Analysis of HIV-1 drug-resistant variants in plasma and peripheral blood mononuclear cells from untreated individuals: implications for clinical management

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SUMMARY

Genotypic resistance analysis on viral DNA and plasma was performed in 56 therapy naïve patients with recent and chronic infection to assess the prevalence of mutations associated with drug resistance and compare cell-free and cell-associated strains.

Direct sequencing of DNA provirus disclosed key mutations to RT inhibitors more frequently than in plasma RNA. In addition, major mutations associated with drug resistance in the PR region were only found in PBMCs. Although our data are limited to a small cohort, they show a different resistance profile between plasma and PBMC compartments and may yield additional information for first-line antiretroviral regimens.

KEY WORDS: HIV resistance mutations, RNA, DNA, Reservoirs, Genotypic discordance

INTRODUCTION

Genotypic antiretroviral resistance testing is a routine tool in the management of human immunodeficiency virus (HIV) patients on highly active antiretroviral therapy (available at http://aidsinfo.nih.gov), but the usefulness of genotypic assay in all drug naïve patients in most settings is under debate (Hecht et al., 2005). It is accepted that transmission of resistant virus is a growing problem (Re et al., 2001 a,b) and the International AIDS Society-USA, the US Department of Health and Human Services, and European guidelines recently recommended antiretroviral resistance testing not only in cases of treatment failure but also for primary HIV infection (Gallant, 2006), because most drug resistance mutations are thought to be overgrown by wild type virus (Re et al., 2003; Derdelinckx et al., 2004; Re et al., 2004; Paraskevis et al., 2005).

In addition, recent studies have focused on the long-term persistence of primary genotypic resistance in untreated patients and on the high stability of certain drug resistant primary mutations (MRPM) (Chew et al., 2005; Barbour et al., 2004; Pao et al., 2004). In clinical practice, genotypic analysis is commonly used on plasma RNA as representative of the viral population (Santoro et al., 2004), but this assay may not detect drug resistant mutations in minor virus variants or variants archived in PBMC DNA. Most plasma virus in untreated patients is produced by active replication in activated CD4+ cells and the error prone process of reverse transcription of HIV genome may generate mutations that can lead to drug resistance (Siliciano, 2005).

However, in the absence of drug pressure, resistant viral variants might revert to fitter wild type...
viruses and resistant variants may be undetectable in treatment-naive patients with chronic infection (Vandamme et al., 2004; Wensing et al., 2005; Descamps et al., 2005).

This study analyzed the emergence of drug resistance in never treated patients to compare cell-free and cell-associated strains for a clearer understanding of the resistance profile between plasma and PBMC compartments (Tagliaferro et al., 2001).

Plasma and PBMC from fifty-six HIV-1 seropositive patients never treated with antiretroviral compounds were sequenced and analyzed for the presence of drug resistant primary mutations (DRM) and for drug susceptibility profile. All patients, 33 with chronic infection and 23 with recent seroconversion, were enrolled after informed consent according the Helsinki declaration of 1975. All sequences were aligned with the CLUSTAL W 1.7 software. Sequence similarity comparisons were performed using the Megalign program (DNASTAR, Inc., Madison, Wis). Pairwise evolutionary distances were estimated with DNADIST (with Kimura's two-parameters method) and phylogenetic trees were constructed by the neighbor-joining method. Bootstrap support was determined by 100 re-samplings of the sequence.

Viral subtypes were determined by comparing HIV POL sequences with the HIV reference sequence using a BLAST database search (http://www.hiv.lan.gov/content/hiv-b/basic_blast/basic_blast.htm). Peripheral blood CD4 lymphocytes were counted by flow cytometry (FACScan, Becton & Dickinson, Mountain View, CA) using commercially available monoclonal antibody (Becton-Dickinson) with a mean of 504 mm³ and the HIV-RNA plasma viremia, quantified by Quantiplex HIV-RNA-3.0 assay (BayerHealthCare, Tarrytown, NY, USA) according to the manufacturer’s instructions, revealed a median of 4,52 x 10⁴ copies/ml. Plasma and PBMC sequences were obtained by the Trugene HIV-1 Genotyping Kit (BayerHealthCare, Tarrytown, NY, USA) and analyzed by the FDA approved OpenGene DNA Sequencing System resistance interpretation algorithm (GuideLines Rules 10.0, BayerHealthCare, Tarrytown, NY, USA; Liu and Shafer, 2006). Forty-one patients showed an overlapping genetic profile in both compartments. Most of them (37 out 41) showed wild type virus, while four out 41 patients showed key mutations, suggesting the transmission of circulating drug-resistant HIV-1 variants.

The phylogenetic analysis of these HIV-1 RT and PR sequences derived from PBMCs and plasma revealed a tight cluster of both viral sequences (Boeri et al., 2004) and an extremely limited genetic evolution (data not shown). All these genotypes, obtained from plasma RNA and cell associated DNA, also concordant for subtype, revealed that most patients were infected with subtype B virus.

Discordant data between the plasma-virus and the PBMC-virus were found in 15 patients but we focused our attention only on the nine patients with major amino-acid changes only in the cellular compartment (Table 1). In particular, proviral DNA analysis in the RT region showed key mutations at the following codons in five patients: G190A/S (2 pts), T215F (1 pt), V106A (2 pts) (Table 1a). These substitutions can compromise the use of a wide variety of drugs also commonly used for first-line therapy like some NNRTIs (G190A/S, V106A) or NRTIs (T215F). Interestingly, DNA study disclosed a primary mutation (G190A) in one case whose sample was not amplified when the analysis was performed on plasma.

### Table 1A - Comparison of resistance mutations in RT gene between plasma HIV RNA and PBMC HIV DNA in some naive patients representative of the study.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>PBMC</th>
</tr>
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<tbody>
<tr>
<td>Not amplified</td>
<td>190G/A</td>
</tr>
<tr>
<td>WT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41M/L, 215T/F</td>
</tr>
<tr>
<td>WT</td>
<td>215T/D</td>
</tr>
<tr>
<td>WT</td>
<td>101K/Q, 106V/A</td>
</tr>
<tr>
<td>WT</td>
<td>106V/A</td>
</tr>
<tr>
<td>WT</td>
<td>215T/S</td>
</tr>
<tr>
<td>WT</td>
<td>190G/S</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT: wild type
Moreover, sequence analysis showed a number of other minor genotypic changes such as T215D/S or M41L detectable only in PBMCs and correlated to a partial resistance to RT inhibitors (Violin et al., 2004).

Amino-acid changes at codon 215 might serve as a marker to distinguish patients infected with viral strains carrying a previous resistance to NRTIs. These mutations are classified as revertant mutations conferring an increasing risk of virological failure in HIV patients, if subsequently treated with AZT and d4T.

In the PR region PBMC-DNA genotyping showed major mutations in four patients, namely D30N, I50V, I84V and M46I able to confer resistance correlated to most protease inhibitors such as nelfinavir/amprenavir/saquinavir/indinavir (Table 1b). The overall frequency of minor mutations in the protease gene was significantly greater than that observed in RT, even though most amino acid changes represented polymorphisms such as 10, 20, 33, 36, 63, 71, 77 and 89, not directly responsible for drug resistance but able to compensate the reduced protease activity caused by primary mutations (Nijhuis et al., 1999) and possibly reflecting a more widespread use of PI inhibitors in recent years. Secondary mutations at specific sites in protease codons (10 and 36) are significantly more likely to fail antiretroviral therapy by 24 weeks (Perno et al., 2001).

In conclusion we found drug resistance in 13 out of 56 naïve patients (about 23%), but this resistance was at least partially evident with a conventional plasma analysis only in four patients (concordant). On the one hand, a different situation in plasma and PBMCs was found in nine HIV-1 infected patients (9 out 13). Interestingly some of these subjects (4 out 9) could be classified as having long-lasting infection. Even if most studies assumed that in the absence of drug-selection pressure, reversion or overtaking by drug-sensitive virus can occur in time, precluding the detection of drug-resistant variants (Vandamme et al., 2004; Wensing et al., 2005; Descamps et al., 2005), a recent super infection or a co-infection by two different viral strains (a wild and a resistant virus) can be hypothesized. This was also confirmed by our phylogenetic analysis showing that HIV RNA in plasma did not cluster with paired HIV DNA extracted from circulating blood cells at the same time point (Ghosn et al., 2006): for example, two out nine discordant genotypes had CRF02 AG subtype in PBMCs and B-subtype virus in plasma sample (data not shown).

As a result of the different fitness of two strains, the resistant variants are overtaken by drug-sensitive virus, that can represent the most representative viral population in plasma.

However the detection of resistance exclusively in peripheral blood cells raises an important question concerning its value and meaning (Re et al., 2006). First, this method detects all forms of intracellular HIV DNA, but what is the exact contribution of integrated provirus in the emergence of drug resistant mutations? Most studies performed on HAART treated subjects (Paolucci et al., 2001; Wang et al., 2000; Bi et al., 2003; Sarmati et al., 2003) to date have demonstrated that infected cells could contribute to the generation of new viral genomes, even when plasma virus is below the limit of detection (Lassen et al., 2004). Secondly, is the virus detected in PBMC a competent-replicative virus or not (Finzi et al., 1997)?

### Table 1B - Comparison of resistance mutations in protease gene between plasma HIV RNA and PBMC HIV DNA in some naïve patients representative of the study.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69K, V77I</td>
<td>L10I, D30N, M36I, M46I, H69K, V77I</td>
</tr>
<tr>
<td>L10V, V77I</td>
<td>L10V, V77I, I84V</td>
</tr>
</tbody>
</table>

*Primary mutations in bold*
Since PBMCs can be reliably used in drug resistance genotyping and different patterns in different compartments can provide different biological information, this study assessed the distribution of reverse transcriptase and protease mutations, adding important new information on the meaning of intracellular mutations. Even if plasma remains the first choice, the detection of amino-acid changes correlated to drug resistance in PBMCs harbouring proviral DNA could be taken into consideration even though their clinical significance remains uncertain. The creation of cellular reservoirs of HIV-1 (Chun et al., 1997), the potential of provirus resulting in replication competent virus and the interaction between cell-free and cell-associated virus in vivo make the determination of drug resistance in cell-free and cell-associated virus an important issue in HIV medicine.

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