DOI: 10.1111/j.1468-1293.2009.00814.x

HIV Medicine (2010), **11**, 483–492

ORIGINAL RESEARCH

HIV-1 proviral resistance mutations: usefulness in clinical practice

B Kabamba-Mukadi,[1] A Duquenne,[1] P Henrivaux,[3] F Musuamba,[2] J Ruelle,[1] J-C Yombi,[3] M Bodéus,[1] B Vandercam[3] and P Goubau[1]

[1]Université Catholique de Louvain, AIDS Reference Laboratory, Brussels, Belgium, [2]Université Catholique de Louvain, LBCM Unit, Brussels, Belgium and [3]Université Catholique de Louvain, Department of Infectious Diseases, Brussels, Belgium

Objectives

Transmitted HIV strains may harbour drug resistance mutations. HIV-1 drug resistance mutations are currently detected in plasma viral RNA. HIV-1 proviral DNA could be an alternative marker, as it persists in infected cells.

Methods

This was a prospective study assessing the prevalence and persistence of HIV-1 drug resistance mutations in DNA from CD4 cells before and after protease inhibitor (PI)- or nonnucleoside reverse transcriptase inhibitor (NNRTI)-based therapy initiation in 69 drug-naïve patients.

Results

Before therapy, 90 and 66% of detected mutations were present in CD4 cells and plasma, respectively. We detected seven key mutations, and four of these (M184M/V, M184M/I, K103K/N and M46M/I) were only found in the cells. When treatment was started, 40 patients were followed; the mutations detected at the naïve stage remained present for at least 1 year. Under successful treatment, new key mutations emerged in CD4 cells (M184I, M184M/I and Y188Y/H).

Conclusions

The proportion of mutations detected in the DNA was statistically significantly higher than that detected in standard RNA genotyping, and these mutations persisted for at least 1 year irrespective of therapy. The pre-existence of resistance mutations did not jeopardise treatment outcome when the drug concerned was not included in the regimen. Analysis of HIV-1 DNA could be useful in chronic infections or when switching therapy in patients with undetectable viraemia.

Keywords: CD4 cells, HIV-1, mutation, naïve patients, provirus, resistance

Accepted 13 November 2009

Introduction

The efficacy of initial therapy for HIV infection may be jeopardized by the presence of drug resistance mutations, which reduce the probability of durable suppression of viral replication. Transmission of resistant HIV-1 strains and reduced drug susceptibility of viruses in untreated patients have been documented [1–4]. Key studies have demonstrated that clinical benefits result from determining viral drug susceptibility before initiating or changing therapy [1,2,5–12]. Based on some publications [13–17] suggesting that testing for drug resistance is even indicated for newly acquired HIV infection, because the proportion of drug-resistant viruses in new HIV infections is increasing, recent international guidelines recommend resistance testing in cases of primary or recent HIV infection [18]. The panel of experts that prepared these guidelines recommends resistance testing when the prevalence of mutations in naïve patients exceeds 5% to 10% or where there is a strong suspicion of transmission of resistance. In other cases, the guidelines suggest that resistance testing should be carefully considered and, if not performed, they recommend storing the earliest available sample so that testing can be conducted at a later date if necessary. Either way, testing should not delay treatment. Importantly, the level of drug resistance and of acquisition of drug-resistant

Correspondence: Mr Benoît Kabamba-Mukadi, Clinique Universitaire St Luc – UCL, Microbiology – Unit of Virology, Avenue Hippocrate 10, 1200 Brussels, Belgium. Tel: + 3227646726; fax: + 3227646933; e-mail: benoit.Kabamba@uclouvain.be

virus may be strongly dependent on the patient's origin, even in a small country such as Belgium, and these factors should be taken into account when considering resistance testing [19–27]. Switching antiretroviral agents for reasons other than virological failure, most frequently to improve convenience or because adverse events require discontinuation, has become standard practice in the management of HIV infection.

At present, HIV-1 drug resistance mutations are detected by analysing plasma viral RNA. However, it is possible that HIV-1 proviral DNA could be used as an alternative marker, as it is known that proviral DNA persists in infected cells, even after prolonged highly active antiretroviral therapy (HAART) that has been demonstrated to be successful on the basis of an undetectable plasma RNA viral load. Data are accumulating regarding the detection of HIV-1 drug resistance mutations in proviral DNA. Some authors have noted the presence of key mutations in proviral DNA which were not present in plasma viral RNA [28–31]. Using direct sequencing, Bona *et al.* [30] assessed the prevalence of mutations associated with drug resistance in cell-free and cell-associated strains in treatment-naïve patients [28–30]. These authors observed that key mutations conferring resistance to reverse transcriptase (RT) inhibitors were found more frequently in proviral DNA than in plasma viral RNA. In addition, major mutations in the protease (PR) region were only found in peripheral blood mononuclear cells (PBMCs). Wang *et al.* [31] showed that drug resistance mutations remained compartmentalized in plasma and PBMCs. In contrast, in therapy-naïve patients, these authors observed a tight concordance between the HIV strains in plasma and PBMCs. Another publication in 2006 [32] described the early establishment of the viral reservoir in patients acquiring resistant strains at primary HIV-1 infection (PHI) and the persistence of resistance-associated mutations with identical profiles in paired HIV RNA and PBMC HIV DNA. Usuku *et al.* [33] followed the changes in drug resistance mutations in a patient receiving HAART. Mutations detected in the plasma were not present or were infrequently present in the proviral DNA. The discrepancy persisted for more than 3 years. It is important to emphasize that the peripheral blood pool of lymphocytes represents about 2% of the total number of lymphocytes in normal young adult men [34]. Schnuda *et al.* [35] showed that the small blood lymphocytes recirculate continuously between the peripheral blood and the lymph nodes in the rat, with each cycle having a duration of less than 3 min.

In this article, we report the results of a prospective study assessing the prevalence and persistence of HIV-1 drug resistance mutations in proviral DNA from purified CD4 cells compared with those in plasma viral RNA before therapy initiation in treatment-naïve patients. We also evaluated the evolution of HIV-1 drug resistance mutations in proviral DNA before and after therapy initiation, and plasma RNA mutation patterns in patients remaining treatment-naïve. As 95 to 99% of infected cells are CD4 cells [36], and in order to confirm the utility of resistance testing in provirus, we used direct sequencing of HIV-1 proviral DNA in purified CD4 cells to follow the evolution of drug resistance mutations in treated and untreated patients and compared the findings to those obtained from HIV-1 viral RNA using the ABI 310 Prism (Applied Biosystems, Foster City, California). We further chose not to use cloning but direct population sequencing as this is routinely used in clinical settings.

Materials and methods

Patients

Between May 2002 and July 2007, genotypic resistance testing was performed on cell-free and cell-associated virus from 69 patients who were not receiving treatment (Table 1). The study was approved by the local ethics committee and informed consent was obtained from each patient. HIV-1 seropositive status was confirmed according to accepted methods. The therapeutic histories of all patients were checked by asking specific questions when they signed the informed consent form and by consulting their clinical records. When documented histories were absent, we contacted the physicians responsible for the patients' care. This confirmed each patient as HIV drug naïve. Checking the therapeutic histories of all patients can be difficult but is important when studying drug mutations in treatment-naïve patients.

Virus was successfully sequenced for 63 of the 69 selected individuals at baseline, both in plasma and in cells. Fifty-eight per cent of the patients were European and 42% non-European, mostly from central Africa. Thirty-nine per cent of the sequenced HIV-1 viruses were subtype B. Thirty-two of the patients subsequently received combination therapy with two nucleoside reverse transcriptase inhibitors (NRTIs) + lopinavir/ritonavir (LPV/r), 12 received two NRTIs + efavirenz (EFV) and 25 remained therapy-naïve. Patients were followed for at least 1 year after initiation of therapy (Table 1).

Purified CD4 cells and reference strain

CD4 cells were isolated from 10 mL of whole blood [collected from patients in ethylenediaminetetraacetic acid (EDTA)] by an immunomagnetic method using anti-CD4 coated magnetic beads (Dynabeads M450 CD4; Dynal AS, Oslo, Norway) according to the manufacturer's protocol

Table 1 Patient characteristics

	All patients	Naïve group	NNRTI group	PI group
Number of patients				
Total	69	25	12	32
> 12 months of follow-up	40	8	10	22
Gender				
Male (%)	49	56	33	50
Female (%)	48	40	67	47
Unknown (%)	3	4		3
Age (years) [mean (range)]	44 (20–68)	41 (27–55)	44 (20–68)	47 (29–65)
Geographic origin				
European (%)	58	48	58	47
Other/unknown (%)	42	52	42	53
Duration of follow-up (months) [mean (range)]*	28 (11–44)	24 (12–41)	34 (17–44)	25 (11–44)
CD4 count (cells/μL)				
Baseline [median (range)]†	338 (6–1460)	616 (177–1111)	238 (168–284)	253 (46–1128)
Follow-up [median (range)]‡	–	476 (245–826)	396 (194–549)	402 (146–1021)
Mean change (range)§	–	− 154 (−533 to 102)	+ 154 (26–365)	+ 209 (−226 to 470)
Wilcoxon test *p*-value for mean change¶		0.07	0.002	0.0001
Plasma HIV RNA (log_{10} copies/mL)				
Baseline [median (range)]†	5.27 (2.6–5.70)	4.00 (1.70–4.82)	4.09 (2.41–4.67)	4.44 (1.70–5.70)
Follow-up [median (range)]‡	–	4.27 (3.47–5.23)	1.80 (1.7–2.75)	1.82 (1.70–4.05)
Mean change (range)§	–	+ 0.08 (− 0.6 to 1.4)	–	–
Wilcoxon test *p*-value for mean change¶	–	0.24	–	–
Patients with viral load < 50 copies/ml (%)	–	–	90	91
HIV-1 subtype (%)				
B	39			
Non-B	61			

*Number of months between the baseline sample and the last follow-up sample sequenced.
†Sample tested at baseline (before therapy initiation for treated patients).
‡Sample tested after at least 12 months of follow-up.
§The changes in CD4 cell count and viral load were calculated per individual in patients with follow-up samples.
¶The critical *p*-value required to reject the null hypothesis (no statistically significant difference) and accept the alternative hypothesis is ≤ 0.05.
NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

and were stored at − 80 °C until use. The purity of the CD4 cell preparation was approximately 99% as estimated using Becton Dickinson FACScan Flow Cytometer technology (Franklin Lakes, New Jersey, USA) (data not shown). The 8E5 cell line was cultured in RPMI-1640 medium and the cells were counted using a Coulter automated haematology analyser, diluted to 10^6 cells per aliquot and stored at − 80 °C. The 8E5 cell-free virus present in the culture supernatant was diluted and stored in aliquots of 30 000 HIV-1 RNA copies/mL.

HIV-1 plasma viral load

The HIV-1 RNA plasma viral load was assessed using the Versant HIV-1 RNA 3.0 assay (Siemens Medical Diagnostics Solutions, Tarrytown, NY, USA) according to the manufacturer's instructions. This assay is based on branched DNA (bDNA) technology. It requires 1 mL of sample and has a dynamic range of 50–500 000 copies/mL. Plasma samples and the 8E5 cell culture supernatant were frozen at − 80 °C until tested for HIV-1 RNA viral load. A viral load result of < 50 copies/mL was regarded as 50 copies/mL when calculating the mean viral load of a given patient group.

Nucleic acid extraction and direct sequencing

DNA was extracted from purified patient CD4 cells diluted in 200 μL of phosphate-buffered saline (PBS), using the High Pure® PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. To concentrate the HIV-1 target gene in patient samples, DNA was eluted in a volume of 50 μL. DNA from 10^6 T-lymphoblastoid 8E5 cells, used as positive control, was eluted in 200 μL. A negative control (in which the template was replaced with nuclease-free water) was included in each polymerase chain reaction (PCR) run. Particular attention was paid to using DNAse- and RNAse-free materials. Depending on the number of samples, the whole DNA purification process required approximately 1 h. HIV RNA was extracted from plasma and from the 8E5 cell culture supernatant using the QIAmp Viral Mini Kit™ (Qiagen, Leiden, the Netherlands) according to the manufacturer's directions. Viral RNA and proviral DNA genotypic antiretroviral drug resistance mutations were identified using an in-house reverse transcriptase–polymerase chain reaction (RT-PCR) method applied to the RT and PR genes (adapted from Schmit *et al.*

[37]). Direct cycle sequencing with BigDye terminator chemistry was carried out on the ABI Prism 310 sequencer (Applied Biosystems). In cases of PCR failure, samples were analysed using a TRUGENE HIV-1 Genotyping Kit (Siemens Medical Diagnostics Solutions). The Stanford mutation list (last updated 09/01/09), excluding polymorphisms and drug-selected mutations without known significance, was used to identify mutations [38].

Statistical analysis

Statistical analysis was performed using JMP statistical software version 7.0.1 (SAS Institute, Cary, NC). The χ^2 test is a nonparametric statistical test used in this case to determine whether the proportion of mutations detected in DNA differed from that detected in RNA or in follow-up DNA samples. In addition, the kappa statistic was used to estimate the agreement between detection of mutations in DNA and detection of mutations in RNA or follow-up DNA samples. Changes in CD4 cell count and viral load were calculated per individual in patients with follow-up samples taken during the study period. The Shapiro test was performed to evaluate the normality of the viral load and CD4 cell count distributions. If the distribution was normal, a paired Student's *t*-test was used to determine whether the mean difference was statistically different from 0. Otherwise, a Wilcoxon nonparametric test was applied. A logistic regression was performed to assess the relationship between the appearance of new mutations and the time elapsed between sample collections. The critical *P*-value required to reject the null hypothesis (that there is no proof of a significant correlation between the variables) and accept the alternative hypothesis was 0.05.

Results

HIV-1 RNA viral load and CD4 cell count

The characteristics of 69 selected patients at the time of inclusion in the study are presented in Table 1. The 69 treatment-naïve patients had a mean viral load of 5.27 (range 2.6–5.70) $\log_{10}$ copies/mL and a mean CD4 lymphocyte count of 338 cells/μL (range 6–1460 cells/μL). Twenty-five patients remained drug-naïve and eight of these had follow-up samples taken during the study period. After a mean follow-up time of 24 (range 12 to 41) months, the mean viral load change was 0.08 (range -0.6 to 1.4) $\log_{10}$ copies/mL, which was not statistically different from 0 (Wilcoxon test associated $P = 0.42$). The mean decrease in CD4 cell count of 174 (median -154; range -533 to 102) cells/mL was not statistically significant by the Wilcoxon test ($P = 0.07$) (Table 1). After EFV-based therapy initiation in the nonnucleoside reverse transcriptase inhibitor (NNRTI) group, 10 patients were followed for at least 12 months and showed a mean increase in CD4 cell count of 173 (median 154; range 26 to 365) cells/mL, which was statistically significant (Wilcoxon test associated $P = 0.002$). Ninety per cent of patients in this group (patient number 16 being the only exception) achieved an undetectable viral load (<50 RNA copies/mL) (Table 1). The protease inhibitor (PI) group comprised 32 individuals, of whom 22 had at least 1 year of follow-up with a mean of 25 months after therapy initiation. The plasma viral load decreased to an undetectable viral load (91% of patients with <50 RNA copies/mL) in 20 of the 22 patients with follow-up. Patients 21 and 37 were exceptions, as viral load was detectable. In this PI group, the mean increase in CD4 cell count was 162 (median 209; range -226 to 470) cells/mL, which was statistically significant (*t*-test associated $P = 0.0001$) (Table 1).

Resistance mutations detectable in plasma and CD4 cells from therapy-naïve patients

Of the resistance mutations detected in the 61 patients with sequenced virus (of the 69 selected patients) at the therapy-naïve stage, 90% were present in CD4 cells and 66% in the plasma. Fifty-five per cent of PR mutations ($n = 20$) and 56% of RT mutations ($n = 9$) were present simultaneously in CD4 cells and plasma. The proportion of mutations detected in the DNA and the proportion detected with standard RNA genotyping were statistically significantly different by the χ^2 test ($P < 0.0001$). We can therefore conclude that the difference in detected mutations is not attributable to chance. The kappa coefficient was 0.71, which means that there was substantial agreement between the two methods in naïve patients [39]. One patient (patient 7) had the M46L PR key mutation in both plasma and cells, while patient 33 had the M46M/I mixed population only in the plasma (3% of 61 patients). The M46I or L mutation confers high resistance to indinavir (IDV). Eight per cent of patients ($n = 61$) had at least one RT mutation in the plasma while 15% had at least one RT resistance mutation in CD4 cells. Seven key mutations were detected in different patients (11.5% of the 61 patients and 10% of all included patients) and four of these (M184M/V, M184M/I, K103K/N and M46M/I) were only found in the cells (data shown for followed patients). For the 40 patients with follow-up samples (see Tables 2 and 4 below), three of the key mutations detected at the naïve stage were present in the RT and PR genes (M46L, M46M/I and K103K/N) of patients 7, 33 and 37. The K103K/N mixed population was not found in the plasma. One treatment-naïve patient (patient 9

Table 2 Evolution of drug resistance mutations in eight of 25 untreated patients detected using a direct sequencing assay of HIV-1 proviral DNA in purified CD4 cells compared with HIV-1 plasma viral RNA

Patient no.	Treatment	Viral load (copies/ml)	CD4 count (cells/μl)	Subtype	Follow-up (months)*	Plasma RT mutations	Proviral RT mutations	Plasma PR mutations	Proviral PR mutations
1	Naïve	4106	†180	C	0	Wild type	Wild type	Wild type	Wild type
1	Naïve	< 50	–		39	Wild type	Wild type	Wild type	Wild type
2	Naïve	170 506	†177	?	0	†Wild type	Wild type	†Wild type	Wild type
2	Naïve	42 534	205		18	Wild type	Wild type	Wild type	Wild type
3	Naïve	59 810	655	B	0	Wild type	†Wild type	Wild type	†Wild type
3	Naïve	25 023	445		12	Wild type	***M184I***	Wild type	Wild type
4	Naïve	60 024	†605	A	0	Wild type	Wild type	L10V	L10V, G73G/S
4	Naïve	54 275	245		41	Wild type	Wild type	L10V	L10V, G73G
5	Naïve	11 574	855	G	0	Wild type	Wild type	Wild type	Wild type
5	Naïve	14 656	479		29	Wild type	Wild type	Wild type	Wild type
6	Naïve	2924	468	B	0	Wild type	Wild type	Wild type	Wild type
6	Naïve	65 804	369		27	Wild type	†Wild type	Wild type	Wild type
7	Naïve	6151	1111	B	0	Wild type	T215L	*M46L*	*M46L*
7	Naïve	1541	578		17	T215L	Not amplified	*M46L*	*M46L*
8	Naïve	28 700	768	B	0	Wild type	†Wild type	Wild type	†Wild type
8	Naïve	22 049	826		14	Wild type	Wild type	Wild type	Wild type

*Elapsed time between two samples, starting from zero for the baseline sample.
†Nucleotide sequence obtained using the Bayer Kit or another sample because of sequencing failure.
Mutations in bold: discrepant mutations between plasma and cells.
Mutations in italic: key mutations conferring high resistance.
PR, protease; RT, reverse transcriptase.

in Table 3) had virus with an RT resistance profile (67N, 70R and 219Q) in both CD4 cells and plasma. Global analysis of the resistance revealed identical results in 93% of CD4 cells and plasma.

Twenty-five patients remained therapy naïve, and eight of these untreated patients were followed. The genotyping results for both the RT and the PR resistance mutations in plasma and CD4 cells from these patients are shown in Table 2. One of the eight patients had one revertant RT resistance mutation (T215L in patient 7), while two patients had a PR mutation, including one key mutation (M46L in patient 7). Although one patient (patient 3) showed a new key RT mutation (M184I) after 12 months, which was present only in the cells, follow-up data for resistance mutations in the plasma and CD4 cells demonstrated stable mutation patterns. Patients 3 and 7 showed mutations in the second sample that were not detected in the first sample; this was probably a result of the known low sensitivity of direct sequencing for detecting minor populations.

Resistance mutations detectable in plasma and CD4 cells in the NNRTI group

The genotyping results for RT and PR resistance mutations in plasma and CD4 cells from the NNRTI-treated patients are shown in Table 3. There were 12 NNRTI-treated patients, most of whom received EFV, and follow-up data were available for 10 of them for a mean time of 34 months (range 17–44 months). Four of these 10 patients had at least one secondary RT resistance mutation (patients 9, 14, 16 and 17), while three patients had one PR mutation in both plasma and cells (patients 11, 13 and 18). All detected PR mutations were secondary mutations, which are not directly relevant for drug resistance. Viruses from patients 9 [mutations D67N, K70R and K219Q, conferring resistance to zidovudine (ZDV) and stavudine (d4T)] and 16 (mutation M41L) showed the same NRTI-correlated resistance mutations in plasma samples and in CD4 cells. Patient 9 was successfully treated with a combination of lamivudine (3TC), tenofovir (TDF) and EFV, and had an undetectable plasma viral load for 30 months. Three patients (patients 12, 13, and 15) showed one or two more mutations in the PR gene in CD4 cells than in plasma. However, follow-up analysis of resistance mutations could only be performed in the provirus as the plasma viral load was undetectable in most cases. All mutations detected at the therapy-naïve stage remained present and there were no additional mutations, with the exception of patients 11 and 17, in whom the key RT mutations Y188Y/H and M184M/I were only detected in the CD4 cells after 42 and 17 months of follow-up, respectively. The Y188Y/H mutation confers resistance to all NNRTIs. Patient 16 was treated with 3TC + TDF + EFV and showed a detectable plasma viral load (550 RNA copies/mL) after 36 months of follow-up without additional mutations. Overall, comparison of the amino acid sequences from the CD4 cells obtained at baseline and the plasma at baseline and the CD4 cells obtained during the follow-up showed comparable mutation patterns, particularly in the RT gene, with two new discrepant key mutations.

Table 3 Evolution of drug resistance mutations in 10 of 12 patients receiving nonnucleoside reverse transcriptase inhibitor (NNRTI)-based highly active antiretroviral therapy (HAART) detected using a direct sequencing assay of HIV-1 proviral DNA in purified CD4 cells

Patient no.	Treatment	Viral load (copies/ml)	CD4 count (cells/μl)	Subtype	Follow-up (months)*	Plasma RT mutations	Proviral RT mutations	Plasma PR mutations	Proviral PR mutations
9	Naïve	46 617	264†		0	D67N, K70R, K219Q	D67N, K70K/R, K219Q	Wild-type	Wild-type
9	3TC + TDF + EFV	< 50	345†		30	–	D67N, K70K/R, K219Q, **T69T/S**	–	Wild-type
10	Naïve	257†	–	B	0	Wild type	Wild type†	Wild type	Wild type†
10	3TC + ABC + EFV	< 50	415		22	–	Wild type	–	Wild type
11	Naïve	29 932	176†	CRF_01 AE	0	Wild type	Wild type	L10L/I	L10L/I
11	3TC + ABC + EFV	< 50	526		42	–	***Y188Y/H***	–	L10L/I
12	Naïve	4260	270	C		Wild type	Wild type	Wild type	Wild type
12	3TC + ABC + NVP	< 50	487†		37	–	Wild type	–	Wild type
13	Naïve	44 990	284	B	0	Wild type	Wild type	L10I	L10L/I
13	3TC + TDF + EFV	< 50	363		18	–	Wild type	–	L10L/I
13	ddI + 3TC + EFV	< 50	239		44	–	Wild type	–	Wild type
14	Naïve	37 904	–	?	0	E44D	E44D	Wild type	Wild type
14	3TC + ABC + EFV	< 50	549		41	–	E44D	–	Wild type
15	Naïve	19 572	279	B	0	Wild type	Wild type†	Wild type	Wild type†
15	3TC + ABC + EFV	< 50	422		34	–	Wild type	–	Wild type
16	Naïve	12 904	–	D	0	M41L	M41L	Wild type	Wild type
16	3TC + TDF + EFV	556	–		36	–	M41L	–	Wild type
17	Naïve	32 941	168†	A	0	T69S	T69S†	Wild type	Wild type†
17	3TC + TDF + EFV	< 50	194		17	–	T69S, ***M184M/I***	–	Wild type
18	Naïve	4051	225†	CRF_02 AG	0	Wild type	Wild type†	L10I	L10I†
18	3TC + ABC + EFV	< 50	389		33	–	Wild type	–	L10I

*Elapsed time between two samples, starting from zero for the baseline sample.
†Nucleotide sequence obtained using the Bayer Kit or another sample because of polymerase chain reaction (PCR) or sequencing failure.
–, not tested because of undetectable plasma viral load or lack of sample.
Mutations in bold: discrepant mutations between plasma and cells.
Mutations in italic: key mutations conferring high resistance.
PR, protease; RT, reverse transcriptase; 3TC, lamivudine; ABC, abacavir; ddI, didanosine; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir.

Resistance mutations detectable in plasma and CD4 cells after PI-based therapy

Table 4 shows the genotyping results for the RT and PR resistance mutations in plasma and CD4 cells from patients undergoing PI-based HAART for whom follow-up data were available. Twenty-two of the 32 patients, most of whom received LPV/r-based therapy, were followed for a mean time of 25 months (range 12–44 months). At the therapy-naïve stage, four (18%) of the 22 patients for whom amino acid sequences were obtained had at least one RT resistance mutation, while nine patients had at least one PR mutation. Patient 37 had a key RT mutation, K103K/N, which was present only in the cells and not in the plasma and which confers high drug resistance to all NNRTIs, while three patients had secondary mutations (T69T/S in the plasma for patient 26 and K70K/R in the cells for patients 24 and 32), which are not relevant for drug resistance. In addition to the K103N mutation conferring resistance to NNRTIs, virus from patient 37 also had the V82V/L mixed population, which confers a reduced response to tipranavir (TPV) boosted with ritonavir (r). One patient of 32 (patient 36) receiving an LPV/r-based regimen showed an increase in plasma RNA viral load from < 50 to 11 300 copies/mL after 18 months of follow-up, with an additional mutation. The additional M184I mutation was observed in the plasma RNA but not in the proviral DNA, and confers high-level resistance to 3TC. This patient was treated with d4T, abacavir (ABC) and LPV/r combination therapy for 1 year before being changed to a 3TC + TDF + LPV/r regimen because of poor compliance. Patient 33 had the M46M/I mixed population in the PR gene at the therapy-naïve stage. The plasma viral load was undetectable under HAART in most cases, but follow-up analysis of the proviral resistance mutations showed the presence of mutations detected at the therapy-naïve stage without additional mutations, except in the sequence from patient 36. Overall, comparison of resistance mutation patterns in CD4 cells with plasma RNA data or follow-up data for CD4 cells revealed similar results for the RT and PR genes, with one or two discrepant mutations.

Analysis of DNA resistance evolution and comparison with RNA

The analysis of DNA resistance evolution in all treated patients showed that the proportion of new mutations was 22% ($n = 6$) ($P < 0.0001$ for the difference from 0), and

Table 4 Evolution of drug resistance mutations in 22 of 32 patients receiving protease inhibitor (PI)-based highly active antiretroviral therapy (HAART) determined using a direct sequencing assay of HIV-1 proviral DNA in purified CD4 cells

Patient no.	Treatment	Viral load (copies/ml)	CD4 count (cells/μl)	Subtype	Follow-up (months)*	Plasma RT mutations	Proviral RT mutations	Plasma PR mutations	Proviral PR mutations
19	Naïve	260 567	289	?	0	Wild type	Wild type	Wild type	Wild type
19	d4T + ABC + LPV/r	150	259		11	–	Wild type†	–	Wild type†
20	Naïve	> 500 000	131†	CRF_03 AB	0	Wild type	Wild type†	L10I	L10I†
20	3TC + ABC + LPV/r	< 50	460		44	–	Wild type	–	L10I, **L10V**
21	Naïve	86 406	323	?	0	Wild type†	Wild type†	Wild type†	Wild type†
21	d4T + ABC + RTV + FPV	80	414		15	–	Wild type	–	Wild type
22	Naïve	2403	347†	CRF_02 AG	0	Wild type	Not amplified	Wild type	Not amplified
22	ZDV + 3TC + LPV/r	< 50	603		27	–	Wild type	–	Wild type
23	Naïve	32 279	50	B	0	Wild type	Wild type	Wild type	Wild type
23	ZDV + 3TC + LPV/r	< 50	146		13	–	Wild type	–	Wild type
23	3TC + TDF + EFV	< 50	185		25	–	Wild type	–	Wild type
24	Naïve	14 856	517†	B	0	Wild type	**K70K/R**	Wild type	Wild type
24	ZDV + 3TC + LPV/r	< 50	291		32	–	Wild type†	–	**Q58E**†
25	Naïve	35 014	45†	B	0	Wild type	Wild type†	Wild type	Wild type†
25	3TC + TDF + LPV/r	< 50	308		42	–	Wild type	–	Wild type
26	Naïve	166 659	1128	?	0	T69T/S	Wild type	**L10L/V**	Wild type
26	ZDV + 3TC + LPV/r	< 50	1021		8	–	Wild type	–	**Q58E**
26	Stops taking treatment	57			32	–	Wild type†	–	**Q58E**†
27	Naïve	20 517	187	A	0	Wild type	Wild type	Wild type	Wild type
27	ZDV + 3TC + LPV/r	< 50	509		13	–	Wild type†	–	Wild type†
28	Naïve	51 614	284†	B	0	Wild type	Wild type	Wild type	Wild type
28	ZDV + 3TC + LPV/r	< 50	185		19	–	Wild type	–	**Q58E**
29	Naïve	192 453	10	CRF02	0	Wild type	Wild type	Wild type	Wild type
29	ZDV + 3TC + RTV + FPV	< 50	204		16	–	Wild type	–	Wild type
30	Naïve	154 697	59†	G	0	Wild type	Wild type	Wild type	Wild type
30	3TC + TDF + RTV + APV	< 50	341		26	–	Wild type†	–	Wild type†
31	Naïve	1614	446	F	0	Wild type	Wild type	L10I, L10V	L10I, L10V
31	ZDV + 3TC + LPV/r	< 50	765		18	–	Wild type	–	L10V
32	Naïve	49 231	211	G	0	Wild type	**K70K/R**	Wild type	Wild type
32	3TC + TDF + RTV + APV	< 50	258		26	–	Wild type	–	Wild type
33	Naïve	457 496	302	CRF01	0	Wild type	Wild type	***M46M/I***	Wild type
33	ZDV + 3TC + RTV + FPV	< 50			19	–	Wild type	–	Wild type
34	Naïve	> 500 000	4	G	0	Wild type	Wild type	L10I	L10I
34	3TC + ABC + LPV/r	< 50	187†		17	–	Wild type	–	L10I
35	Naïve	215 771	225		0	Wild type	Wild type	Wild type	Wild type
35	3TC + ABC + LPV/r	< 50	501		37	–	Wild type	–	Wild type
36	Naïve	> 500 000	51	CRF_02	0	Wild type	Wild type	L10V	Wild type
36	d4T + ABC + LPV/r	< 50	386		13	–	Wild type	–	L10V
36	3TC + TDF + RTV + APV	11 319	362		18	***M184I***	Wild type	L10V	L10V
37	Naïve	44 934	272	CRF_06 CPX	0	Wild type	***K103K/N***	Wild type	**V82V/L**
37	3TC + d4T + LPV/r	< 50	496		11	–	Wild type	–	Wild type
38	Naïve	5872	200	B	0	Wild type	Wild type	Wild type	Wild type
38	3TC + ZDV + LPV/r	< 50	472		21	–	Wild type	–	Wild type
39	Naïve	56 117	64	B	0	Wild type	Wild type	Wild type	Wild type
39	3TC + TDF + LPV/r	< 50	534		42	–	Wild type	–	Wild type
40	Naïve	1119	334	CRF_02 AG	0	Wild type	Wild type	Wild type	Wild type
40	TDF + FTC + LPV/r	3618	347†		33	Wild type	Wild type	Wild type	Wild type
40	TDF + FTC + LPV/r	< 50	–		35	–	Wild type	–	Wild type

*Elapsed time between two samples, starting from zero for the baseline sample.
†Nucleotide sequence obtained by Bayer Kit or on another sample due to sequencing failure.
–, not tested because of undetectable plasma viral load or lack of sample.
Mutations in bold: discrepant mutations between plasma and cells.
Mutations in italic: Key mutations conferring high resistance.
ABC, abacavir; APV, amprenavir; ddI, didanosine; d4T, stavudine; EFV, efavirenz; FPV, fosamprenavir; FTC, emtricitabine; LPV/r, lopinavir boosted with ritonavir; NVP, nevirapine; PR, protease; RT, reverse transcriptase; RTV, ritonavir; TDF, tenofovir; 3TC, lamivudine; ZDV, zidovudine.

these included three new key mutations. However, the appearance of new mutations was not correlated with the time elapsed between sample collections. A logistic regression was performed and a P value of 0.34 [unitary odds ratio (OR) 1.03; global OR 3.24] was obtained. All the other covariates (patient characteristics and use of antiretroviral therapy) were found not to influence the incidence rate of new mutations. The comparison of pre-HAART RNA

genotyping with post-treatment DNA sequencing gave calculated prevalences of detected mutations of 59 and 78%, respectively. The proportion of detected mutations (19%) in the DNA was significantly higher than in the pre-HAART RNA by the χ^2 test ($P<0.0001$), with moderately good agreement between the two methods in terms of the number of detected mutations (kappa coefficient 0.56). A kappa coefficient of 0.50 indicated moderately good agreement in terms of predicted drug activity between the pretreatment RNA and pretreatment DNA mutation profiles, and a kappa coefficient of 0.40 indicated only fairly good agreement between the pretreatment RNA and post-treatment DNA mutation profiles, as a result of the accumulation of new mutations.

Discussion

Genotyping for HIV-1 drug resistance mutations is routinely performed on a plasma sample. At present, guidelines do not recommend HIV-1 drug resistance testing on cellular proviral DNA. The proviral compartment archives the various strains, either wild-type or drug-resistant, that arise during infection. The long-term persistence of archived drug-resistant DNA may jeopardize the efficacy of targeted drugs, and represents the 'resistance potential' profile of a patient [40]. This is important when switching antiretroviral agents or initiating treatment in patients without available historical data or conserved samples. Only a small percentage of lymphocytes (about 2% of all lymphocytes in the body) stay in the blood for a short time, before migrating to lymphoid and nonlymphoid organs. Eighty-five percent of re-circulating lymphocyte pool cells entering the lymph system are from the blood while about 15% are from the lymph. These data are mostly derived from animal experiments [34]. They underline the fact that an absence of resistance mutations in blood lymphocytes does not exclude the possibility that resistance is present. There is an increasing body of literature on the possible utility of assessing drug resistance mutations in the provirus. Our data are in accordance with previous observations and indicate the practical feasibility of sequencing the provirus. As reported by Bona *et al.* [30], we also found more drug resistance mutations, particularly key mutations, in the cell proviral DNA than in the plasma. Based on the Stanford mutation list, excluding polymorphisms and drug-selected mutations with no known significance, the proportion of mutations detected in the DNA was significantly higher than the proportion detected using standard RNA genotyping by the χ^2 test. At the therapy-naïve stage, we detected seven key mutations in the RT and PR genes in different patients (10% of all included patients), and four of these (M184M/V, M184M/I, K103K/N and M46M/I) were only found in the cells. Three key mutations (K103K/N, M46L and M46M/I) were found in different patients, for whom the follow-up was possible (4.3% of 69 patients included in the study). The K103K/N was not found in the plasma. At the time of study inclusion, 8% of patients had at least one RT mutation in the plasma, while 15% had at least one RT resistance mutation in CD4 cells. One therapy-naïve patient had virus with an RT resistance profile (67N, 70R and 219Q) in both CD4 cells and plasma. Before initiating treatment, PR gene sequencing showed that the percentage of patients with viruses carrying at least one PR mutation was 25% for CD4 cells and 23% for plasma. Wang *et al.* [31] and recently Ghosn *et al.* [32] reported a tight concordance of resistance profiles in paired HIV RNA and PBMC HIV DNA. Our own results demonstrate that at baseline only 55% of PR mutations and 56% of RT mutations were simultaneously present in CD4 cells and plasma, with substantial agreement between the two methods as assessed using kappa statistics. In their study, Usuku *et al.* [33] noted the persistence of a discrepancy between plasma and PBMCs for more than 3 years. In this study, the comparison between pretreatment amino acid sequences from CD4 cells and the plasma compartment and the comparison between pretreatment CD4 cell samples and follow-up CD4 cell samples showed a statistically significant proportion of new mutations of 22%, although the appearance of new mutations was not correlated with the time elapsed between tests. One of the 40 patients with follow-up samples had a key RT resistance mutation present in cells but not in plasma. Thirty-eight per cent of patients had RT mutations and 17 of the 40 patients with follow-up (43%) had one or two PR mutations in cells and plasma, but most of these were not relevant for drug resistance. In the NNRTI group, two patients (patients 11 and 17) of 10 who received at least 12 months of EFV-based HAART showed new key mutations (Y188Y/H and M184M/I), while one (patient 36) in the PI group and one naïve patient (patient 3) had a new key RT mutation (M184I). All new key mutations except one (in patient 36) were only present in the CD4 cells. Patient 36, who received d4T, ABC and LPV/r combination therapy for 1 year before changing to a 3TC, TDF and LPV/r regimen, showed a new key mutation (M184I) after 18 months of follow-up in the plasma RNA but not in the proviral DNA. Thus, monitoring of the evolution of drug resistance mutations in treated patients by direct sequencing of HIV-1 proviral DNA in purified CD4 cells revealed new mutations, with moderately good agreement between pre- and post-treatment DNA mutation patterns. In patients who remained treatment-naïve, almost no evolution was observed in mutations detected in plasma RNA or cell DNA. After therapy initiation we noted the persistence of HIV-1 drug resistance mutations in proviral DNA from purified

CD4 cells compared with plasma viral RNA at baseline. In our small cohort, 30 of 32 treated patients showed an undetectable plasma viral load after at least 12 months and up to 44 months of follow-up. Patients with pre-existing resistance mutations had a good response to all types of HAART, but none of them underwent combination therapy with the targeted drug. One interesting question was whether the DNA test might be useful to guide therapy switches in patients with suppressed viral load. This was addressed by comparing the prevalences of detected mutations in pretreatment RNA and post-treatment DNA (59 and 78%, respectively). A statistically significant proportion of mutations (19%) were detected in the DNA compared to the pretreatment RNA. The data demonstrated that sequencing DNA is possible and the recommended RNA sequencing might miss some mutations. In the comparison of pretreatment RNA with post-treatment DNA using kappa statistics, a moderately good agreement was found in terms of mutations detected and only a fairly good agreement in terms of predicting drug activity because of the accumulation of new mutations in the DNA. In patients with detectable viraemia, no new DNA mutations were detected and the viral loads were too low to enable RNA genotyping to be performed (patients 16, 19 and 21 with 556, 150 and 80 copies/mL, respectively). Therefore, we could not conclude that the standard method had underestimated the accumulation of mutations as the test was only possible on cell DNA samples. Transmission of drug-resistant HIV-1 strains and reduced susceptibility of viruses derived from untreated patients have been documented. Although wild-type virus may outgrow any initially resistant virus in patients with chronic asymptomatic HIV infection, some key mutations were consistently detected in this study. More resistance mutations were detected in the provirus in CD4 cells than in the virus in plasma and these mutations persisted for at least 1 year of follow-up with or without therapy, but the overall pattern of resistance was fairly similar in plasma and cells. HIV-1 proviral DNA would in our hands be most useful for making decisions, when changing therapy, on the best alternative treatment for patients with undetectable plasma viral load.

References

1 Boden D, Hurley A, Zhang L *et al*. HIV-1 drug resistance in newly infected individuals. *JAMA* 1999; **282**: 1135–1141.

2 Little SJ, Daar ES, D'Aquila RT *et al*. Reduced antiretroviral drug susceptibility among patients with primary HIV infection. *JAMA* 1999; **282**: 1142–1149.

3 Vercauteren J, Derdelinckx I, Sasse A *et al*. Prevalence and epidemiology of HIV type 1 drug resistance among newly diagnosed therapy-naive patients in Belgium from 2003 to 2006. *AIDS Res Hum Retroviruses* 2008; **24**: 355–362.

4 Wensing AMJ, Vercauteren J, van de Vijver DA *et al*. for the SPREAD Programme. Transmission of drug-resistant HIV-1 in Europe remains limited to single classes. *AIDS* 2008; **22**: 625–635.

5 Angarano G, Monno L, Appice A *et al*. Transmission of zidovudine-resistant HIV-1 through heterosexual contacts [Letter]. *AIDS* 1994; **8**: 1013–1014.

6 Yerly S, Vora S, Rizzardi P *et al*. Acute HIV infection: impact on the spread of HIV and transmission of drug resistance. *AIDS* 2001; **15**: 2287–2292.

7 Conlon CP, Klenerman P, Edwards A *et al*. Heterosexual transmission of human immunodeficiency virus type 1 variants associated with zidovudine resistance. *J Infect Dis* 1994; **169**: 411–415.

8 Hecht FM, Grant RM, Petropoulos CJ *et al*. Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. *N Engl J Med* 1998; **339**: 307–311.

9 Ippolito G, Del Poggio P, Arici C *et al*. Transmission of zidovudine-resistant HIV during a bloody fight [letter]. *JAMA* 1994; **272**: 433–434.

10 Imrie A, Beveridge A, Genn W *et al*. Transmission of human immunodeficiency virus type 1 resistant to nevirapine and zidovudine: Sydney Primary HIV Infection Study Group. *J Infect Dis* 1997; **175**: 1502–1506.

11 Veenstra J, Schuurman R, Cornelissen M *et al*. Transmission of zidovudine-resistant human immunodeficiency virus type 1 variants following deliberate injection of blood from a patient with AIDS: characteristics and natural history of the virus. *Clin Infect Dis* 1995; **21**: 556–560.

12 Yerly S, Kaiser L, Race E *et al*. Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet* 1999; **354**: 729–733.

13 Baxter JD, Mayers DL, Wentworth DN *et al*. Final results of CPCRA 046: a pilot study of antiretroviral management based on plasma genotypic antiretroviral resistance testing (GART) in patients failing antiretroviral therapy. *Antivir Ther* 1999; **4** (Suppl 1): 43.

14 Baxter JD, Mayers DL, Wentworth DN *et al*. A randomized study on antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing antiretroviral therapy. *AIDS* 2000; **14**: F83–F93.

15 Clevenbergh P, Durant J, Halfon P *et al*. Persisting long-term benefit of antiretroviral genotypic guided treatment for HIV-infected patients failing HAART. *Antivir Ther* 1999; **4** (Suppl 1): 42.

16 Cohen C, Hunt S, Sension M *et al*. for the VIRA3001 Study Team. A randomized trial assessing the impact of phenotypic resistance testing on antiretroviral therapy. *AIDS* 2002; **16**: 579–588.

17 Tural C, Ruiz L, Holtzer C *et al*. Clinical utility of HIV-1 genotyping and expert advice: the Havanna trial. *AIDS* 2002; **16**: 209–2018.

18 Hirsch MS, Günthard HF, Schapiro JM *et al.* Antiretroviral drug resistance testing in adult HIV-1 infection: 2008 recommendations of an international AIDS society–USA panel. *Clin Infect Dis* 2008; **47**: 266–285.
19 Clumeck N, Pozniak A, Raffi F and the EACS Executive Committee. European AIDS Clinical Society (EACS) guidelines for the clinical management and treatment of HIV-infected adults. *HIV Med* 2008; **9**: 65–71.
20 Grant RM, Hecht FM, Warmerdam M *et al.* Time trends in primary HIV-1 drug resistance among recently infected persons. *JAMA* 2002; **288**: 181–198.
21 Hammer SM, Saag MS, Schechter M *et al.* Treatment for adult HIV infection, 2006 recommendations of the international AIDS society–USA panel. *JAMA* 2006; **296**: 827–843.
22 The Euro Guidelines Group for HIV resistance. Clinical and laboratory guidelines for the use of HIV-1 drug resistance testing as part of treatment management: recommendations for the European setting. *AIDS* 2001; **15**: 309–320.
23 Vandamme AM, Sonnerborg A, Ait-Khaled M *et al.* Updated European recommendations for the clinical use of HIV drug resistance testing. *Antivir Ther* 2004; **9**: 829–848.
24 Wensing AMJ, van de Vijver DA, Angarano G *et al.* Prevalence of drug resistant HIV-1 variants in untreated individuals in Europe: implications for clinical management. *J Infect Dis* 2005; **192**: 958–966.
25 Descamps D, Flandre P, Calvez V *et al.* Mechanisms of virologic failure in previously untreated HIV-infected patients from a trial of induction-maintenance therapy. Trilege (Agence Nationale de Recherches sur le SIDA 072) Study Team. *JAMA* 2000; **283**: 205–211.
26 Havlir DV, Hellmann NS, Petropoulos CJ *et al.* Drug susceptibility in HIV infection after viral rebound in patients receiving indinavir-containing regimens. *JAMA* 2000; **283**: 229–234.
27 International AIDS Society-USA Resistance Mutations Project Panel. Update of the drug resistance mutations in HIV-1. Available at www.iasusa.org/resistance_mutations (accessed 3 February 2006).
28 Riva E, Pistello M, Narciso P *et al.* Decay of HIV type 1 DNA and development of drug-resistant mutants in patients with primary HIV type 1 infection receiving highly active antiretroviral therapy. *AIDS Res Hum Retroviruses* 2001; **17**: 1599–1604.
29 Chew CB, Potter SJ, Wang B *et al.* Assessment of drug resistance mutations in plasma and peripheral blood mononuclear cells at different plasma viral loads in patients receiving HAART. *J Clin Virol* 2005; **33**: 206–216.
30 Bona I, Gibellini D, Borderi M *et al.* Genotypic resistance in plasma and peripheral blood lymphocytes in a group of naive HIV-1 patients. *J Clin Virol* 2007; **38**: 313–320.
31 Wang YM, Dyer WB, Workman C *et al.* Drug resistance and viral evolution in plasma and peripheral blood cells during structured treatment interruption (STI) and non-interrupted HAART. *Curr HIV Res* 2007; **5**: 235–250.
32 Ghosn J, Pellegrin I, Goujard C *et al.* for the French PRIMO Cohort Study Group (ANRS CO 06). HIV-1 resistant strains acquired at the time of primary infection massively fuel the cellular reservoir and persist for lengthy periods of time. *AIDS* 2006; **20**: 159–170.
33 Usuku S, Noguchi Y, Sakamoto M *et al.* Analysis of a long-term discrepancy in drug-targeted genes in plasma HIV-1 and PBMC HIV-DNA in the same patient. *Jpn J Infect Dis* 2006; **59**: 122–125.
34 Pabsta R, Rosenbergb YJ. Interpreting data on lymphocyte subsets in the blood of HIV patients – organ distribution, proliferation and migration kinetics are critical factors. *Pathobiology* 1998; **66**: 117–122.
35 Schnuda ND. Circulation and migration of small blood lymphocytes in the rat. I. Kinetics of lymphocyte circulation in the lymphoid organs. *Am J Pathol* 1978; **93**: 623–638.
36 Girard PM, Katlama Ch, Pialoux G. *VIH*. Paris: Doin, 2001.
37 Schmit JC, Cogniaux J, Hermans P *et al.* Multiple drug resistance to nucleoside analogues and nonnucleoside reverse transcriptase inhibitors in an efficiently replicating human immunodeficiency virus type 1 patient strain. *J Infect Dis* 1996; **174**: 962–968.
38 The Stanford mutation list version 6.0.3. Available at http://hivdb.stanford.edu/pages/algs/sierra_mutation.html (accessed 1 September 2009).
39 Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**: 159–174.
40 Noë A, Plum J, Verhofstede C. The latent HIV-1 reservoir in patients undergoing HAART: an archive of pre-HAART drug resistance. *J Antimicrob Chemother* 2005; **55**: 410–412.