiple rounds of viral replication necessary to sustain significant levels of plasma HIV RNA were easily reignited, presumably from a population of cells infected prior to the initiation of the original therapy.

We observed that early combination antiretroviral therapy can result in arrest of the maturation, and subsequent decay, of HIV-1–specific immune responses, both humoral and cellular. We have documented reduction in both HIV-specific and -nonspecific B cell responses after initiation of antiretroviral therapy in cohorts of both newly and chronically HIV-infected persons [39]. Reduction in HIV-specific B cell activity is likely

Figure 4. Cytotoxic T lymphocyte precursor (CTLp) levels/10$^6$ peripheral blood mononuclear cells (PBMC) to HIV env (green), gag (blue), pol (yellow), and nef (red) in 6 subjects treated with antiretroviral therapy >12 months.
due to the reduction in viral antigen load. The observed reduction in hypergammaglobulinemia and the frequency of circulating IgG antigen–secreting cells associated with antiviral therapy may reflect reduction in cellular activation dependent on continuous HIV-1 replication, although this explanation remains hypothetical.

Evidence has accumulated that the activity of HIV-specific CTL is important in controlling HIV-1 replication. The resolution of high levels of viremia observed during primary infection is temporally associated with the appearance of HIV-specific CTL [8, 9]. We recently reported an inverse correlation between CTL activity and plasma HIV RNA in untreated HIV-1–infected subjects [40]. In a study of 33 persons with untreated primary HIV-1 infection, Musey et al. [41] reported the persistence of broad CTL activity in association with control of plasma viremia. In our treated subjects, we frequently observed the opposite phenomenon. Early pharmacologic control of viremia was generally associated with decreasing levels of CTL activity. We hypothesize that the reduction in these HIV-specific cellular responses results from a reduction in antigen load, below a level necessary to sustain immunologic stimulation at a maximal level. However, a direct inhibitory effect of antiviral therapy on CTL activity could not be ruled out. While these findings are encouraging in indicating the extent of virus suppression mediated by the therapy, it may be prudent to enhance HIV-specific immune responses with vaccination strategies before stopping the antiviral regimen. This patient population, that is, those treated early in infection, is ideal for such manipulations, since HIV-1–specific CD4 T helper activities are likely to be well-preserved [42]. What remains less clear is the most appropriate HIV-1 vaccine to use for effective immunization strategies [43].

In summary, combination therapy with multiple drugs targeting both HIV-1 RT and protease can potently and durably suppress HIV-1 replication in vivo. However, it is equally important to note that while this regimen was highly effective virologically, it was not well-tolerated. The use of antiretroviral agents in this setting must therefore include a consideration of issues surrounding patient adherence and tolerability. When applied early after infection, control of viral replication is associated with preservation of normal absolute CD4 cells, increases in the CD4-to-CD8 cell ratio, preservation of lymphoid architecture, and a dramatic reduction in HIV-1 in blood and tissue, along with waning HIV-1–specific immune responses. However, multiple lines of evidence indicate that replication-competent HIV-1 remains in persons whose infection has been suppressed for a prolonged period. This conclusion is consistent with recent reports that, after prolonged combination therapy, replication-competent HIV-1 remains detectable in PBMC [28–30], probably resident in resting CD4 T cells. Thus, the eradication of HIV-1 from infected persons will not be simple [44]. Nevertheless, this study provides virologic and immunologic insights to aid in planning future proof-of-concept clinical trials. New therapeutic approaches must also include strategies to immunologically activate resting CD4 T cells that latently harbor residual HIV-1. Also, it may be necessary to stimulate HIV-1–specific immunity with vaccine strategies before contemplating the withdrawal of antiviral therapy.

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