Intermittent HIV-1 Viremia (Blips) and Drug Resistance in Patients Receiving HAART

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TREATMENT OF HUMAN IMMUNODEFICIENCY virus type 1 (HIV-1) infection with highly active antiretroviral therapy (HAART) can suppress viremia to below the limit of detection of available clinical assays. The current goal of antiretroviral therapy is suppression of viremia to below 50 copies/mL of HIV-1 RNA, the limit of detection of the most sensitive available clinical assay. Suppression to this level is necessary to prevent drug resistance, the major cause of treatment failure. After achieving suppression, many patients experience intermittent episodes of detectable viremia (“blips”). Blips may raise concern that resistance is developing and complicate management by increasing patient anxiety, triggering costly repeat measurements of viral load, and generating uncertainty regarding drug resistance.

Context Many patients infected with human immunodeficiency virus type 1 (HIV-1) and receiving highly active antiretroviral therapy experience intermittent episodes of detectable viremia (“blips”), which may raise concerns about drug resistance, lead to costly repeat measurements of viral RNA, and sometimes trigger alterations in therapy.

Objective To test the hypothesis that blips represent random biological and statistical variation around mean steady-state HIV-1 RNA levels slightly below 50 copies/mL rather than biologically significant elevations in viremia.

Design, Setting, and Patients Between June 19, 2003, and February 9, 2004, patients receiving therapy underwent intensive sampling (every 2-3 days) over 3 to 4 months to define the frequency, magnitude, and duration of blips and their association with drug levels and other clinical variables. Blips were defined as HIV-1 RNA measurements greater than or equal to 50 copies/mL preceded and followed by measurements less than 50 copies/mL without a change in treatment. To determine whether blips result from or lead to drug resistance, an ultrasensitive genotyping assay was used to detect drug resistance mutations before, during, and after blips. Patients were 10 HIV-1–infected asymptomatic adults recruited by clinicians and followed up in the Moore Clinic at the Johns Hopkins Hospital. Patients had suppression of viremia to below 50 copies/mL while receiving a stable antiretroviral regimen for 6 months or longer.

Main Outcome Measures At each time point, plasma HIV-1 RNA levels were measured in 2 independent laboratories and drug resistance mutations were analyzed by clonal sequencing.

Results With the intensive sampling, blips were detected in 9 of 10 patients. Statistical analysis was consistent with random assay variation around a mean viral load below 50 copies/mL. Blips were not concordant on independent testing and had a short duration (median, <3 days) and low magnitude (median, 79 copies/mL). Blip frequency was not associated with demographic, clinical, or treatment variables. Blips did not occur in relation to illness, vaccination, or directly measured antiretroviral drug concentrations. Blips were marginally associated (P = .08) with reported episodes of nonadherence. Most importantly, in approximately 1000 independent clones sequenced for both protease and reverse transcriptase, no new resistance mutations were seen before, during, or shortly after blips.

Conclusion Most blips in this population appear to represent random biological and statistical variation around mean HIV-1 levels below 50 copies/mL rather than clinically significant elevations in viremia.
INTERMITTENT HIV-1 VIREMIA (BLIPS) AND DRUG RESISTANCE

garding whether therapy should be altered.

The nature and significance of blips are unclear. Blips may result from transiently reduced drug concentrations (due to suboptimal adherence, decreased bioavailability, or increased clearance). They may also result from heightened immune activation, as during vaccination or illness. Work by Havlir et al and subsequent studies by others have suggested that isolated blips are not associated with virological failure. However, blips have been associated with an increased risk of clinical failure, viral evolution, and slower decay of viral reservoirs. Of greatest concern are reports of the evolution of drug resistance during blips.

Dornadula et al provided insights into the nature of blips by documenting continuous release of virions into the plasma even in patients receiving HAART who had plasma virus levels below the limit of detection of clinical assays. Using special methods, they found a mean level of 15 to 20 copies/mL in these patients. Confirmation of ongoing virus production came from direct cloning and genotyping of this low-level plasma virus, which was shown to be generally devoid of new resistance mutations. Whether this low-level viremia reflects release of virus from stable reservoirs, continuing cycles of ongoing replication, or both is unclear. Regardless of the mechanism, it is clear that HAART suppresses viremia to a new steady state slightly below the clinical limit of detection.

Given that effective HAART regimens produce steady-state viremia slightly below 50 copies/mL, we hypothesized that most blips represent random biological and statistical variation around these steady-state levels rather than biologically significant elevations in viremia. This hypothesis involves several predictions: (1) Blip frequency will depend on the steady-state level in a given patient. (2) The number of blips detected will depend on the number of measurements made. (3) For steady-state levels at or below 20 copies/mL, paired measurements on the same sample will rarely be concordantly positive, so that blips will not be reproducible. (4) Similarly, consecutive measurements will rarely be both positive, so that the apparent duration of blips will be short. (5) The magnitude of blips will be low and clustered toward the detection limit since they represent fluctuations around a mean below this limit. (6) Blips will not generally correlate with adherence, drug levels, or intercurrent illnesses. (7) Most importantly, genotypic analysis during and after a blip will generally show no new resistance mutations.

To determine the clinical significance of blips, we tested these predictions in a prospective study in which 10 patients with suppression of viremia while receiving HAART underwent more intensive sampling than has been used in previous studies. At each time point, plasma virus and antiretroviral drug levels were determined, and genotypic resistance was analyzed using a method more sensitive than commercial assays. The goal of the study was to provide new insights into the nature and clinical significance of blips.

METHODS

Patient Population and Study Design

Between June 19, 2003, and February 9, 2004, we studied asymptomatic HIV-1–infected adults who had achieved suppression of viremia to below 50 copies/mL while receiving a stable HAART regimen for 6 months or longer and who were willing and able to make regular study visits required for the study. A total of 13 patients were recruited by Moore Clinic clinicians at the Johns Hopkins Hospital, where the patients were followed up. Three patients subsequently decided not to participate, either because of the number of visits required or frequent blood draws. Data from these 3 patients were not included in the study. Prior antiretroviral exposure from the time of diagnosis to study entry was ascertained by chart review and patient interview. Patients were not excluded for a history of blips. Blips were defined as HIV-1 RNA measurements greater than or equal to 50 copies/mL preceded by measurements below 50 copies/mL and followed by a return to below 50 copies/mL without a change in treatment. Volunteers donated 100 mL of blood for genotyping of the virus in the plasma and in the cellular reservoir in resting CD4 cells. Beginning 1 month thereafter, participants donated 17 mL of blood 3 times weekly (Monday, Wednesday, and Friday) for 36 total study visits. Due to holidays, most participants required more time than the minimum 12 weeks to complete the 36 visits, with a mean of 3.3 gaps in consecutive study visits per patient. However, in all cases the 36 visits were completed by 127 days (mean, 99.4 days).

The protocol was approved by a Johns Hopkins institutional review board. If participants expressed interest in the study and provided consent to their clinician, they were contacted by a study investigator (R.E.N.) who discussed the study design with the patient. If patients decided to participate, they were provided with a written combined institutional review board/Health Insurance Portability and Accountability Act informed consent form approved by the Johns Hopkins University institutional review board. The features of the study were then explained to the patient by an investigator (R.E.N.). Written informed consent was obtained for all aspects of the study involving blood drawing and analysis of personal health information from the patients or their medical records. Because the patients are not identified in the study herein, written informed consent for publication in a medical journal was not specifically obtained. However, the consent form did indicate that health information obtained in the course of the study would be used and/or given out as needed after the study to develop new strategies for fighting HIV infection. These patients were included in other reports having different research questions.
cians caring for the patients. Three participants were white and 7 were black. Race/ethnicity was used in the study herein for description of the study cohort and to determine whether blip frequency was greater in any particular racial/ethnic category.

Plasma HIV-1 RNA Quantification

Viral RNA was quantified using the ultrasensitive Roche Amplicor Monitor System version 1.5 assay (Roche Molecular Systems Inc, Blanchburg, NJ), which has a lower limit of quantification of 50 copies/mL. Assays were performed in 2 independent laboratories: a Clinical Laboratory Improvement Amendments–certified laboratory that performs this measurement for clinical management of a large patient population and a research laboratory that has performed this assay for numerous studies.\(^{24,34-37}\) Recommended negative, low, and high RNA controls, as well as quantitation standards (Roche Amplicor Monitor System version 1.5), were included in each assay. There were no false-positive results on HIV-negative plasma in either laboratory. In general, study visits for each patient occurred at the same time of day (typically early morning), thus likely avoiding any effects of diurnal variation. There is little evidence that diurnal variation has any significant role in viral load. Diurnal variation in viral load has been evaluated in 2 reports\(^ {38,39}\) and was not identified in either study.

Amplification and Sequencing the pol Gene From Plasma and Reservoir Virus

To allow consistent amplification and sequencing of the small number of viral genomes present in the plasma of patients receiving suppressive HAART regimens, plasma virus was first pelleted by ultracentrifugation; reverse transcriptase (RT) and protease genes were then separately analyzed by RT polymerase chain reaction (PCR), cloning, and sequencing using a previously described ultrasensitive genotyping method.\(^ {25,26}\) Briefly, blood was collected using an acid-citrate-dextrose anticoagulant, and the plasma was separated on a Ficoll gradient and filtered or spun to remove any contaminating cells. Plasma was then ultracentrifuged at 25 200g for 2 hours at 4°C to pellet the virions. The virions were lysed under denaturing conditions, and the viral RNA was isolated using a standard commercial silica-gel membrane–binding method (QIAamp Viral RNA MiniKit; Qiagen, Valencia, Calif). The isolated RNA was treated with DNase I to ensure that amplified HIV-1 sequences were derived from viral RNA and not from contaminating DNA. A complementary DNA fragment was synthesized from viral RNA and amplified separately for both the protease and RT segments of the pol gene using a commercial 1-step reverse transcriptase PCR (Superscript II RNase H-Reverse Transcriptase with High Fidelity Platinum Taq DNA Polymerase; Invitrogen Corp, Carlsbad, Calif). The first PCR product was then diluted 1:4 and used in a nested reaction. The DNA products from the outer PCR reaction were amplified using 0.5 units of High Fidelity Platinum Taq DNA polymerase. The DNA from this PCR reaction was then separated on a 1.5% agarose gel, purified using the QIAquick Gel Extraction Kit, and was not a visit in which a blip occurred. This sequence was only 1 transition mutation away from the hub of the network. Genetic diversity at each time point (theta) was assessed using a rank-sum test according to the methods of Rozas et al.\(^ {46}\) Sequences were analyzed for drug resistant mutations using the Los Alamos\(^ {47}\) and Stanford\(^ {48}\) databases.

Drug Concentration Monitoring

At each time point, random drug levels were obtained for the protease and nonnucleoside RT inhibitors using modified, validated, high-performance liquid chromatography methods.\(^ {49,50}\) For lopinavir, ritonavir, and saquinavir, the mobile phase was 0.1% trifluoroacetic acid, acetonitrile, and methanol (53:42.5). Analytes were separated isocratically followed by a step gradient wash at 30°C using a reverse-phase Beckman C18 column and
detected at 220 nm (lopinavir) and at 239 nm (ritonavir and saquinavir). The internal standard, A-86093.0, was supplied by Abbott Laboratories (Abbott Park, Ill). Calibration standards ranged from 100 to 15000 ng/mL for lopinavir and ritonavir and from 87 to 13 121 ng/mL for saquinavir. For nelfinavir, the nelfinavir active metabolite M8, and efavirenz, plasma proteins were precipitated with acetonitrile and the supernatant was dried. M8 levels were measured because M8 is the major metabolite of nelfinavir and has equivalent virological activity. Samples were dissolved in mobile phase, applied to a C18 reverse-phase column at 30°C, separated isocratically in 0.1% trifluoroacetic acid (pH 5.0):acetonitrile: methanol (47:48:5), and detected at 253 nm. Calibration standards ranged from 100 to 20000 ng/mL. For nevirapine, plasma samples were applied to a Waters HLB reverse-phase cartridge, washed with an acid/base series, eluted, dried, and dissolved in mobile phase (63% 25 mM phosphate buffer [pH 6.0] and 5.2 mM 1-butanesulfonic acid, 21.5% methanol and 15.5% acetonitrile). Analytes were separated isocratically followed by a step gradient wash at 30°C using a reverse-phase Supelco LC-8 column and detected at 280 nm. Calibration standards ranged from 25 to 10000 ng/mL. For all assays, quality control samples were interspersed between unknown samples. Mean correlation coefficients for calibration curves were greater than 0.998 (SD, 0.001). Although diurnal variation in antiretroviral drug levels has been reported, plasma drug levels should be most greatly affected by the time of last dose, the dosing interval, differential absorption rates, and individual drug pharmacodynamics.

**Patient Questionnaires**

At each study visit, patients completed a questionnaire recording adherence, the date and time of the last dose of each antiretroviral drug, recent vaccination, and physical illness. No participant changed antiretroviral drugs during the course of the study.

**Statistical Analysis**

More than 99% of the expected viral load data were successfully collected. A total of 720 viral load measurements were expected (36 time points × 2 independent measures × 10 patients). Of these, 713 were collected (99.03%). For 5 of the missing viral load measurements, the other independent assay at the same time point was successfully completed, and this value was included in the study. At 1 time point, for patient 113, viral load measurements were missing from both laboratories because venipuncture could not be performed at that visit. All viral load calculations were made using 713 measurements as the denominator. More than 98% (98.33%) of the planned assays for plasma drug concentration were completed. The time points for which drug concentration data were missing were excluded from all analyses of the correlation between drug levels and blips.

Continuous variables were summarized as medians and ranges, and group comparisons were made using the Wilcoxon rank-sum test. Dichotomous data were summarized as frequencies and proportions, and groups were compared using the Fisher exact test. The agreement between laboratories assaying the same sample was calculated using the κ statistic.

The proportion of predicted RNA measurements greater than or equal to a specified level (eg, 50 copies/mL) was estimated by the probability of obtaining measurements above that level. We assumed that the distribution of the RNA measurements was normal on the log_{10} scale, and the SD was based on the coefficient of variation (CV) for 25 copies/mL (90%), the lowest viral RNA measure for which a CV was reported in assay validation. The CV supplied by the assay package insert was not based on the log values. However, the CV equates to the SD on the natural log scale and was converted to the log_{10} scale by dividing by 2.3026, resulting in the SD of 0.3909. This SD was slightly greater than the SD of 0.25 calculated on the log_{10} scale for 50 copies/mL, which is the lowest level reported in the company’s earlier version of the assay (Amplicor 1.0). In assessment of predicted blip characteristics (assuming a constant viral load of 10, 20, or 30 copies/mL, distribution of viral load measurements as normal on the log_{10} scale, and independence of all assays), the same SD was used for all 3 levels. Because of the assumptions made on the distribution of the viral loads and the reliance on the estimate of the CV from the package insert, we did not perform formal comparisons of the distributions of the observed and predicted blip sizes. Drug concentrations were compared with therapeutic levels and dichotomized to any subtherapeutic levels vs all therapeutic levels at each time point. These were cross-tabulated with viral load (detectable vs not detectable) at the same time point. χ² analysis was used to test for the association of blips with subtherapeutic drug levels.

Analyses were performed using SAS version 9.0 (SAS Institute Inc, Cary, NC). All reported P values are 2-sided, and P<.05 was considered significant. A 1-tailed test would be more conservative when reporting negative results; however, for the analyses herein, 2-tailed tests were preferred to limit type 1 error that can occur when a number of tests are being performed. For the association of drug concentration and blips, the 2-tailed P value was .22 and would thus be .11 for a 1-tailed test. The only 2-tailed P value between .05 and .10, for which the significance would be affected by performing a 1-tailed test, is for the association of a blip within 7 days after self-reported nonadherence. For this comparison, the P value of .08 is reported herein and is considered marginally significant.

**RESULTS**

**Patient Demographics and Treatment Histories**

To analyze blips, we studied 10 patients receiving HAART who had stable suppression of viremia to below 50 copies/mL (Table 1). The patients ranged in age from 39 to 59 years. Seven were men and 3 were women; 7 were black and 3 were white. The study was carried out between June 19, 2003, and
February 9, 2004. Most patients had started HAART after reaching low CD4 nadirs with high viral RNA levels. Some had received prior nonsuppressive antiretroviral therapy, but all were eventually started on a HAART regimen that produced prolonged suppression of viremia to below 50 copies/ml (median, 34 months; range, 11-79 months). With their current regimens, 4 patients had blips detected during routine clinical care prior to entry, and there were 6 total blips out of 125 prior viral RNA measurements (4.8%). These blips were detected a mean of 15.7 months (range, 2-30 months) before study entry, and in all cases viral loads returned to below 50 copies/ml without a change in therapy. The median prior blip magnitude was 94 copies/ml (range, 61-108). In these patients, viral load measurements were generally performed every 3 months as part of routine clinical care and were not influenced at all by participation in the study because these viral load measurements predated study participation.

**Blip Frequency and Dynamics**

To capture as many blips as possible, we obtained plasma samples every 2 to 3 days for 3 to 4 months for duplicate HIV-1 RNA measurements in 2 independent laboratories. The higher value was used because any value above 50 copies/ml may be considered a blip by clinicians and we wanted to capture as many blips as possible. All patients completed 36 study visits. Patients were permitted to take breaks from the study protocol when the General Clinical Research Center was closed for holidays and when patients requested time for out-of-town travel. In general, we attempted to gather data in a “3-visit” or “weekly” clustered fashion to minimize isolated study visits. For study conclusions, it was considered more important to have a large number of study visits clustered within a relatively short time vs having strictly consecutive study visits. The mean and median times to completion of the 36 study visits were 99.4 days and 97.5 days, respectively (range, 88-127 days). The minimum possible time to complete the study was 82 days. Mean and median numbers of gaps in consecutive study visits were 3.3 and 3, respectively.

Blips were detected in 9 of 10 patients (Figure 1 and Table 1). Of 713 viral RNA measurements, 26 (3.6%) were above 50 copies/ml. Together these constituted 18 total blips, with consecutive positive measurements counted as a single blip. Patients experienced a median of 2 blips (range, 0-5). The observed proportion of positive assay results was consistent with random variation around a mean level of 10 to 20 copies/ml (Table 2). Nine blips were detected by one laboratory, 8 by the other laboratory, and 1 by both. Thus, although there was no difference in the sensitivity of assays used by the 2 laboratories, concordance was poor (k=+4.4%). This result is expected if blips result from random variation around a mean substantially below 50 copies/ml.

Frequent sampling also allowed us to better estimate the true duration and magnitude of blips. Fifteen of 18 blips represented isolated measurements above 50 copies/ml, with the subsequent measurement negative. Thus, the typical blip was brief (median duration, 2.5 days; range, 2-11.5 days). Only 1 patient (patient 154) experienced blips that persisted for more than 1 consecutive study visit. Blips were low in magnitude (median, 79 copies/ml; range, 51-201 copies/ml), with a clustering of val-

### Table 1. Clinical Characteristics, Current Antiretroviral Regimens, and Blip Frequencies

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>CD4 Cell Count</th>
<th>Duration of HIV-1 Infection</th>
<th>Maximum Pretreatment Viral Load</th>
<th>CD4 Cell Count at Enrollment</th>
<th>Current Antiretroviral Regimen</th>
<th>Duration of Viral Suppression (mo)</th>
<th>No. of Prior Blips/Plasma Viral Load Measurements (% Positive)</th>
<th>No. of Blips During Study (Viral Load, copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99</td>
<td>1</td>
<td>64</td>
<td>&gt;750,000</td>
<td>451</td>
<td>d4T, 3TC, TDF, LPV</td>
<td>15</td>
<td>2/9 (22)</td>
<td>2 (80, 105)</td>
</tr>
<tr>
<td>113</td>
<td>4</td>
<td>61</td>
<td>152,000</td>
<td>306</td>
<td>3TC, ABC, NFV</td>
<td>36</td>
<td>1/8 (13)</td>
<td>2 (63, 91)</td>
</tr>
<tr>
<td>134</td>
<td>129</td>
<td>117</td>
<td>457,000</td>
<td>394</td>
<td>3TC, TDF, EFV</td>
<td>11</td>
<td>0/6 (0)</td>
<td>1 (56)</td>
</tr>
<tr>
<td>135</td>
<td>65</td>
<td>170</td>
<td>548,000</td>
<td>445</td>
<td>AZT, 3TC, SQV, RTV</td>
<td>79</td>
<td>0/27 (0)</td>
<td>2 (58, 61)</td>
</tr>
<tr>
<td>136</td>
<td>Unavailable</td>
<td>49</td>
<td>Unavailable</td>
<td>696</td>
<td>AZT, 3TC, EFV</td>
<td>39</td>
<td>0/15 (0)</td>
<td>2 (81, 201)</td>
</tr>
<tr>
<td>139</td>
<td>7</td>
<td>97</td>
<td>Unavailable</td>
<td>475</td>
<td>EFV, APV, LPV</td>
<td>33</td>
<td>2/14 (14)</td>
<td>2 (51, 78)</td>
</tr>
<tr>
<td>140</td>
<td>6</td>
<td>79</td>
<td>431,000</td>
<td>433</td>
<td>AZT, 3TC, NFV</td>
<td>69</td>
<td>0/23 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>147</td>
<td>23</td>
<td>148</td>
<td>&gt;750,000</td>
<td>502</td>
<td>TDF, EFV, SQV, RTV</td>
<td>11</td>
<td>1/4 (25)</td>
<td>1 (81)</td>
</tr>
<tr>
<td>154</td>
<td>18</td>
<td>208</td>
<td>645,000</td>
<td>167</td>
<td>AZT, 3TC, ABC, TDF, LPV</td>
<td>17</td>
<td>0/7 (0) (68/144/86, 110/58/69/53/69, 67/62, 64, 134)</td>
<td>2 (79)</td>
</tr>
</tbody>
</table>

**Abbreviations:** ABC, abacavir; APV, amprenavir; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; HIV-1, human immunodeficiency virus type 1; LPV, lopinavir; NA, not applicable; NFV, nelfinavir; NVP, nevirapine; RTV, ritonavir; SQV, saquinavir; TDF, tenofovirdisoproxil fumarate; 3TC, lamivudine.

*Viral load measurements at time of acute HIV infection excluded.
†On the current regimen of highly active antiretroviral therapy.
‡A blip was defined as a viral load ≥50 copies/ml preceded and followed by measurements <50 copies/ml, without a change in therapy. For duplicate measurements, the higher value was used in this analysis. Consecutive positive measurements were counted as a single blip.
§Value separated by slashes indicate consecutive positive measurements within each blip.
||Mean value shown (median of 0 blips/10.5 viral load measurements).
Plasma human immunodeficiency virus 1 (HIV-1) RNA levels, plasma concentrations of protease and nonnucleoside reverse transcriptase inhibitors, and suggested minimum target trough concentration (as provided by drug manufacturers), for each of the 10 patients. Data markers below the black dotted line indicate undetectable levels of plasma HIV-1 RNA (<50 copies/mL). Patients were sampled 36 times. For each sample, viral RNA was measured in 2 independent laboratories. For each time point, the higher value was plotted. A total of 720 viral load measurements were expected (36 time points × 2 independent measurements × 10 patients). Of these, 713 were collected (99.03%). Of planned assays for plasma drug concentration, 98.33% were completed. M8 is the measurable active metabolite of nelfinavir. EFV indicates efavirenz; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; RTV, ritonavir; SQV, saquinavir.
ues toward the 50 copies/mL limit and only 1 value above 200 copies/mL. Brief blips of low magnitude are also consistent with the random-variation hypothesis.

**Association of Blips With Immune Activation, Adherence, and Drug Concentrations**

Blip frequency was not associated with demographic parameters such as sex, race, and age. There was no association with clinical parameters such as CD4 cell nadir, CD4 cell count at entry, pretreatment viral load, duration of infection, duration of virological suppression, and number of prior blips. Blips were not associated with therapeutic variables such as the number of drugs in the current regimen (Table 3). Blips were not observed with intercurrent illnesses (pharyngitis/sinusitis, cold/upper respiratory tract infection, gout flare, oral herpes outbreak, or gastrointestinal tract upset), or influenza vaccination (given during the study to 9 of 10 patients). Blips were marginally (P = .08) associated with patient-reported nonadherence (Table 4).

To determine whether blips were temporally associated with decreased drug concentrations, the protease

<p>| Table 2. Comparison of Observed Blip Characteristics With Predicted Characteristics Assuming Random Variation Around a Mean Plasma HIV-1 RNA Level of 10 to 30 Copies/mL |
|----------------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>10 Copies/mL</th>
<th>20 Copies/mL</th>
<th>30 Copies/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of viral load measurements</td>
<td></td>
<td>.036†</td>
<td>.035</td>
</tr>
<tr>
<td>≥50 copies/mL (blips)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concordance between independent assays</td>
<td></td>
<td>.077</td>
<td>.035</td>
</tr>
<tr>
<td>on a given sample‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blip duration§</td>
<td>.17</td>
<td>.07</td>
<td>.30</td>
</tr>
<tr>
<td>Blip magnitude (proportion &gt;200%)</td>
<td>.038</td>
<td>.011</td>
<td>.036</td>
</tr>
</tbody>
</table>

*Predicted blip characteristics assuming a constant viral load of 10, 20, or 30 copies/mL and that the distribution of viral load measurements is normal on the log_{10} scale and that all assays are independent. The reported coefficient of variation of the assay was converted to the SD of 0.3909 (see "Methods" section). The same SD was used for all 3 viral load levels. Because we were not able to measure viral load level values of <50 copies/mL, we would not be able to assess whether the distribution of viral load levels in individual patients would still conform to the normal distribution. For patient 136, who had a viral load level of 201 copies/mL at 1 time point, it was shown that this was not an implausible level. For patient 154, the most likely explanation for the number of detectable measurements is that the patient’s viral load was in the range of 30-40 copies/mL, rather than <20 copies/mL. This is consistent with the fact that a larger number of viral clones were obtained per time point from the plasma of this patient than from any other patient.

†Calculated as the number of viral load measurements ≥50 copies/mL (26) out of the total number of assays performed (713).
‡Given a viral load measurement ≥50 copies/mL on 1 assay, the probability that an independent assay on the same sample will be ≥50 copies/mL.
§Proportion of viral load measurements that are positive after the previous measurement was positive.

**Table 3. Association Between Blip Frequency and Baseline Demographic, Clinical, and Treatment Characteristics**

<table>
<thead>
<tr>
<th>No. of Blips During Study Period</th>
<th>0</th>
<th>1</th>
<th>≥2</th>
<th>( P ) Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of patients</strong></td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>&gt;.99</td>
</tr>
<tr>
<td><strong>Sex, No. (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1 (100)</td>
<td>2 (67)</td>
<td>4 (67)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Female</td>
<td>0</td>
<td>1 (33)</td>
<td>2 (33)</td>
<td></td>
</tr>
<tr>
<td><strong>Race, No. (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>0</td>
<td>2 (67)</td>
<td>5 (83)</td>
<td>.46</td>
</tr>
<tr>
<td>White</td>
<td>1 (100)</td>
<td>1 (33)</td>
<td>1 (17)</td>
<td></td>
</tr>
<tr>
<td><strong>Age, median (IQR), y</strong></td>
<td>59</td>
<td>43 (39-48)</td>
<td>48 (42-49)</td>
<td>.21</td>
</tr>
<tr>
<td><strong>HIV-1 acquisition risk factor, No. (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men having sex with men</td>
<td>1 (100)</td>
<td>2 (67)</td>
<td>1 (17)</td>
<td>.57</td>
</tr>
<tr>
<td>Intravenous drug use</td>
<td>0</td>
<td>0</td>
<td>3 (50)</td>
<td></td>
</tr>
<tr>
<td>Heterosexual contact</td>
<td>0</td>
<td>1 (33)</td>
<td>2 (33)</td>
<td></td>
</tr>
<tr>
<td><strong>CD4 cell count nadir, median (IQR), cells/µL</strong></td>
<td>6</td>
<td>32 (23-129)</td>
<td>7 (4-18)</td>
<td>.18</td>
</tr>
<tr>
<td><strong>CD4 cell count at study enrollment, median (IQR), cells/µL</strong></td>
<td>433</td>
<td>502 (394-670)</td>
<td>448 (306-475)</td>
<td>.68</td>
</tr>
<tr>
<td><strong>Pretreatment viral load, median (IQR), copies/mL†</strong></td>
<td>431 000</td>
<td>484 000 (457 000-750 000)</td>
<td>596 500 (350 000-697 500)</td>
<td>.55</td>
</tr>
<tr>
<td><strong>Duration of HIV-1 infection, median (IQR), mo</strong></td>
<td>79</td>
<td>148 (111-184)</td>
<td>81 (61-170)</td>
<td>.44</td>
</tr>
<tr>
<td><strong>Duration of HIV-1 RNA suppression, median (IQR), mo</strong></td>
<td>69</td>
<td>35 (11-56)</td>
<td>35 (17-39)</td>
<td>.45</td>
</tr>
<tr>
<td><strong>No. of medications included in current HAART, No. (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1 (100)</td>
<td>2 (67)</td>
<td>3 (50)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1 (33)</td>
<td>2 (33)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1 (17)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; IQR, interquartile range.

*By Fisher exact test for categorical characteristics and Kruskal-Wallis test for continuous characteristics.
†Viral load measurements at time of acute HIV infection are excluded.

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inhibitor and nonnucleoside RT inhibitor concentrations in plasma were measured at each time point in each patient (Figure 1). Large intrapatient fluctuations in drug concentrations were noted in some patients (patients 99, 136, and 148). Importantly, there was no association between low drug concentrations and blips (P=.22 by \( \chi^2 \) test). Most blips (78%) occurred when drug levels were above the suggested trough concentrations (Figure 1).

**Genotypic Analysis of Blips**

Although plasma virus levels were below 50 copies/mL at most time points, the protease and RT regions of plasma viral RNA were successfully amplified and sequenced before, during, and after blips in 9 of the 10 patients (Figure 2). An average of 4 to 5 clones were obtained per time point, for a total of 455 independent protease sequences (830 in nonblip samples, 121 in blip samples) and 1079 independent RT sequences (50 copies/mL in either plasma and the cellular reservoir or in plasma samples obtained at time points prior to the blip. These results are compatible with the idea that there may be no accumulation of new drug resistance mutations associated with blips.

Phylogenetic analysis and genotypic data for a representative patient (136) are shown in Figure 3. Clones obtained from the resting CD4+ cell reservoir at baseline and from the 36 plasma samples clustered together away from sequences from other patients. Virus present during a blip was not phylogenetically distinct from nonblip samples, indicating a lack of viral evolution during blips. Some blip sequences were identical to sequences in the cellular reservoir. Phylogenetic analysis using network trees also failed to show increased divergence of blip sequences (Figure 4). Analysis of genetic diversity (theta) at each time point did not show increased diversity during blips. The median theta for all patients was 0.0051 (interquartile range, 0.002-0.010) for nonblip visits and 0.0061 (interquartile range, 0.003-0.012) for blip visits (P=.37). Most importantly, no drug resistance mutations were detected in any of the sequences from this patient, who had received no prior nonsuppressive therapy before starting HAART (Figures 2 and 3).

In contrast to patient 136, the remaining 8 patients had received prior nonsuppressive therapy, and resistance mutations attributable to the nonsuppressive therapy could be detected (Figure 2). However, no new drug resistance mutations were seen in the 121 protease sequences and the 163 RT sequences obtained during blips in this study. All blip sequences were either wild type or contained mutations that were seen prior to the blip (Figure 2). Blip samples did not have a higher proportion of resistant clones. The degree of resistance was not associated with blip magnitude or frequency. The patient with the blip of greatest magnitude (patient 136) had only wild-type clones, and the patient with the most frequent blips (patient 154) had only 1 major protease mutation (I84V) and no RT mutations that would confer resistance to the current regimen. The K103N mutation detected during 1 blip was selected by prior therapy with efavirenz. During the study period, the patient was not receiving any drug that would select for this mutation.

The genotypic analysis also suggested that blips do not lead to resistance. New resistance mutations were not found immediately after blips. In 455 independent protease sequences and 575 independent RT sequences obtained in the 30 days following a blip, no new resistance mutations were detected. In 1 patient (patient 90), the protease mutation M46I appeared 8 weeks after a blip and then disappeared. Given the patient’s history of poor adherence and prior exposure to multiple protease inhibitors, this is likely to be an archival mutation not detected in baseline sampling. Taken together,
these results refute the notion that resistance arises during or immediately after blips.

**COMMENT**

Given that in adherent patients HAART decreases viremia to a new steady-state level slightly below 50 copies/mL (eg, 15-20 copies/mL), we hypothesized that normal biological and statistical variation would result in occasional values above 50 copies/mL without a clinically significant elevation in plasma HIV-1 RNA levels. Our analysis of the frequency, magnitude, reproducibility, duration, clinical associations, and genotypic consequences of blips are all consistent with this hypothesis.

With respect to frequency, blips were detected in a greater percentage of the patients than in previous studies, probably because of the intensive sampling used here. In previous studies, blips were detected in 11% to 46% of patients. Since the sampling interval we used (every 2-3 days) is on the same time scale as the decay rate of virus-producing cells (t1/2, approximately 1 day), it is unlikely that significant elevations in viremia were

**Figure 2. Lack of New Genotypic Resistance Mutations in Plasma Virus During or Immediately After Blips**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Prior ART Exposure</th>
<th>Current HAART</th>
<th>Genotype of Virus Detected in Plasma by Visit Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI NRTI NNRTI</td>
<td>PI NRTI NNRTI</td>
<td>WT/ Mutation B</td>
</tr>
<tr>
<td>99</td>
<td>RTV/EFV</td>
<td>RTV/EFV</td>
<td>WT</td>
</tr>
<tr>
<td>113</td>
<td>AZT NVP</td>
<td>AZT NVP</td>
<td>WT</td>
</tr>
<tr>
<td>134</td>
<td>d4T EFV</td>
<td>d4T EFV</td>
<td>WT</td>
</tr>
<tr>
<td>135</td>
<td>d4T EFV</td>
<td>d4T EFV</td>
<td>WT</td>
</tr>
<tr>
<td>136</td>
<td>d4T EFV</td>
<td>d4T EFV</td>
<td>WT</td>
</tr>
<tr>
<td>139</td>
<td>NFV SQV RTV</td>
<td>NFV SQV</td>
<td>WT</td>
</tr>
<tr>
<td>147</td>
<td>d4T EFV</td>
<td>d4T EFV</td>
<td>WT</td>
</tr>
<tr>
<td>148</td>
<td>d4T EFV</td>
<td>d4T EFV</td>
<td>WT</td>
</tr>
<tr>
<td>154</td>
<td>d4T EFV</td>
<td>d4T EFV</td>
<td>WT</td>
</tr>
</tbody>
</table>

At all points other than those at which the plasma HIV-1 RNA level was ≥50 copies/mL, the viral load was undetectable. Up to 7 independent clones were obtained at each time point. Note that no new mutations conferring drug resistance appeared during the blips. All mutations detected during or within 30 days after blips were present at baseline (B) or were seen in plasma samples taken prior to the blips. Baseline mutations were identified in either plasma or resting CD4 cell reservoir samples (peripheral blood). The resting cell reservoir was sampled only at baseline. "Prior ART exposure" refers to other antiretroviral drugs the patient has taken that are not part of the current regimen. ABC indicates abacavir; ADF, adefovir; APV, amprenavir; ART, antiretroviral therapy; AZT, zidovudine; ddC, zalcitabine; ddI, didanosine; d4T, stavudine; EFV, efavirenz; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NVP, nevirapine; PI, protease inhibitor; RTV, ritonavir; SQV, saquinavir; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; WT, wild type.
The L substitutions at position 210 are not associated with significant resistance to zidovudine. There were no missing data for this analysis except as indicated above. AA indicates amino acid; AZT, zidovudine; EPV, efavirenz; HIV, human immunodeficiency virus; 3TC, lamivudine.
missed. Based on the statistical arguments presented above, the number of blips detected is likely to be directly related to the frequency of sampling. However, the fraction of positive viral RNA measurements will be related to the patient’s steady-state level of viremia regardless of sampling frequency. Blips occurred with a frequency (3.6% of measurements) that was similar to that observed prior to entry (4.8%) and that was consistent with random variation around a steady-state viral RNA level of 10 to 20 copies/mL. Therefore, blips below 200 copies/mL, any pair of duplicate or consecutive measurements would rarely be concordantly positive (probability ≈ 2.2% [derived from data in Table 2]), consistent with the poor reproducibility (κ = 4.4%) and short duration (median, 2.5 days) observed herein.

The intensive sampling used herein allowed a more precise definition of blip duration. Most of the blips consisted of a single measurement preceded and followed by measurements below 50 copies/mL. The short duration of most blips documented herein is in contradistinction to longer estimates made with mathematical models that infer the shape of blips from clinical data with sparse sampling. The magnitude of observed blips was low (median, 79 copies/mL), with clustering of values close to the detection limit. This is consistent with the tail of a normal log10 distribution centered around 20 copies/mL. Blip magnitude correlated poorly between laboratories. The poor reproducibility of blips below 200 copies/mL has been noted previously. At a true viral RNA level of 20 copies/mL, any pair of duplicate or consecutive measurements would rarely be concordantly positive (probability ≈ 2.2% [derived from data in Table 2]), consistent with the poor reproducibility (κ = 4.4%) and short duration (median, 2.5 days) observed herein.

There was no association between blips and demographic, treatment, or HIV-associated clinical factors. Furthermore, blips were unrelated to intercurrent illnesses, vaccination, or decreases in antiretroviral drug concentrations.

However, blips were marginally associated with self-reported nonadherence (P = .08). Again, these findings are consistent with the hypothesis presented above. Recent work by Miller et al. also found no link between blips and nonadherence. Extensive analysis of drug concentrations over time revealed wide interpatient variation but no correlation between drug concentrations and blips, raising concerns about the usefulness of therapeutic drug monitoring in the management of patients experiencing blips.

Despite previous reports that blips represent resistant virus, our analysis of a total of 951 and 1079 independent clones for the protease and RT regions, respectively, failed to identify any new genotypic resistance before, during, or immediately after blips. One potential explanation for the discrepancy is that the extensive sampling used here allowed a more precise definition of preexisting resistance so that the appearance of new mutations could be more accurately assessed.

These findings provide an explanation for the work of Havlir et al. and others, demonstrating that blips do not predict virological failure. We suggest that isolated low-level positive viral RNA measurements may not be cause for clinical concern. Of course, consistently detectable viremia can be associated with resistance and further studies will be needed to define when detectable viremia should trigger a change in therapy. Given a steady-state level of 20 copies/mL, 96.4% of blips due to random variation will fall below 200 copies/mL. Therefore, blips with a magnitude of greater than 200 copies/mL or blips that are detected in at least 2 independent or consecutive measurements may be more of a cause for concern.

In conclusion, among patients with suppression of viremia to below 50 copies/mL, most blips appear to represent normal biological and statistical variation around mean levels that are below 50 copies/mL rather than clinically significant elevations in the level of viral replication. These conclusions are based on an intensive study of a small group of patients and may not be representative of all patients.
patients studied had started therapy with low CD4 cell counts and high viral load levels, and it will be important to confirm these results in patients who start therapy earlier in the course of infection.

**Author Contributions:** Dr Siliciano had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Nettles, Kieffer, Quinn, Flexner, Carson, Ray, Siliciano. Acquisition of data: Nettles, Kieffer, Kwon, Monroe, Han, Parsons, Cofrancesco, Quinn, Jackson, Flexner, Persaud, Siliciano. Analysis and interpretation of data: Nettles, Kieffer, Parsons, Gallant, Quinn, Jackson, Flexner, Carson, Ray, Persaud, Siliciano. Drafting of the manuscript: Nettles, Kwon, Parsons, Flexner, Carson, Ray, Siliciano. Critical revision of the manuscript for important intellectual content: Kieffer, Monie, Han, Cofrancesco, Gallant, Quinn, Jackson, Flexner, Ray, Persaud, Siliciano. Statistical analysis: Nettles, Quinn, Carson, Ray. Obtaining funding: Nettles, Carson, Siliciano. Administrative, technical, or material support: Kwon, Monie, Han, Parsons, Cofrancesco, Jackson, Flexner, Ray. Study supervision: Kieffer, Flexner, Persaud, Siliciano. Drs Nettles and Kieffer contributed equally to this article.

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**Previous Presentation:** Preliminary data representing some of the work described herein were presented as an abstract (651) at the 11th Conference on Retroviruses and Opportunistic Infections; February 8-11, 2004; San Francisco, Calif; and as an abstract (H1134) at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy; October 30-November 2, 2004; Washington, DC.

**Acknowledgment:** We acknowledge James Johnson and assistance for drug concentration measurements.

**REFERENCES**


Perhaps the most valuable result of all education is the ability to make yourself do the thing you have to do, when it ought to be done, whether you like it or not; it is the first lesson that ought to be learned; and however early a man’s training begins, it is probably the last lesson that he learns thoroughly.

—Thomas H. Huxley (1825-1895)