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Both Human Immunodeficiency Virus Cellular DNA Sequencing and Plasma RNA Sequencing Are Useful for Detection of Drug Resistance Mutations in Blood Samples from Antiretroviral-Drug-Naive Patients▽

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Genotypic antiretroviral testing is recommended for newly infected drug-naive subjects, and the material of choice is plasma RNA. Since drug resistance mutations (DRMs) may persist longer in cellular DNA than in plasma RNA, we investigated whether the use of peripheral blood mononuclear cell (PBMC) human immunodeficiency virus (HIV) DNA increases the sensitivity of genotypic testing in antiretroviral-drug-naive subjects. We compared the rate of primary drug resistance in plasma RNA and PBMC DNA in 288 HIV type 1-infected drug-naive persons tested at a single clinical virology center from June 2004 to October 2006. Resistance in the plasma compartment to at least one drug was detected for 64 out of 288 (22.2%) naive patients and in the PBMC compartment for 56 (19.4%) patients. Overall, DRMs were found in 80 out of 288 (27.8%) patients. PBMC DRMs were present in plasma RNA from 16 subjects with wild-type virus infections. Another nine patients had additional DRMs in PBMCs with respect to those detected in plasma RNA. On the other hand, extra plasma DRMs were detected in PBMCs for 24 and 8 subjects with wild-type and drug-resistant virus, respectively. Resistance to more than one class of antiretroviral drug was detected by plasma and PBMC analysis for 25.0% and 36.2% of the subjects, respectively. Our data support the potential utility of genotypic resistance testing of PBMC DNA in conjunction with the currently recommended plasma RNA analysis.

Transmission of drug-resistant human immunodeficiency virus type 1 (HIV-1) to newly infected subjects is well recognized. In a European study evaluating the 1996-to-2002 time period, resistant variants were found in 13.5% of recently infected patients and in 8.7% of chronically infected subjects (29). In a study conducted in the United States during 1997 to 2001 among 1,082 drug-naive persons who had been diagnosed as being infected with HIV during the previous 12 months, 8.3% had reverse transcriptase (RT) or major protease (PR) mutations associated with reduced antiretroviral-drug susceptibility (28). Similarly, in another U.S. study conducted between 1999 and 2001 among chronically infected patients, the overall estimated prevalence of resistance mutation was 8.8% (14). In a very recent contribution from the United States, an overall prevalence of resistance of 18% among 192 HIV-infected naive patients tested in 2003 and 2004 was reported (6).

Indeed, current guidelines recommend the use of antiretroviral resistance testing of drug-naive subjects who are either acutely or chronically infected, particularly in geographic areas where primary resistance has been consistently documented (8). Infection with a virus already resistant to antiretroviral drugs has been reported to have a negative impact on the initial response to highly active antiretroviral therapy (HAART) and to shorten the time to first virological failure (10). However, recent evidence suggests that the impact of transmitted drug resistance may be short term provided that HAART is guided by antiretroviral resistance testing (15, 21). While plasma RNA is the recommended material for drug resistance testing, little is known about the persistence of drug resistance mutations (DRMs) acquired during primary infection in the plasma of patients not subjected to early therapy. In principle, drug-resistant variants in the absence of therapy should be readily outcompeted by possibly coinfecting wild-type virus or should slowly back mutate to the wild type. In fact, transmitted DRMs in plasma RNA from drug-naive subjects have been shown to be detectable for up to 3 years (1, 17). A reasonable hypothesis is that DRMs persist at detectable levels longer in PBMC DNA than in plasma RNA due to the different rates of turnover of the virus in the two compartments (20). Indeed, discrepancies between drug resistance mutations in virus populations harbored in plasma RNA and PBMC DNA have been reported for subjects failing therapy as well as following cessation of treatment (24, 27). Furthermore, drug resistance mutations were virtually identical in plasma RNA

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TABLE 1. Patient characteristics

Characteristic	All patients	Chronically infected patients	Patients with primary or recent infection
Patients no.	288	231 (80.2%)	57 (19.8%)
Age	40 (SD, ±11)	40.5 (SD, ±11)	38 (SD, ±10)
Male	237 (82.2%)	185 (80%)	52 (91.2%)
No. of viral subtype B strains	227 (78.8%)	180 (77.9%)	47 (82.4%)
CD4 cell count (cells/mm^3)	394 (SD, ±263)	362 (SD, ±251)	539 (SD, ±269)
CD4 cell percentage	21 (SD, ±10)	20 (SD, ±9.5)	26 (SD, ±12)
HIV RNA load log (copies/ml)	5.165 (SD, ±5.152)	5.179 (SD, ±5.173)	5.129 (SD, ±5.113)

and PBMC DNA in the only study published so far on drug-naive subjects (5). Nevertheless, detection by clonal analysis of early archivation of DRMs in a patient with primary infection was recently reported (18).

In order to further investigate whether sequences obtained from PBMCs provide information on transmitted resistance that is better than or complementary with the information provided by sequences obtained from plasma for drug-naive patients with either chronic or acute infection, we performed a prospective analysis of a large number of subjects attending five different infectious diseases units that refer to a single laboratory for antiretroviral drug resistance testing.

MATERIALS AND METHODS

Study population. A total of 301 drug-naive HIV-1-infected persons attending five infectious diseases units located in Veneto in northeastern Italy were consecutively recruited from 15 June 2004 to 31 October 2006 after their written, informed consent to the study was obtained.

Eligibility criteria included age >18 years and antiretroviral-drug-naive status according to personal interview and a review of the history of infection from the first positive serological test; this was always performed in the enrolling hospital.

Blood samples were submitted to the Laboratory of Virology at the University Hospital of Padua, stored within 6 h of collection, and subsequently analyzed.

Primary or recent HIV infection was defined according to the presence of either of following criteria: (i) a negative or indeterminate HIV antibody enzyme-linked immunosorbent assay result associated with a positive plasma HIV RNA result, or (ii) an initially negative test for HIV antibody followed by a positive serology result within 18 months.

Genotypic analysis of plasma and PBMCs. Blood collected in EDTA was separated into plasma and cells by Ficoll-Paque Plus density gradient centrifugation. Aliquots of plasma and 2×10^6 PBMCs in dry pellets were stored at −80°C until use.

Plasma (1.5 ml) was centrifuged at 4°C for 1 h at 21,000 × *g*. The resulting pellet was processed by use of a QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany), with processing performed according to the instructions provided by the manufacturer, and resuspended in 60 μl of RNA diluent. RT-PCR was carried out on these extracts by use of Superscript One-Step reverse transcriptase (Invitrogen, Carlsbad, CA) and primers designed in house (19). The products from these reactions were amplified by a nested PCR performed in house (19), generating amplicons that encompass the entire HIV PR coding region and the first 324 codons of the RT coding region. DNA was extracted from 2×10^6 PBMCs from patients by use of a QIAamp DNA Blood Biorobot kit (QIAGEN, Hilden, Germany), with the process performed according to the instructions provided by the manufacturer. HIV DNA was amplified as described above, with the exclusion of the RT step.

A TruGene HIV-1 genotyping kit (Bayer Health Care LLC, Tarrytown, NY) was used to identify mutations. All sequences with DRMs in plasma and/or PBMCs were reanalyzed by use of a ViroSeq HIV-1 genotyping system (Celera Diagnostics, Alameda, CA) to confirm the first result.

Prediction of susceptibility and subtype analysis. Assessment of the possible impact of DRMs on the response to HAART was performed by the use of the Stanford HIVdb drug-resistance algorithm, version 4.2.6 (available at http://hivdb.stanford.edu). The Stanford Database algorithm assigns a drug-specific score to each DRM detected. The final score obtained from the combination of all DRMs observed in a single viral strain is translated into one of five levels of susceptibility: susceptibility, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance (22). In this study, reduced susceptibility was scored when the Stanford HIVdb system indicated the presence of at least low-level resistance, allowing only clinically relevant mutation patterns to be considered.

Subtyping was performed through use of the Rega Institute subtyping tool, version 2.0, available at http://dbpartners.stanford.edu/RegaSubtyping/.

RESULTS

Patient characteristics. Of the 301 subjects enrolled in the study, 288 were included in the results. For 13 individuals, we could not obtain a sequence from PBMCs ($n = 11$), from plasma ($n = 1$), or from both plasma and PBMCs ($n = 1$), yielding a 95.7% success rate in amplification in total for both plasma RNA and PBMC DNA. Table 1 shows the main characteristics of the 288 patients analyzed, less than 20% of whom had had a primary or recent infection when tested.

DRMs and inferred drug susceptibility. Major DRMs were detected in plasma RNA and/or PBMC DNA for 80 out of 288 (27.8%) patients (Table 2). The rates of detection of any DRM in plasma RNA versus PBMC DNA were not statistically different (22.2% versus 19.4%), but the discrepancy between the results for the two compartments was appreciable. Indeed, of the 80 patients showing plasma RNA and/or PBMC DNA resistance, there were 24 and 16 patients for whom DRMs were detected only in plasma and only in PBMCs, respectively, in the paired compartment in the context of a wild-type virus.

TABLE 2. Patients expected to be resistant to at least one drug based on analysis of plasma RNA, PBMC DNA, or either or both

Analysis category	No. of patients expected to show resistance to indicated drug class							
	NRTI	NNRTI	PI	NRTI plus NNRTI	NRTI plus PI	NNRTI plus PI	All three classes	Total
Plasma RNA	21	15	12	6	4	1	5	64
PBMC DNA	16	12	7	5	9	2	5	56
Plasma RNA and/or PBMC DNA	23	19	16	6	9	2	5	80

TABLE 3. Patients with archived DRMs only and wild-type strains or minor mutations in plasma

Patient code	PR DRM(s)		RT DRM(s)	
	Plasma	PBMC	Plasma	PBMC
9	M36I L63P	L10IV K20IM M36I M46IM I54IV L63P V77IV V82CFGV		M41LM D67DN A98AP V118IV M184MV L210LW T215NSTY
19	M36L L63P	L10IL M36IL M46IM I54IV L63P V77IV V82CFGV		M184MV T215ST
28	M36I V77I	M36I V77I		Y181HY
32	K20KM M36I L63A I93L	K20KM D30DN M36I M46IM D60DN L63A I93L		T215ST P225PT
41	M36IM L63Q	L10I K20I M36I M46IM I54IV L63HLQ V77IV V82CFGV		M41LM D67DN V118N M184MV L210LW T215NSTY
62	L63P I93L	K20R L23I L63P I93L	V118I	V118I
64	M36L	M36L		G190EG
65	L63H V77I I93L	M46I L63H V77I I93L		K219I M230LM K238I
83	K20KR M36L L63P	K20R M36L L63P L90LM		
97	I54IN L63A	M46LM I50IN I54IN L63AT		
122	L63P V77I I93L	L63P V77I I93L		M41L T69S
138	L10I L33V I93L	L10I L33V M46IM I93L		
159	L63G	L63G	M41K	G190E M230V
193	M36I L63P I93L	M36I L63P I93L		V179ILM M184IMR
200	K20R M36IL L63P A71T	L33I L63P V77I I93L		D67N
234	L33V	L33V	T215A	G190EG T215A

Table 3 shows the details for the 16 patients with DRMs only in PBMCs which would have gone undetected using standard plasma RNA sequencing. Furthermore, nine other patients had additional mutations only in the PBMC compartment besides those present both in plasma and PBMCs. The additional DRMs were mutations conferring resistance to nucleoside reverse transcriptase inhibitors (NRTIs) in two cases, to non-NRTIs (NNRTIs) in one case, to protease inhibitors (PIs) in three cases, and to NRTIs plus PIs in three cases. On the other hand, only eight patients had additional DRMs only in plasma RNA in the context of other DRMs detected in both compartments.

All sequences with DRMs in plasma and/or PBMCs were reanalyzed starting from the amplicon as described above. For the primary DRMs complete concordance was observed.

Only the samples from 16 patients with archived DRMs were resequenced, and the results were always concordant.

Interestingly, there was a trend towards a higher rate of inferred two-class resistance in PBMC DNA than in plasma RNA (36.2% versus 25.0%; $P = 0.16$) despite an overall lower number of patients with results showing resistance in PBMC DNA. For the whole subset of patients with any evidence of drug resistance both in plasma and in PBMC, the number of DRMs was not significantly larger in DNA than in RNA (94 versus 88). Thus, taking into account all of the discordant DRM results, different mutation patterns originating from paired sequences were detected for 55 patients.

Regarding the predicted loss of treatment options, 48 of 64 (75.0%), 35 of 56 (97.2%), and 58 of 80 (72.5%) patients had resistance to drugs of only one class according to the results of analysis of plasma, PBMCs, and combined plasma and PBMCs, respectively.

Looking at patients with resistance mutations in PBMCs only, inclusion of low-resistance mutations allowed us to characterize 10 subjects with single-class mutations, 5 with two-class mutations, and 1 with three-class mutations; by contrast, when only intermediate-resistance and high-resistance mutations are considered, the same figures are 5, 2, and 1 for single-class, two-class, and three-class mutations, respectively.

Looking to other discordant subjects with more DRMs in PBMCs than in plasma, inclusion of low-resistance mutations allowed us to characterize only one more subject with class 2 resistance; the other eight patients had intermediate- or high-resistance class 1, class 2, and class 3 mutations in one, six, and one cases, respectively.

The most frequent DRMs detected were at codons 41 (8.3%) and 215 (11.1%) in the RT region and at codon 46 (8.3%) in the PR region (Table 4). Although RT mutations M184I/V and L210W and PR mutations I54V and V82A/F/T/S were detected in PBMC DNA at a rate more than twofold higher than in plasma RNA, none of these differences was statistically significant due to the low number of occurrences. Mutations at codon 215 were more often amino acids representative of partial transition from the resistant Y and F than the Y/F itself (23 versus 9 cases; $P = 0.0003$), suggesting an ongoing process of reversion of resistant strains to the wild type for most patients.

Subtype characterization. Subtype B was the most prevalent HIV clade in this file, being detected in 227 (78.8%) subjects. The non-B subtype included 7 clade A, 4 C, 3 D, 9 F, 5 G, 6 CRF01_AE, and 27 CRF02_AG virus strains. Of these, 33 (54.1%) were detected in Italian citizens. There were no statistically significant differences in the rates of plasma or PBMC DRMs between B and non-B subtypes. Taken together, 17 out of 61 (27.8%) non-B type strains were resistant.

Primary or recent infections. Fifty-seven (19.8%) subjects in the entire cohort were identified as having primary or recent infection at the time of testing. Eighteen (31.5%) subjects with primary or recent infection had DRMs in plasma and/or PBMCs compared to 62 (26.8%) subjects among 231 subjects with chronic infections, this difference being not statistically significant. Among those with primary or recent infections, 17 subjects had DRMs conferring resistance to drugs of a single class, and a single patient had a complex picture of a triple-class

TABLE 4. Distribution of major DRMs detected in plasma only, PBMCs only, or both

DRM	No. of concordant plasma and PBMC results	No. of results for plasma only	No. of results for PBMCs only	No. (%) of results for all patients	% of results for patients with DRMs
Protease					
D30N		1	1	2 (0.69)	2.5
M46IL	4	7	13	24 (8.33)	30
I54V		1	5	6 (2.08)	7.5
V82AFTS	1	1	4	6 (2.08)	7.5
I84V	1		1	2 (0.69)	2.5
L90M	3		1	4 (1.38)	5
Reverse transcriptase					
M41L	16	4	4	24 (8.33)	30
D67GN	4	4	4	12 (4.16)	15
K70R	2	2		4 (1.38)	5
K103NE	9	1	2	12 (4.16)	15
V106A	2	1		3 (1.04)	18.75
M184VI		3	7	10 (3.47)	12.5
Y188L		2		2 (0.69)	2.5
G190AES	3		4	7 (2.43)	8.75
L210W	4	2	5	11 (3.81)	13.75
T215YF	2	3	4	9 (3.12)	11.25
T215CDEISV	14	4	5	23 (7.98)	28.75
K219QS	6	3		9 (3.12)	11.25

resistance, mainly archived in DNA. Four (7%) subjects with primary or recent infection had only PBMC DRMs, two of which were related to NRTI resistance, one to NNRTIs, and one to PIs. Of the 18 DRMs, 2 were from non-B subtype and the remaining 16 from B subtype strains.

Overall, there were no statistically significant differences in the rates of DRMs in patients with primary or recent infection compared to patients with chronic infection. Likewise, we did not observe significant differences in the distribution of DRMs in plasma and PBMCs between patients with primary or recent infection and patients with chronic infection. By contrast, multiclass resistance was found in 21 of 62 (33.8%) chronically infected patients with DRMs compared to 1 of 18 (5.5%) patients with DRMs and primary or recent infection. The difference in the percentages of multiclass resistance detected in patients with primary or recent infections versus chronically infected patients was statistically significant ($P = 0.03$).

DISCUSSION

We demonstrated a 22.2% prevalence of DRMs conferring at least low-level resistance to at least one drug for plasma RNA from drug-naive patients tested in 2004 to 2006 in a northeastern region of Italy (Veneto). A relevant number of subjects had DRMs related to different drug classes: 16 out of 64 (25%) resistant strains when looking at the plasma compartment, and 22 out of 80 (27.5%) taking into account DNA also. Thus, 22 out of 288 (7.6%) patients were found to have multi-class-resistant viral strains. Variable rates of primary drug resistance have been reported for untreated individuals. The prevalence found in this study, conducted between 2004 and 2006, is higher than the prevalence reported in previous studies (28, 29). Data from a study conducted in 1997 to 2001 in the United States show a 1.3% rate of multi-class-resistant variants among naive patients (28). Likewise, a study from Europe for the period 1996 to 2002 reports a 2.0% rate of these resistant variants (29).

The most relevant result of this study is the underestimation of transmitted resistance derived by routine plasma analysis that is revealed by the examination of the virus population archived in PBMCs. As many as 25 subjects were shown to carry in their PBMCs a more extensive set of DRMs, resulting in a worse drug sensitivity score with respect to what predicted by routine analysis of plasma RNA. It must be emphasized that PBMC DNA sequencing cannot be proposed as a substitute for plasma RNA sequencing. Indeed, a larger number of patients had DRMs in the plasma than in the PBMC compartment.

Recently, the results of a study aimed at comparing cell-free and cell-associated resistance have been published (2). The study, conducted with 31 naive patients, showed that direct sequencing of DNA provirus disclosed key mutations in samples from seven patients and that five of these mutations were not detected by routine plasma analysis. The authors concluded that, due to the small size of the study, further observations on this subject are required. To this end, our report adds more evidence from a large number of patients with respect to the importance of comparative evaluation of resistance profiles in plasma and PBMC.

The use of PBMC DNA in addition to plasma RNA is expected to confer the highest sensitivity to detection of primary resistance via population sequencing. In this study, using both sources would have detected 16 additional cases, a 25% increase with respect to routine plasma RNA analysis. Since separate analyses of plasma RNA and PBMC DNA would translate to doubling the cost and time involved, alternative approaches could be tested, including combining plasma RNA and PBMC DNA extracts or using mixed nucleic acids obtained from PBMCs.

A potential bias that could have weakened our findings is

incorrect sample sequencing in the discordant cases due to the fluctuating representation of drug-resistant minority species at the threshold of sensitivity of population sequencing. To control for this possibility and minimize its impact, we resequenced all the discordant cases and confirmed the results. This suggests that the results derived from our analysis are not artifactual, although the relative amounts of the different genomes in the two compartments could be clarified only by clonal analysis (18).

The issue of the limited sensitivity of routinely used and Food and Drug Administration-cleared genotypic drug resistance testing is inherent in population-sequencing technology and has been shown to result in an underestimation of transmission of drug-resistant variants (12). Fluctuations over time have been reported for the proportion of recent HIV-1 seroconverters harboring drug-resistant strains as well as for the proportion of patients with chronic HIV-1 infection with undetectable viral load (4). Moreover, potential transmitters include subjects with no prior exposure to therapy who may even be unaware of their HIV status and patients failing therapy with or without a drug-resistant virus infection. Therefore, an inverse correlation has been assumed between the proportions of virological suppression in chronic HIV-1 carriers associated with the use of HAART and rates of transmission of drug-resistant viruses among individuals with a new HIV-1 infection in a given community (4). It will be interesting for future studies to address this issue by means of combined plasma and cellular resistance analysis.

The clinical significance of drug-resistant minority species remains to be fully assessed, but preliminary evidence suggests that the presence of minor variants which may be missed by standard plasma RNA genotyping can lead to the failure of subsequent treatments (3, 13, 23). The inadequacy of standard sequencing of the bulk PCR product for detecting low-frequency DRMs was demonstrated by single-genome sequencing and serial dilution of plasma RNA (16), and many strategies are being explored to improve the detection of low-frequency DRMs to assess the potential impact of different codons, viral heterogeneity, and the emergence and decay of minor mutants (7, 11).

The discordance between plasma and PBMC resistance patterns may have several explanations depending on whether the infection is in the acute, early, or chronic phase. Patients infected by mixtures of drug-resistant and wild-type strains are expected to experience an overgrowth of the wild-type virus in the absence of any drug pressure due to the frequently decreased replicative capacity consequent to selection of most major drug resistance mutations. The time required for the decrease of the drug-resistant species to undetectable levels is a function of the relative proportions of the two competing species in the virus inoculum and the difference in replicative capacity. Infection with a drug-resistance strain in the absence of the wild-type virus should result in a longer detectability of DRMs, since true reversion to the wild type requires a stochastic process of variation and selection. The small number of tested patients with primary or recent infection in our case file did not allow us to detect any differences with respect to chronically infected patients either in the overall prevalence of DRMs or in the ratio of plasma RNA to PBMC DNA DRMs. However, the resistance patterns found in recently or acutely infected subjects appeared to be less extensive than those found in chronically infected patients, mostly involving only one drug class. Similar findings were reported in 2004 for 14 out of 17 newly infected patients (26). In theory, a more prolonged time of HIV infection could allow multiple rounds of transmission of different drug-resistant viruses, resulting in more extensive resistance patterns in drug-naive subjects tested at later stages of infection. However, findings pertaining to the relatively rare occurrence of HIV superinfection (9, 25) do not seem to support this hypothesis. Multiple parallel testing of plasma and PBMCs at different time points could elucidate the kinetics of transmitted resistance over time. Low-resolution cross-sectional sequencing of the bulk PCR products obtained from plasma and PBMCs does not provide a detailed view of the natural history of primary resistance. However, our data demonstrate that PBMC DNA analysis in conjunction with the currently recommended plasma analysis has the potential to increase the sensitivity of the detection of drug resistance in drug-naive subjects. Further studies of larger HIV populations are warranted to define the role of DNA genotyping in both clinical and research settings.

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