Long-Term Control of Human Immunodeficiency Virus-1 Replication Despite Extensive Resistance to Current Antiretroviral Regimens: Clonal Analysis of Resistance Mutations in Proviral Deoxyribonucleic Acid

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Archived resistance mutations compromise antiretroviral treatment. We have investigated 3 selected aviremic patients who had extensive historical resistance to their current regimen. All 3 patients underwent unstructured treatment interruptions associated to the re-emergence of wild-type virus before starting their current suppressive regimes. Almost all historical resistance mutations detected in plasma were found in circulating proviral deoxyribonucleic acid. None of the clones analyzed was fully resistant to the current antiretroviral regimen.

Keywords. HIV; proviral DNA; resistance; single genome sequencing.

Resistance mutations selected by antiretroviral therapy (ART) of human immunodeficiency virus (HIV)-1 infection are archived in the reservoir of latently infected cells as integrated cellular deoxyribonucleic acid (DNA) [1, 2]. These archived mutations are thought to be the cause of subsequent treatment failures if the patient receives antiretroviral drugs compromised by historical resistance [3, 4].

We have recently seen 3 patients with long-term virological suppression who were receiving antiretroviral drugs that should not be effective considering resistance mutations detected during prior virological failures. Due to poor adherence, all 3 patients had undergone unstructured treatment interruptions before starting their current antiretroviral regimen.

To try to understand how the current antiretroviral regimens of these 3 patients are effective, we have performed a clonal analysis by single-genome sequencing (SGS) [5] searching for resistance mutations in circulating proviral DNA. This method involves the isolation and sequencing of individual viral genomes and makes possible the detection of mutations present in frequencies below the limit of routine bulk sequencing methods. To the best of our knowledge, this is the first time that clonal analysis of proviral DNA is performed in patients who receive effective ART after treatment interruptions.

METHODS

The historical bulk genotyping was done in plasma viral ribonucleic acid (RNA) using Trugene HIV-1 Genotyping Kit (Siemens). One sample of whole blood from each patient was collected during virologic suppression. To perform the clonal analysis by SGS total DNA was obtained, serially diluted, and tested by endpoint polymerase chain reaction (PCR) to select the dilution with 30% or less positive wells. According to a Poisson distribution, those positive wells contain amplicons derived from a single DNA molecule more than 80% of the time. A region encompassing the protease gene and the first 213 codons of RT gene was amplified by nested PCR. DNA bulk sequencing was done using the same nested PCR with undiluted DNA (see Supplementary Data for additional details).

Bulk and clonal proviral sequences of PCR products were assembled, aligned, and compared with the HIV-1 subtype B consensus sequence (HXB2). In clonal analysis, sequencing reactions containing more than 1 genome were discarded. Drug-resistance genotypes and hypermutated sequences (present an inordinate number of identical transitions) were characterized using Stanford database, version 7.0 (http://hivdb.stanford.edu/).

CASE 1

Patient no. 1 had 1 historical plasma population genotype—4 years before starting ART—showing M184V, L10V, V32I, M46I, A71V, V82A, L63P, and L90M while he was receiving didanosine, stavudine, and nelfinavir (Figure 1). Due to poor compliance, the patient underwent an unstructured treatment interruption, lasting 45 months. At the end of this interruption, another plasma population genotype showed re-emergence of wild-type virus. The patient subsequently started treatment with tenofovir disoproxil fumarate (TDF)/emtricitabine (FTC) and lopinavir/ritonavir (400 mg/100 mg BID). After 4 months of treatment, the patient achieved suppression despite the fact...
that considering the historical genotype results, only TDF was active in this regimen. The patient remained suppressed with this regimen for 41 months. Due to diarrhoea, lopinavir/ritonavir was changed to darunavir/ritonavir (800 mg QD), keeping virological suppression for another 22 months. According to historical genotype, darunavir/ritonavir had low-level
resistance. He was finally switched to TDF/FTC and rilpivirine and remained suppressed. In total, he has remained aviremic for 72 months. Clonal analysis of proviral DNA showed that of 27 clones, 3 contained major resistant mutations (Figure 2). These mutations were the same that had been detected in plasma.

**CASE 2**

Patient no. 2 had 5 historical plasma population genotypes (last 1 performed 33 months before restarting ART) showing among other mutations K65R, Y115F, Y181C, and M184V (Figure 1). The patient received multiple regimens including dual therapy with TDF and lamivudine or FTC to try to preserve immune status without compromising other therapeutic options. She underwent an unstructured treatment interruption during 6 months, before being admitted to a rehabilitation center due to chronic alcoholism. Neither CD4 count nor resistance testing was performed after this interruption. During her stay in the rehabilitation center, the patient started treatment with TDF/FTC and efavirenz. With this regimen, she has been virologically suppressed for the last 60 months, despite the fact that only efavirenz was partially active. In proviral DNA, of 34 clones, 1 had K65R, V90I, Y115F, and M184V (Figure 2). The Y181C mutation was not found in any clone. In addition, we detected 8 hypermutated clones. Those clones would be replication-incompetent due to the introduction of several stop codons [6].

**CASE 3**

Patient no. 3 had 2 historical population genotypes (the last 1 performed 7 years before restarting ART) showing K103N and M184V (Figure 1). The patient was poorly compliant with her antiretroviral regimen due to psychiatric disease. After several unstructured treatment interruptions—lasting a total of 9 months—associated to re-emergence of wild-type virus in plasma population genotyping, the patient started TDF/FTC and raltegravir. Of these 3 drugs, only TDF and raltegravir were active given prior historical genotypes. The patient has been suppressed for the last 24 months. Of 23 clones, 1 clone had the K103N mutation, 1 had M184V, and 1 had both K103N and M184V (Figure 2).

**RESULTS**

By bulk DNA genotyping, we detected wild-type sequences in patient no. 1 and no. 3. In patient no. 2, we detected a hypermutated genotype because the frequency of hypermutation of total clones was 23% (Figure 2). In patient no. 3, we detected I13V and V77I.

In our samples, the fraction of archived resistance mutations detected in proviral DNA by SGS with respect to the resistance mutations previously detected in viral RNA was 100%, 75%, and 100% for patient no. 1, no. 2, and no. 3, respectively (Figure 2). In contrast, the fraction of archived mutations detected by bulk genotyping of proviral DNA was 0% in patient no. 1, 27.27% in
patient no. 2, and 33.33% in patient no. 3. Patient no. 2 harbored V77I, V90I, and M184I in more than 20% of the analyzed clones, and 8 of 34 (23.53%) clones were hypermutated. These 2 findings were detected in bulk genotyping of proviral DNA. Patient no. 3 also harbored I13V and V77I mutations in more than 20% of the clones, and these were detected in bulk proviral DNA. In all 3 patients, we detected by clonal analysis resistance mutations that had not been revealed by prior historical RNA genotypes (Figure 2).

**DISCUSSION**

These 3 patients underwent unstructured treatment interruptions before starting their current—“theoretically ineffective”—ART. In 2 of the patients, plasma genotyping showed that treatment interruption led to re-emergence of wild-type virus. Structured treatment interruptions in patients with extensive resistance have been tried in the past to induce the re-emergence of a more sensitive viral population. This strategy was abandoned because treatment interruptions were associated with greater progression of disease without conferring virological benefits [7].

It is possible that treatment interruptions associated to re-emergence of wild-type virus contributed to the unexpected efficacy of ART in these 3 cases. More than a decade ago Deeks et al [8] suggested that although drug-resistant virus can persist at low levels after the interruption of therapy, durable suppression may be achieved if wild-type virus re-emerges as the predominant population. In our patients, the majority of clones evaluated in proviral DNA were wild type, and, most importantly, all of those clones showing resistance mutations were sensitive to at least 1 of the drugs included in the regimen. Boucher et al [9] studied the provirus of 4 patients at the end of treatment interruptions, before starting salvage therapy. Two of them had reversion to wild-type HIV-1 at the end of the interruption. In contrast with our results, in the Boucher et al [9] study, 2 patients with minority resistant variants detected in proviral DNA but without protease inhibitor mutations had subsequent virological failures despite receiving lopinavir/ritonavir. These 2 patients did not develop mutations to lopinavir/ritonavir, suggesting that non-adherence might have caused failure.

It is interesting to note that patient no. 2 did not have a fully active agent included in her regimen if we consider historical mutations. Efavirenz could be considered as only partially active because of the history of Y181C. However, our clonal analysis

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**Figure 2.** Comparison of resistance detected mutations by bulk ribonucleic acid (RNA) plasma genotyping, bulk proviral deoxyribonucleic acid (DNA) genotyping, and clonal analysis of proviral DNA by single-genome sequencing. Abbreviations: D, detected; ND, nondetected; PR, protease mutation; RT, reverse transcriptase mutation.
did not find this mutation in proviral DNA, suggesting a very low frequency (95% confidence interval, 0%–8.8%). It has been shown that reverse-transcriptase, inhibitor-resistant variants archived in cellular DNA fade from detection with time [10, 11].

Several prior studies [1, 12–15] have not found concordance between population-based sequencing of provirus and results from prior plasma population genotyping. In accordance with these results, population DNA genotyping failed to identify almost all archived mutations. However, as in prior studies [1, 9], our analysis of proviral DNA by SGS was able to identify almost all mutations presented in prior plasma population genotypes. Of note, clonal analysis showed that resistance mutations were dispersed in different clones and not associated into a single genotype, possibly explaining the success of the current treatment regimen [16].

CONCLUSIONS

Our study has some limitations. The viral mutations found could be present in a proviral HIV-1 genome without ability to produce infectious virus. The small sample size limits the relevance of our findings. Moreover, due to unavailability of samples, sequencing was not performed on plasma or proviral DNA immediately before reinitiating treatment. However, we think that our cases prove the concept that under certain circumstances, sequencing can be more widely applicable.

Supplementary Material

Supplementary material is available online at Open Forum Infectious Diseases (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

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