NEW MICROBIOLOGICA, **30**, 313-317, 2007

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

Isabella Bon[1], Federica Alessandrini[1], Marco Borderi[2], Roberta Gorini[1], Maria Carla Re[1]

[1]Section of Microbiology; [2]Infectious Diseases, Department of Clinical and Experimental Medicine, University of Bologna, Italy

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

Corresponding author
Isabella Bon
Department of Clinical and Experimental Medicine
Section of Microbiology
University of Bologna
St. Orsola Hospital
Massarenti, 9, 40138 Bologna, Italy
E-mail: isabella.bon@unibo.it

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, matched for sex, age and risk factors, 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 mm^3 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 10^4 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, BayerHealth Care, 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 naïve patients representative of the study.*

Plasma	*PBMC*
Not amplified	**190G/A**
WT*	41M/L, **215T/F**
WT	215T/D
WT	101K/Q, **106V/A**
WT	**106V/A**
WT	215T/S
WT	**190G/S**

WT: wild type

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.*

Plasma	*PBMC*
H69K, V77I	L10I, **D30N**, M36I, **M46I**, H69K, V77I
G16E, L63P, H69K, V77I	G16E, L63P, H69K, V77I, **I50V**
I13V, K20R, M36I, L89I	I13V, K20R, M36I, L89I, **M46I**
L10V, V77I	L10V, V77I, **I84V**

Primary mutations in **bold**

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)?

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.

ACKNOWLEDGEMENTS
This work was supported by Funds from Fondazione Cassa di Risparmio Bologna, Italy (n° 2006.0035, June 2006), "AIDS projects" of the Italian Ministry of Health, funds for selected research topics of the University of Bologna and MURST 60%,
We thank Dr. Ileana Turrini and Dr. Massimo Ciscato (Bayer, Italy) for a grant dedicated to drug resistance testing.
We also thank Ms Anne Collins for editing the manuscript.

REFERENCES

Barbour, J.D., Hecht, F.M., Wrin, T., Liegler, T.J., Ramstead, C.A., Busch, M.P. (2004). Persistence of primary drug resistance among recently HIV-1 infected adults. *AIDS* **18**, 1683-1689.

Bi, X., Gatanaga, H., Ida, S., Tsuchiya, K., Matsuoka-Aizawa, S., Kimura, S., Oka, S. (2003). Emergence of protease inhibitor resistance-associated mutations in plasma HIV-1 precedes that in proviruses of peripheral blood mononuclear cells by more than a year. *J. Acquir. Immune Defic. Syndr*. **34** (1), 1-6.

BLAST database available at http://www.hiv.lan.gov-content/hiv-b/basic_blast/basic_blast.htm

Boeri, E., Canducci, F., Grasso, MA., Presi, S., Carrera, P., Racca, S., Clementi, M. (2004). Phylogenetic internal control for HIV-1 genotypic antiretroviral testing. *New Microbiol.* **27** (2), 105-109.

Chew, C.B., Potter, S.J., Wang, B., Wang, Y.M., Shaw, C.O., Dwyer, D.E., saksena, N.K. (2005). Assessment of drug resistance mutations in plasma and peripheral blood mononuclear cells at different plasma viral loads in patients receiving HAART. *J. Clin. Virol.* **33** (3), 206-216.

Chun, T.W., Stuyver, L., Mizell, S.B., Ehler, L.A., Mican, J.A., Baseler, M., Lloyd, A.L., Nowak, M.A., Fauci, A.S. (1997). Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. USA* **94**, 13193-13197.

Derdelinckx, I.K., Van Laethem, B., Maes, Y., Schrooten, S., De Wit, E., Florence, K. (2004).Current levels of drug resistance among therapy-naive HIV-infected patients have significant impact on treatment response. *J. Acquir. Immune Defic. Syndr.* **37**, 1664-1666.

Descamps, D., Chaix, M.L., Andre, P., Brodard, V., Cottalorda, J., Deveau, C. (2005). French national sentinel survey of antiretroviral drug resistance in patients with HIV-1 primary infection and in antiretroviral-naive chronically infected patients in 2001-2002. *J. Acquir. Immune Defic. Syndr*. **38**, 545-552.

Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, R.E., Quinn TC, Chadwick K, Margolick J, Brookmeyer R, Gallant J, Markowitz M, Ho DD, Richman DD, Siliciano R.F. (1997). Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278** (5341), 1295-1300.

Gallant, J.E. (2006). Antiretroviral drug resistance and resistance testing. *Top HIV Med.* **13**, 138-142.

Ghosn, J., Pellegrin, I., Goujard, C., Deveau, C., Viard, J.P., Galimand, J., Harzic, M., Tamalet, C., Meyer, L., Rouzioux, C., Chaix, M.L. (2006). HIV-1 resistant strains acquired at the time of primary infection massively fuel the cellular reservoir and persist for lengthy periods of time. *AIDS* **20**, 159-170.

Guidelines for the use of antiretroviral agents in HIV infected Adult and Adolescent. Developed by the DHHS panel on antiretroviral guidelines for adults and adolescents - A working group of the office of AIDS research advisory councils OARAC. Available at http://aidsinfo.nih.gov

Hecht, F.M., Grant, R.M. (2005). Resistance testing in drug-naïve HIV-infected patients: is it time? *Clin. Infect. Dis*. **41**, 1324-1325.

Lassen, K., Han, Y., Zhou, Y., Siliciano, J., Siliciano, R.F. (2004). The multifactorial nature of HIV-1 latency. *TREND in Mol. Med.* **10** (11), 525-531.

Liu, T.F., Shafer, R.W. (2006). Web resources for HIV type 1 genotypic-resistance test interpretation. *Clin. Infect. Dis.* **42**, 1608-1618.

Nijhuis, M., Schuurman, R., de Jong, D., Erickson, J., Gustchina, E., Albert, J. (1999). Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* **13**, 2349-2359.

Pao, D., Andrady, U., Clarke, J., Dean, G., Drake, S., Fisher, M. (2004). Long term persistence of primary genotypic resistance after HIV-1 seroconversion. *J. Acquir. Immune Defic. Syndr.* **37**, 1570-1573.

Paolucci, S., Baldanti, F., Campanini, G., Zavattoni, M., Cattaneo, E., Dossena, L., Gerna, G. (2001). Analysis of HIV drug-resistant quasispecies in plasma, peripheral blood mononuclear cells and viral isolates from treatment-naive and HAART patients. *J. Med. Virol.* **65** (2), 207-217.

Paraskevis, D., Magiorkinis, E., Katsoulidou, A., Hatzitheodorou, E., Antoniadou, A., Papadopoulos, A. Hellenic Multi-centre study on HIV-resistance (2005). Prevalence of resistance-associated mutations in newly diagnosed HIV-1 patients in Greece. *Virus Res.* **112**, 115-122.

Perno, C.F., Cozzi-Lepri, A., Balotta, C., Forbici, F., Violin, M., Bertoli, A. Italian Cohort Naive Antiretroviral (I.CO.N.A.) Study Group (2001). Secondary mutations in the protease region of human immunodeficiency virus and virologic failure in drug-naive patients treated with protease inhibitor-based therapy. *J. Infect. Dis.* **184**, 983-991.

Re, M.C., Bon, I., Monari, P., Gorini, R., Schiavone, P., Gibellini, D., La Placa, M. (2003). Drug failure during HIV-1 treatment. New perspectives in monitoring drug resistance. *New Microbiol.* **26** (4), 405-413.

Re, M.C., Monari, P., Bon, I., Borderi, M., Chiodo, F. and HIV-1 Emilia Romagna Infectious Disease Study Group (2004). Conflicting interpretations of the prevalence of mutations associated with drug resistance in antiviral naive HIV-1 patients with acute and chronic infection. *Int. J. Antimicrob. Agents* **23** (2), 164-168.

Re, M.C., Monari, P., Bon, I., Gibellini, D., Vitone, F., Borderi, M., La Placa, M. (2001 b). Analysis of HIV-1 drug resistant mutations by line probe assay and direct sequencing in a cohort of therapy naive HIV-1 infected Italian patients. *BMC Microbiol.* **1**, 30.

Re, M.C., Monari, P., Borderi, M., Tadolini, M., Verucchi, G., Vitone, F., Spinosa, S., La Placa, M. (2001a). Presence of genotypic resistance to antiretroviral drugs in a cohort of therapy-naive HIV-1-infected Italian patients. *J. Acquir. Immune Defic. Syndr.* **1** (3), 315-316.

Re, M.C., Vitone, F., Bon, I., Schiavone, P., Gibellini, D. (2006). Meaning of DNA detection during the follow-up of HIV-1 infected patients: a brief review. *New Microbiol.* **29**, 81-88.

Santoro, M., Ceccherini-Silberstein, F., Gori, C., Svicher, V., Forbici, F., Bellocci, MC., d' Arrigo, R., Bertoli, A. (2004). Temporal change in the use of genotypic resistance testing over the years 1999-2003. *New Microbiol.* **27** (1), 141-144.

Sarmati, L., Nicastri, E., Uccella, I., D'Ettorre, G., Parisi, S.G., Palmisano, L., Galluzzo, C., Concia, E., Vullo, V., Vella, S., Andreoni, M. (2003). Drug-associated resistance mutations in plasma and peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected patients for whom highly active antiretroviral therapy is failing. *J. Clin. Microbiol.* **41** (4), 1760-1762.

Siliciano, R.F., M.D., PhD. (2005). Scientific rationale for antiretroviral therapy in 2005: viral reservoir and resistance evolution. *Top HIV Med.* **13** (3), 96-100.

Tagliaferro, L., Menegazzi, P., Cataldini, S., De Simone, M., Chiriacò, P., Varnier, O.E. (2001). Drug resistance testing in PBMCs of HIV infected patients. *New Microbiol*. **24** (4), 325-332.

Vandamme, A,M,, Sonnerborg, A., Ait-Khaled, M., Albert, J., Asjo, B., Bacheler, L., Banhegyi, D., Boucher, C., Brun-Vezinet, F., Camacho, R., Clevenbergh, P., Clumeck, N., Dedes, N., De Luca, A., Doerr, H.W., Faudon, J.L., Gatti, G., Gerstoft, J., Hall, W.W., Hatzakis, A., Hellmann, N., Horban, A., Lundgren, J.D., Kempf, D., Miller, M., Miller, V., Myers, T.W., Nielsen, C., Opravil, M., Palmisano, L., Perno, C.F., Phillips, A., Pillay, D., Pumarola, T., Ruiz, L., Salminen, M., Schapiro, J., Schmidt, B., Schmit, J.C., Schuurman, R., Shulse, E., Soriano, V., Staszewski, S., Vella, S., Youle, M., Ziermann, R., Perrin, L. (2004). Updated European recommendations for the clinical use of HIV drug resistance testing. *Antivir. Ther*. **9** (6), 829-848.

Violin, M., Cozzi-Lepri, A., Velleca, R., Vincenti, A., D'Elia, S., Chiodo, F., Ghinelli, F., Bertoli, A., d'Arminio-Monforte, A., Perno, C.F., Moroni, M., Balotta, C. (2004). Risk of failure in patients with 215 HIV-1 revertants starting their first thymidine analog-containing highly active antiretroviral therapy. *AIDS* **18**, 227-235.

Wang, Y.M., Dyer, W.B., Workman, C., Wang, B., Sullivan, J.S., Saksena, N.K. (2000). Molecular evidence for drug-induced compartmentalization of HIV-1 quasispecies in a patient with periodic changes to HAART. *AIDS* **14**, 2265-2272.

Wensing, A.M., van de Vijver, D.A., Angarano, G., Asjo, B., Balotta, C., Boeri, E., Camacho, R., Chaix, M.L., Costagliela, D., De Luca, A., Derdelinckx, I., Grossman, Z., Hamouda, O., Hatzakis, A., Hemmer, R., Hoepelman, A., Horban, A., Korn, K., Kucherer, C., Leitner, T., Loveday, C., MacRae, E., Maljkovic, I., de Mendoza, C., Meyer, L., Nielsen, C., Op de Coul, E.L., Ormaasen, V., Paraskevis, D., Perrin, L., Puchhammer-Stockl, E., Ruiz, L., Salminen, M., Schmit, J.C., Schneider, F., Schuurman, R., Soriano, V., Stanczak, G., Stanojevic, M., Vandamme, A.M., Van Laethem, K., Violin, M., Wilbe, K., Yerly, S., Zazzi, M., Boucher, C.A.; SPREAD Programme (2005). Prevalence of drug-resistant HIV-1 variants in untreated individuals in Europe: implications for clinical management. *J. Infect. Dis.* **192** (6), 958-966.