HIV-1 Drug Resistance Profiles in Children and Adults With Viral Load of <50 Copies/mL Receiving Combination Therapy

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Context The continued release of human immunodeficiency virus type 1 (HIV-1) into plasma at very low levels during highly active antiretroviral therapy (HAART) can be detected using specialized techniques, but the nature and significance of this low-level viremia, especially as related to acquisition of drug resistance mutations, are unclear.

Objective To determine genetic resistance profiles of low-level plasma HIV-1 in patients with prolonged viral suppression (<50 copies/mL of plasma HIV-1 RNA) while receiving HAART.

Design and Setting Cross-sectional study conducted at a US academic hospital from November 1999 to February 2001 using a novel method for amplification of low levels of viral genomes in plasma.

Patients Eighteen HIV-1–infected patients (7 children and 11 adults), enrolled in a longitudinal study of HIV-1 reservoirs, who had suppression of viral replication while receiving protease inhibitor–containing combination therapy. Two patients (1 adult and 1 child) with less optimal suppression of viral replication were included to assess virus predominating when plasma HIV-1 RNA levels are low but detectable (<1000 copies/mL). Follow-up analyses were conducted in 3 patients.

Main Outcome Measure Detection of drug resistance mutations in clones amplified from low-level plasma virus.

Results Viral sequences were amplified from 8 of the 18 patients with simultaneous plasma HIV-1 measurements of less than 50 copies/mL and from 2 patients with 231 and 50 copies/mL. Clones from 3 treatment-naive patients with less than 50 copies/mL of plasma HIV-1 RNA showed continued release, for as long as 42 months, of wild-type drug-sensitive virus. The 7 patients with prior nonsuppressive therapy, with viral loads below 50 copies/mL and during “blips” to 231 and 64 copies/mL, had only resistance mutations consistent with pre-HAART therapy (although reverse transcriptase inhibitor mutations may have continued to occur). New HAART-related mutations were seen in a control patient with prior viral load levels of about 400 to 1000 copies/mL. For phylogenetic analysis, sequences were available for both resting CD4+ T cells and plasma HIV for 7 of 10 patients and showed patient-specific clustering of sequences and a close relationship between virus in the plasma and the latent reservoir.

Conclusions Based on the samples that could be amplified, low-level viremia in children and adults receiving HAART with prolonged suppression of viremia to less than 50 copies/mL of HIV-1 RNA may result primarily from archival, pre-HAART virus, reflecting earlier treatment conditions, and does not appear to require development of new, HAART-selected mutations reflecting partial resistance to therapy. Low-level viremia below 50 copies/mL may represent less of a concern regarding impending drug failure of current HAART regimens. However, the archival drug-resistant virus may be relevant regarding future treatment strategies.
positive plasma HIV-1 RNA determinations (“blips”).12,16,17 The source of this low-level viremia in the setting of seemingly effective HAART remains unclear. In principle, it might result from the release of drug-sensitive virus from some source where the drugs cannot fully suppress replication or from the presence of virus that has acquired sufficient resistance to permit low-level replication in the presence of the drugs.

Understanding whether the continued release of virus into the plasma of patients with “undetectable” plasma HIV-1 RNA is associated with the gradual evolution of drug resistance is important in the design of treatment strategies. The acquisition of new drug resistance mutations in this setting would have major implications for the management of patients with low-level or intermittent viremia. Alternatively, stasis in the evolution of drug resistance despite several years of continuous treatment with protease inhibitor–containing regimens would suggest that in the optimal situation, responses to HAART might be very durable and limited principally by toxicity. The present study was designed to determine whether the low level of viremia that can be detected with specialized methods in patients receiving HAART reflects the presence of initial resistance mutations to one of the drugs in the current regimen (“initial” refers to the earliest mutations conferring some degree of resistance to a drug in the regimen, with breakthrough being prevented by the other drugs or by the lack of fitness of the virus having the initial mutation). Pioneering studies by Gunthard and colleagues8,9 have shown that resistance can develop when viremia is consistently in the low but detectable range (20-400 copies/mL). In addition, the plasma virus that initially appears during failure of lamivudine-containing combination therapy regimens has the M184V mutation.7,8 These results suggest that partially resistant virus might be responsible for the low level of ongoing virus production seen in patients taking suppressive HAART that ultimately might allow for the further development of drug resistance. An alternative explanation is that the low-level viremia simply reflects the release of archival drug-sensitive virus following the activation of latently infected resting CD4+ T cells, which are known to persist in infected adults and children despite suppression of viremia to undetectable levels with HAART for as long as 4 years.13,19,24

Determining the significance of the persistent low-level viremia seen in patients receiving HAART had been hampered by the failure of current methods of resistance testing to operate at plasma virus levels below about 1000 copies/mL. Thus, we developed a method for amplifying and sequencing the HIV-1 pol gene from the very small number of virus particles in the plasma of patients receiving HAART who have a plasma viral load of less than 50 copies/mL. To determine whether low-level viremia requires the development of drug resistance mutations, we used this method in a cross-sectional analysis of a group of patients who had prolonged suppression of viral replication with HAART and less than 50 copies/mL of HIV-1 RNA at the time of study.

**METHODS**

**Patients**

Adults and children infected with HIV-1 were eligible based on 2 criteria.

**Previous Study Participation.** The first criterion was participation in previous studies of latent reservoir resting CD4+ cells, which involved highly compliant patients receiving HAART. This allowed validation of sequences obtained from low-level plasma virus by comparison with latent reservoir sequences from the same patient. Patients were from 2 previously characterized cohorts receiving HAART: a cohort of HIV-1–infected adults (ages 34-54 years)20,21 and a cohort of children (ages 4-11 years)24 with perinatally acquired HIV-1 infection. The adult cohort was derived from a group of 22 individuals,21 of whom 19 were in the Baltimore, Md, area. Of the 19 individuals, 8 were included in sequencing analyses. Eleven were not studied because of treatment interruption (n=4), failed treatment (n=1), malignancy (n=1), relocation to a different state (n=1), and scheduled visit not coinciding with the November 1999 to February 2001 sampling period (n=4). Four individuals from a larger cohort23 were from the Baltimore area but not included because of lack of viral suppression. Those with residence elsewhere were not included in the study in part due to concerns about the quality of the plasma samples following shipping. There were 2 more individuals recently added to the latent reservoir study who were included (1 has been followed-up for more than 2 years, and the other for about a year) and 1 patient not in the latent reservoir study from the Johns Hopkins clinic population who met the criterion of long-term suppression. One adult patient having viremia (see below) from the Johns Hopkins clinic population was added for control purposes to assess the nature of virus present during blips. Thus, a total of 12 adults were studied. Of the pediatric cohort, all 8 individuals in the cohort24 were studied. One child did not have optimal suppression (described below) but was included for control purposes.

**Suppression of Replication.** The second criterion was prolonged suppression of viral replication with HAART for at least 12 months without failure defined as 2 consecutive plasma virus measurements >500 copies/mL. However, because the purpose of the study was to understand the nature of the low-level viremia that persists in patients receiving HAART, patients were not excluded for isolated blips. For comparison, 2 individuals (C11 and A57) who had several positive plasma HIV-1 RNA levels in the range of 400 to 1500 copies/mL were also studied. Repeat analyses were carried out on all the pediatric patients in the study (a program for analysis of samples from adults is being planned). In 3 children (C2, C12, and C22) for whom samples became available due to the timing of routine scheduled follow-up visits, on repeat analysis a mean of 5.2 months after the initial
analysis, amplification of viral sequences was achieved; the plasma virus level was less than 50 copies/mL at the time of sampling. In C22, viral sequences were amplified at a third time point during a blip to 64 copies/mL. There were 24 opportunities for sampling for the follow-up sequencing analyses at scheduled clinic visits (including cancelled or missed appointments) during the study period for the 8 children in the study. One parent refused consent for additional study. For the remaining 7 children, 16 samples were obtained and virus was amplified from 4 samples from 3 children. In the other 4 children amplification did not yield product for reasons described in the “Results” section. Inability to obtain samples was mostly due to missed clinic visits and intercurrent illness.

The mothers of the children included in the study did not receive zidovudine during pregnancy. Although patient C2 was born after neonatal post-exposure prophylaxis with zidovudine became standard care, it is unclear whether zidovudine was administered by the patient’s care providers.

Eligibility was not restricted with respect to the stage of disease at which therapy was started because good responses to therapy can be observed in compliant patients even when therapy is started at late stages of disease. Sampling was conducted a mean of 32.3 months (range, 16.7-49.0 months) after the initiation of HAART and coincided with routine clinic visits for children and for adults, with visits associated with the ongoing study of persistence of virus in the latent reservoir, occurring at intervals of about 3 to 6 months. Written informed consent approved by the institutional review board was obtained from adult subjects or from the parent or guardian of children.

**Plasma Viral Load Measurement**

The plasma HIV-1 RNA assays were carried out using the standard (detection limit, 200 and 400 copies/mL) and ultrasensitive (detection limit, 50 copies/mL) Roche Amplicor Monitor system (Roche Diagnostics, Nutley, NJ).

**HIV-1 Virion RNA Isolation and Amplification and Sequencing of HIV-1**

Virus particles were pelleted from up to 40 mL of plasma by ultracentrifugation as described by Dornadula et al. Isolated RNA was reverse transcribed, and the pol gene was PCR amplified using a nested version of a previously described pol amplification scheme with a proof-reading polymerase. Polymerase chain reaction products were cloned into PCR-BluntII-TOPO (Invitrogen Corp, Carlsbad, Calif) and sequenced using a fluorescent dideoxy terminator method of cycle sequencing on an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif) following manufacturer protocols.

**Sequence Validation and Statistical Considerations**

Sequence validation was carried out by recommended methods. Algorithms were used to distinguish PCR errors from polymorphisms and resistance mutations and to establish the independence of clones obtained from the same patient. Sequence changes at positions associated with drug resistance were considered polymorphisms if the treatment history was negative for drugs that select those changes or inconsistent with reported patterns of ordered accumulation of resistance mutations. Clones obtained from different PCR reactions were considered independent. Clones obtained from the same PCR reaction were only considered independent if they differed by drug resistance mutations, had 4 or more nonsynonymous differences, or had 2 or more synonymous differences. The latter 2 criteria were based on the predicted frequencies of PCR-induced mutation. Basic local alignment search tool (BLAST) searches of Genbank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html) revealed that none of the sequences matched laboratory strains or other patient isolates. Phylogenetic trees were inferred from nucleotide sequences using PAUP* version 4.0 (Sinauer Associates Inc, Sunderland, Mass). The HKY-85 model of evolution was used. All trees were constructed using the neighbor-joining method, and internal node support was verified using the bootstrap method with 1000 replicates. Trees were also inferred using 2 other models (maximum parsimony and maximum likelihood). The most parsimonious tree was sought using a heuristic search procedure with 100 random-addition sequence replicates and tree bisection-reconnection branch swapping. These sequences have been submitted to Genbank (accession Nos. AF383879 and AF383925). Plasma sequences have not been previously published. Previously published latent reservoir sequences were used to validate the patient-specific character of the plasma sequences presented here and to carry out a novel phylogenetic analysis of the relationship between plasma and latent reservoir sequences.

**RESULTS**

Analysis of low-level viremia was undertaken in 20 patients. The 18 patients with suppression of viral replication maintained suppression throughout the course of study, for a mean total time of 42.8 months (range, 28.7-54.0 months). The characteristics of and viral load data for the 8 patients whose samples could be amplified and the 2 controls with amplified samples (see below) are given in the TABLE and in FIGURE I. Most patients had plasma HIV-1 RNA levels that were consistently below the limit of detection of the ultrasensitive assay (50 copies/mL). Several patients had isolated positive readings in the range of 50 to 500 copies/mL. Four patients (A3, C12, A21, and C22) started therapy with late-stage disease and had 5- to 10-fold increases in CD4+ cell counts during HAART. Measurement of genotypic drug resistance via amplification and sequencing of HIV-1 was attempted in 12 adults and 8 children receiving HAART. Of the 10 adults from the latent reservoir cohort that were studied, 4 had samples that could be amplified (FIGURE 2 and FIGURE 3). A sample from the adult with
suppression not in the reservoir cohort could not be amplified. Amplification was achieved in the adult (A57) with suboptimal suppression (50 copies/mL). Of the 8 children there were 7 with viral suppression, and samples were amplified in 4 individuals. A sample from the 1 child (C11) with suboptimal suppression (231 copies/mL) was also amplified. In 3 children (C2, C12, and C22), repeat analyses were performed a mean of 5.2 months after the initial analysis involving amplification of viral sequences; the plasma virus level was less than 50 copies/mL at the time of sampling. In C22, viral sequences were amplified at a third time point during a blip to 64 copies/mL. We were unable to amplify viral sequences from 10 patients. This may reflect levels of viremia so low that no virus particles were present in the volume of plasma analyzed (approximately 3-40 mL), polymorphisms at primer binding sites, or other factors affecting the sensitivity of the assay, such as inhibitory factors for PCR present in the plasma. No patients were excluded from the original pediatric and adult cohorts for viral rebound. All of the patients except A57 continued to have suppression of viral replication on subsequent follow-up for a mean of 10.5 months, indicating that none of the patients had reversion to high levels of virus.

### Table

<table>
<thead>
<tr>
<th>Prior Therapy†</th>
<th>Patient‡</th>
<th>Current Age, y</th>
<th>Sex/Race</th>
<th>Prior Therapy Regimens (mo)</th>
<th>HAART Regimen (mo)</th>
<th>Total Time Receiving HAART, mo§</th>
<th>CD4⁺, cells/µL (%)</th>
<th>CD8⁺, cells/µL (%)</th>
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<tbody>
<tr>
<td>Group 1: None</td>
<td>C2</td>
<td>4</td>
<td>F/AA</td>
<td>None</td>
<td>Zidovudine-lamivudine-ritonavir</td>
<td>40.8</td>
<td>1900 (32)</td>
<td>1179 (25)</td>
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<td></td>
<td>A3</td>
<td>54</td>
<td>M/W</td>
<td>None</td>
<td>Zidovudine-lamivudine-ritonavir (27.1)</td>
<td>48.0</td>
<td>547 (34)</td>
<td>484 (30)</td>
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<tr>
<td></td>
<td>A11</td>
<td>42</td>
<td>M/W</td>
<td>None</td>
<td>Stavudine-ritonavir-saquinavir (16.8)</td>
<td>51.3</td>
<td>989 (39)</td>
<td>960 (38)</td>
</tr>
<tr>
<td>Group 2: RT inhibitors</td>
<td>C11</td>
<td>10</td>
<td>F/AA</td>
<td>Zidovudine (50.2) Zidovudine-didanosine (27.7)</td>
<td>Zidovudine-lamivudine-nelfinavir</td>
<td>32.0</td>
<td>1000 (32)</td>
<td>1129 (37)</td>
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<tr>
<td></td>
<td>C12</td>
<td>11</td>
<td>F/AA</td>
<td>Zidovudine (57.8) Zidovudine-lamivudine (22.3)</td>
<td>Stavudine-ritonavir</td>
<td>44.7</td>
<td>1457 (34)</td>
<td>847 (21)</td>
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<td>C15</td>
<td>11</td>
<td>M/AA</td>
<td>Zidovudine (72.2) Zidovudine-lamivudine (25.2) Didanosine-stavudine-delavirdine (1.9)</td>
<td>Zidovudine-lamivudine-nelfinavir-ritonavir</td>
<td>28.7</td>
<td>626 (21)</td>
<td>1183 (39)</td>
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<td>A21</td>
<td>53</td>
<td>M/W</td>
<td>Zidovudine (56.8) Stavudine (5.7)</td>
<td>Zidovudine-lamivudine-inclavir</td>
<td>59.0</td>
<td>787 (30)</td>
<td>1127 (44)</td>
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<td></td>
<td>C22</td>
<td>8</td>
<td>F/AA</td>
<td>Zidovudine (29.0) Zidovudine-lamivudine (6.9) Zidovudine-lamivudine-ritonavir (3.4) Zidovudine-lamivudine-nelfinavir (0.6)</td>
<td>Delavirdine-didanosine-ritonavir-saquinavir</td>
<td>30.7</td>
<td>1635 (34)</td>
<td>1536 (32)</td>
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<td>Group 3: Protease inhibitors</td>
<td>A9</td>
<td>34</td>
<td>M/AA</td>
<td>Ritonavir (15.3)</td>
<td>Ritonavir-zidovudine-lamivudine-saquinavir (31.9)</td>
<td>51.9</td>
<td>1339 (47)</td>
<td>743 (26)</td>
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<td>Group 4: RT and protease inhibitors</td>
<td>A57</td>
<td>54</td>
<td>F/AA</td>
<td>Zidovudine (3.0) Zidovudine-lamivudine (5.0) Zidovudine-lamivudine-saquinavir (2.0)</td>
<td>Zidovudine-lamivudine-ritonavir (15.2) Zidovudine-lamivudine-nelfinavir-efavirenz (9.2)</td>
<td>52.2</td>
<td>342 (23)</td>
<td>697 (46)</td>
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</table>

*HAART indicates highly active antiretroviral therapy; C, child; F, female; AA, African American; A, adult; M, male; W, white; and RT, reverse transcriptase.
†Prior therapy refers to treatment with single or multiple drug regimens that failed to suppress viral replication to undetectable levels prior to the initiation of suppressive HAART.
‡Patient ID numbers for adults and children are consistent with those used in earlier publications.19,20,22,29
§Total number of months of continuous treatment with the indicated HAART regimen(s) with effective suppression of viral replication.
¶Patient C22 received 2 different protease inhibitor-containing regimens but compliance with the protease inhibitor part of the regimen was poor prior to initiation of 4-drug HAART.
¶¶Had intensification with abacavir at 45.3 months following 2 positive HIV-1 plasma RNA measurements.
detection of low-level viremia in this study did not correspond to impending drug failure. Over this period there was a mean of 2.7 (median, 3.0) additional measurements of plasma HIV-1 RNA. For patient A57, addition of abacavir brought the viral load to less than 50 copies/mL.

Genotypic analysis of extremely low levels of plasma virus presents a number of problems. First, because the number of target molecules of viral RNA is so small, extensive amplification by PCR is required before cloning and sequencing. Under these conditions, contamination and PCR error become potential problems. We thus initially studied patients for whom we had previously determined pol sequences of virus in the latent reservoir in resting CD4+ cells.21,24,30 Sequences from both cells and plasma were available for 7 of the 10 patients (FIGURE 4 and FIGURE 5). This analysis showed patient-specific clustering of sequences from plasma and resting CD4+ cells, confirming the expected origin of the sequences and the close relationship between virus in the plasma and the latent reservoir. In each case, control PCR reactions set up without the initial reverse transcriptase (RT) step were negative, excluding contaminating DNA as a source for the amplified sequences. As described in the “Methods” section, algorithms were used to distinguish PCR errors from polymorphisms and resistance mutations. In addition, particular care was taken to establish the independence of clones obtained from the same patient. Clones from the same patient were considered independent only if they were derived from independent amplifications or had substantial sequence differences that could not be explained as PCR-induced mutations. A total of 47 independent plasma sequences were obtained from 10 patients.

A second critical issue is related to sampling. Because of the extremely low concentration of plasma virus in these patients, only small numbers of viral genomes can be obtained from individual patients. The clones studied represent a partial picture of the complex
Figure 2. HIV-1 Reverse Transcriptase Sequences Amplified From Plasma

HAART indicates highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; AZT, zidovudine; ddl, didanosine; 3TC, lamivudine; d4T, stavudine; ABC, abacavir; DLV, delavirdine; EFV, efavirenz; RTV, ritonavir; NFV, nefinavir; SQV, saquinavir; and IDV, indinavir. For protease drug resistance color codes, see Figure 3. Accessory mutation refers to a genetic variant that alone does not confer high-level resistance but can occur in association with resistance mutations and may contribute to resistance or viral fitness.

*Indicates insertion sequences.
viral quasi species in each patient. Although the absence of particular types of sequences in the samples studied cannot be used to rule out the existence of such sequences, the presence of particular types of sequences can be used to assemble a general picture of the virus present in the plasma in these patients. Of particular interest are general patterns that are consistent across a diverse patient population. Because of the low number of sequences obtained from each patient, conclusions were based on general patterns shared by all of the patients studied.

Of the 47 independent pol clones, 10 had no drug resistance mutations in either protease or the first 220 amino acids of RT, the region of RT reported to encode the major drug resistance mutations (Figures 2 and 3). Resistance mutations were absent in all 9 independent clones obtained from the 3 patients in the study who had had no prior therapy before starting HAART (Figures 2 and 3, Group 1). Interestingly, HAART regimens for all 3 patients included lamivudine, a drug for which a single nucleotide change at codon 184 in RT can confer a high degree of resistance.31,32 While the presence of low levels of drug-resistant virus in these patients cannot be excluded, the results demonstrate that wild-type virus sensitive to all 3 drugs in a HAART regimen continues to be released into the plasma as the dominant form in the circulating virus pool even in patients who have been receiving HAART for as long as 42 months with suppression of viral replication to less than 50 copies/mL. In patient C2, wild-type virus was detected again in the plasma at 34 months, 6 months after detection of wild-type virus in the initial analysis.

The remaining patients had sequences with drug resistance mutations (Figures 2 and 3). In 5 patients who had each had extensive prior nonsuppressive treatment with zidovudine and other RT inhibitors for as long as 8 years (Figures 2 and 3, Group 2), resistance mutations consistent with the prior therapy were found; although the history of prolonged zidovudine therapy with high levels of viremia strongly suggests that mutations were selected by prior therapy, we cannot definitively exclude the possibility that some RT inhibitor mutations were continuing to occur. Zidovudine resistance mutations...
were present in 27 of 32 independent clones obtained from these patients. The other 5 clones had the M184V mutation associated with lamivudine resistance. These were obtained from patient C22 who had received lamivudine for 19.7 months before starting HAART. Patient C15 also had the M184V, E44D, and V118I substitutions in RT that confer lamivudine resistance. These mutations were present in association with zidovudine resistance mutations. Patient C15 had been pretreated with zidovudine and lamivudine for 25.2 months before HAART and continues to receive these drugs as part of his HAART regimen. In addition, patient C15 also had 2 distinct viral clones with the K103N and Y181C substitutions that are selected by delavirdine and other non-nucleoside RT inhibitors (NNRTI). Prior to starting HAART, this patient received therapy with delavirdine for only 1.9 months. Despite 17 months of treatment with a HAART regimen that does not contain an NNRTI, drug-resistant viruses harboring the K103N and Y181C substitutions were still being released into the plasma. In patient C11, for whom pre-HAART sequences from replication-competent virus in the latent reservoir were available, zidovudine resistance mutations were demonstrated to exist in this compartment prior to the initiation of HAART.

Among all patients who were naive to protease inhibitors at the time HAART was initiated, no primary protease inhibitor resistance mutations were detected in the 41 independent clones analyzed (Figures 2 and 3, Groups 1 and 2). The “secondary” mutations observed in protease in patient C11 appear to represent natural polymorphisms rather than the development of new resistance to nelfinavir. Latent reservoir sequences obtained prior to the initiation of combination therapy with nelfinavir showed the L63P and A71T substitutions in protease. The V82A mutation was seen in clone 4.2. Although this mutation confers resistance to certain other protease inhibitors, it has not been observed in association with nelfinavir treatment in vivo and is associated with minimal resistance to nelfinavir in vitro. Similarly, patient C12 had an isolated substitution at position 84 in protease, which alone does not confer significant resistance to ritonavir. Further evidence for the ability of the HAART regimen to prevent accumulation of new mutations is provided by the durability of suppression of viral replication in settings in which the HAART regimen may be compromised. For 2 patients (C15 and A21), the HAART regimens included drugs that were part of previous nonsuppressive regimens and for which resistance mutations were present. In addition, in 2 other patients (C11 and C12), the effectiveness of the stavudine component of the HAART regimen may be compromised by preexisting zidovudine resistance mutations or the multinucleoside RT inhibitor insertion sequence at codon 69. Nevertheless, these...
patients maintained suppression of viral replication. In patient C12, virus with the T69SSG and SVT insertions was again detected at 42 months, 8 months after detection of the insertions in the initial analysis.

To summarize, patients who had prior nucleoside analogue therapy had protease sequences that were wild type and RT sequences with mutations selected by prior therapy (although, as mentioned previously, we cannot rule out continuing mutation representing resistance to RTIs in some cases). A reciprocal pattern was observed in a single patient (A9) who had received prior monotherapy with a protease inhibitor (Figures 2 and 3, Group 3). In this case, the RT sequences were all wild type; resistance was only seen in the protease gene. One of 4 plasma virus clones had the characteristic protease substitutions V82A, I54V, V77I, and L63P, which confer high-level resistance to ritonavir.38,41 Phylogenetic analysis (Figure 5) revealed that this clone coclustered with a group of ritonavir-resistant sequences isolated from the latent reservoir in resting CD4+ cells in this patient.30 Plasma clone 1.1 was most closely related to 2 clones obtained from the latent reservoir 32 months previously.30 These reservoir clones showed a 28-fold resistance to ritonavir on phenotypic analysis.30 Two other plasma clones had only the protease V82F substitution. These clones coclustered with a latent reservoir sequence obtained 21 months previously that had the V82F mutation and only negligible (1.1-fold) phenotypic resistance to ritonavir. Interestingly, the fourth plasma clone lacked any drug resistance mutations. Thus, despite the fact that the patient developed high-level ritonavir resistance 50 months previously and had continued taking ritonavir through the time of study, 3 of 4 clones isolated from the plasma lacked mutations associated with high-level ritonavir resistance.

In 3 of the 6 other patients from whom 1 or more sequences from the latent reservoir were available, the same commingling of plasma-derived sequences with the cellular sequences was observed (Figure 4), suggesting that the same sequences dominated both the plasma and archival latent virus pools. Because the archival latent pool was sampled years earlier than the plasma, continued evolution might have resulted in consistent temporal clustering. Instead, sequences obtained from temporally distinct plasma and cellular specimens from patients A9, C2, C12, and C22 commingled, and the branches that joined them were no longer than the branches linking sequences obtained contemporaneously.

In a single patient who had clearly documented prior nonsuppressive therapy with inhibitors of both RT and protease (Figures 2 and 3, Group 4), new mutations conferring resistance to drugs in the HAART regimen were detected. This patient (A57) differs from the majority of the patients in this study.

Figure 5. Phylogenetic Clustering of pol Sequences From Low-Level Plasma Virus in a Patient Receiving HAART.
in that she has had intermittent detectable viremia between 400 and 1000 copies/mL during HAART. New nefinnavir and ritonavir resistance mutations were detected at a time when her viral load was 50 copies/mL. This result confirms that drug resistance mutations can be detected when the viral load is as low as 50 copies/mL with the methods used here. Nevertheless, in the patients who had better suppression of viral replication, new resistance mutations clearly attributable to drugs in the HAART regimen were not detected as dominant in the low level of plasma virus that continues to be released during long-term HAART.

**COMMENT**

Although HAART can suppress plasma virus levels to below the limits of detection of currently available ultrasensitive assays in many patients, we show here that it is possible not only to detect but also to characterize virus that continues to be produced at low levels in such patients. Standard methods for the analysis of drug resistance require plasma HIV-1 RNA levels of greater than 1000 copies/mL. However, by the use of appropriate centrifugation and PCR methods, we were able to amplify and clone HIV-1 pol genes from 8 of 18 patients who had less than 50 copies/mL of HIV-1 RNA, permitting us to determine whether the low-level viremia present in patients on effective HAART regimens results from the development of partial drug resistance. We found that this low-level plasma virus was predominately wild type (drug sensitive) by genotypic analysis in the case of previously untreated individuals. In patients who had prior nonsuppressive therapy, both wild-type and archival drug-resistant viruses continued to enter the plasma. Overall, the sequences obtained from plasma were similar to those from virus that persists in the latent reservoir in resting CD4+ cells. We did not find evidence of the emergence in plasma of new resistance mutations clearly attributable to the HAART regimen. Thus, in patients receiving suppressive HAART, low-level virus production can occur over the course of several years without selection of resistance mutations to any of the drugs in the regimen. On the other hand, if resistant virus was selected by prior nonsuppressive therapy with particular drugs, there is continued production of this resistant virus whether or not the patient continues taking those drugs. These findings have important implications for the mechanism of viral replication and persistence during HAART, the durability of HAART regimens, and the concept of “recycling” drugs.

With respect to the mechanism of continued virus production during effective HAART, 2 general theories might be considered. The first involves continuous cycles of virus production and de novo infection of additional susceptible cells. This replication may occur in sanctuary sites not exposed to fully inhibitory concentrations of the drugs and may occur at such a low level that drug resistance mutations are not readily selected by the suboptimal drug concentrations. Alternatively, the plasma virus might derive from long-lived cells infected prior to the initiation of therapy, including latently infected CD4+ cells.

Several findings in the present study argue against continuous ongoing cycles of viral replication as the sole mechanism for the low-level plasma viremia detected during HAART. First, new resistance mutations were not found in the plasma virus, even under conditions that could readily select for such mutations and despite the fact that resistance can develop when the level of viremia is in the 20 to 400 copies/mL range. In all 24 sequences obtained from 7 lamivudine-naïve patients who took lamivudine as part of their HAART regimen, we did not detect the single-base M184V mutation. This mutation commonly appears as the initial resistance mutation in patients failing lamivudine-containing HAART regimens. We show here that low-level virus production can occur over several years without selection of this initial drug resistance mutation and with continued antiretroviral suppression. Second, consistent with the absence of new lamivudine resistance mutations in the low-level plasma virus was the absence of any other dominant mutations conferring resistance to the protease inhibitors in the HAART regimen, even in patients whose prior therapy had selected mutations that might be expected to diminish the effectiveness of the HAART regimen. In 2 patients who had extensive prior therapy with zidovudine and who continued taking it during HAART (patients C15 and A21), the detection of zidovudine-resistant mutants in the plasma did not represent impending drug failure. One might expect that continuous rounds of replication would allow this zidovudine-resistant virus to acquire additional mutations resulting in the loss of virologic suppression, but this was not observed. Similarly, the S1 insertion at codon 69 of RT, which is known to confer high-level resistance to all nucleoside RT inhibitors, was detected in the plasma of 1 individual (C12) who continues to have suppression of viral replication with a 2-drug regimen of stavudine and ritonavir.

It is important to emphasize that the sampling of a limited set of sequences from a small number of patients does not allow us to exclude ongoing viral evolution in patients who have sustained suppression of viral replication with HAART. It is possible that virus with new resistance mutations will eventually arise in some patients with sustained suppression. In studies of virus in the cellular reservoirs, evolution in env sequences has been noted in a subset of patients receiving HAART. New drug resistance mutations can clearly arise in cases when the level of viremia rises into the detectable range. However, the consistent pattern of no new HAART-selected mutations in the patients studied here strongly suggests that ongoing release of virus in patients with less than 50 copies/mL of HIV-1 RNA does not require partial drug resistance to current regimens. Our results also indicate that this low-level viremia does not necessarily lead to dominant populations with partial drug resistance over the course of 3 to 4 years in all patients. This conclusion is strengthened by the fact that our cohort was not limited to patients who have optimal sup-
pression of viremia. Like many patients receiving HAART, several of our patients had had prior blips, and yet no virus with new resistance mutations was detected in the plasma. Also, in those patients for whom amplification could not be accomplished, it is possible that significant mutations may have occurred; however, the lack of viral breakthrough would suggest that this may be less likely.

Another plausible mechanism for the continued production of low-level plasma virus in the setting of effective HAART is the intermittent release of virus from long-lived compartments such as the latent reservoir in resting CD4+ cells. Previous studies have shown that latently infected CD4+ cells harboring replication-competent virus persist in these patients despite prolonged HAART. Recent studies have found that in some patients there are striking similarities in the env gene between the rebound virus detected following discontinuation of HAART and those recovered from latently infected resting CD4+ cells or those present prior to HAART. In our study, the virus obtained from the plasma showed a close phylogenetic relationship to virus found in the latent reservoir, with substantial intermingling of plasma and latent reservoir sequences from individual patients. When substantial numbers of latent reservoir sequences were available, the intermingling of plasma and latent reservoir sequences was especially apparent, as in patient A9 (Figure 5). In addition, the presence in the plasma of virus that should be strongly selected against or outcompeted in the current treatment setting is suggestive of release from the latent reservoir. One striking example is the persistence of wild-type virus in the plasma of patient A9 coexisting with virus having previously selected ritonavir-resistant mutations in the setting of continued ritonavir treatment. Both forms persisted in the latent reservoir in this patient. Among virus cultured from the cellular latent reservoir of HIV-1 in this patient, small minority subpopulations with an additional L90M mutation were transiently noted. These viruses could have been selected by prior ritonavir monotherapy or may have been selected by the saquinavir component of the HAART regimen, and could represent an issue of sampling a complex archive. In any event, these viruses did not become dominant over time. Taken together, these results suggest random release of archival virus rather than a continuous linear evolutionary process resulting in increasingly resistant virus. Likewise, in other patients, virus with mutations conferring resistance to drugs that were part of failed regimens continued to enter the plasma for at least 1 to 2 years after discontinuation of the drugs. While small effective population size may limit competitive effects, strong selection at drug resistance sites may overcome small population effects.

In conclusion, we were able to amplify and characterize the virus present in the plasma at extremely low levels (<50 copies/mL) in patients treated with prolonged HAART. This virus lacked new resistance mutations clearly attributable to selection under HAART. In heavily pretreated patients, virus with drug resistance mutations possibly attributable to the prior therapy was found in the plasma but was not indicative of impending drug failure. However, the persistence of this “archival” drug-resistant virus, even in the absence of continued treatment with the relevant drug, argues against the idea of recycling drugs that were part of prior non-suppressive regimens if loss of suppression should occur during long-term HAART. In treatment-naive patients receiving HAART, only wild-type virus was found, demonstrating that low levels of virus production can occur over several years without the selection of increasingly drug-resistant virus. One likely source of this low-level plasma viremia is the latent reservoir in resting CD4+ cells. The data are less consistent with the idea that this viremia reflects continuous cycles of de novo infection of susceptible cells. Perhaps the most plausible explanation is intermittent release from the latent reservoir or some localized drug sanctuary site with only limited additional replication at a level that is insufficient to generate new resistance mutations. Developing approaches for eliminating the source of this residual viremia is clearly a prerequisite to virus eradication.

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