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Comparison of Oligonucleotide Ligation Assay and Consensus Sequencing for Detection of Drug-Resistant Mutants of Human Immunodeficiency Virus Type 1 in Peripheral Blood Mononuclear Cells and Plasma

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Drug-resistant mutants of human immunodeficiency virus type 1 (HIV-1) recede below the limit of detection of most assays applied to plasma when selective pressure is altered due to changes in antiretroviral treatment (ART). Viral variants with different mutations are selected by the new ART when replication is not suppressed or wild-type variants with greater replication fitness outgrow mutants following the cessation of ART. Mutants selected by past ART appear to persist in reservoirs even when not detected in the plasma, and when conferring cross-resistance they can compromise the efficacy of novel ART. Oligonucleotide ligation assay (OLA) of virus in plasma and peripheral blood mononuclear cells (PBMC) was compared to consensus sequence dideoxynucleotide chain terminator sequencing for detection of 91 drug resistance mutations that had receded below the limit of detection by sequencing of plasma. OLA of plasma virus detected 27.5% (95% confidence interval [CI], 19 to 39%) of mutant genotypes; consensus sequencing of the PBMC amplicon from the same specimen detected 23.1% (95% CI, 14 to 34%); and OLA of PBMC detected 53.8% (95% CI, 44 to 64%). These data suggest that concentrations of drug-resistant mutants were greater in PBMC than in plasma after changes in ART and indicate that the OLA was more sensitive than consensus sequencing in detecting low levels of select drug-resistant mutants.

Drug-resistant viruses pose a major obstacle for the effective treatment of human immunodeficiency virus type 1 (HIV-1) infection (17). When antiretroviral therapy (ART) does not suppress viral replication, drug-resistant variants are selected (17). Changes in ART result in the selection of alternative mutants or, if stopped, wild-type viruses predominate (6, 11) due to their greater replication capacity (18). Drug-resistant mutants appear to persist in PBMC after either primary infection with drug-resistant virus or after selection by drug therapy, even when not detected in the plasma (2, 8, 16, 21). Mutants from PBMC and often from other viral reservoirs may be rapidly selected once ART is started or resumed (17). Drug-resistant viruses can compromise the efficacy of ART by effectively reducing the number of active antiretroviral drugs (20). Therefore, detecting low levels of drug-resistant mutants within the viral population should assist in prescribing effective therapy.

Tests for drug-resistant HIV-1 in clinical settings commonly utilize consensus assays that sequence the region of virus that encodes protease (PR) and reverse transcriptase (RT) (17) and generally are applied to virus from plasma. Minor genotypes, those at concentrations that are ≤50% of the viral population, are not consistently detected by consensus sequencing (24). An oligonucleotide ligation assay (OLA) can detect mutant genotypes present at concentrations as low as 5% among wild-type virus (5, 14). Because PBMC appear to act as an archive of HIV-1 replication-competent genotypes selected in the past (16, 21), we hypothesized that application of the OLA to PBMC would identify mutants that had receded below the level of detection by consensus sequencing of plasma. To evaluate the relative sensitivity of the OLA compared to that of consensus sequencing and to test the sensitivity of PBMC versus that of plasma, both assays were applied to the same PCR amplicons derived from the PBMC and plasma of specimens from which mutant viruses had receded below the limit of detection as determined by consensus sequencing.

MATERIALS AND METHODS

Study design and patient specimens. Plasma specimens submitted for HIV-1 genotypic resistance testing at the University of Washington's Clinical Virology Laboratory underwent PCR amplification and consensus dideoxynucleotide chain terminator sequencing of HIV-1 *pol* for determination of mutant genotypes. Sequence was determined without reference to results from the individual's previously submitted specimens. Before sequence results were reported, chromatograms from specimens that had apparently lost a mutation were reviewed for any evidence of the lost drug resistance mutation. Specimens were selected for this study if a mutation detected in the patient's penultimate specimen was confirmed on three independent reviews of the chromatogram to be absent in the present specimen. DNA was extracted from the PBMC of the

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selected specimens, and the consensus dideoxynucleotide sequence was directly determined from PCR-amplified HIV-1 *pol*. The plasma and PBMC amplicons that had been sequenced were also evaluated by the OLA.

Nucleic acid extraction. RNA, extracted from 1 or 2 ml of plasma (viral load, >50 copies/ml) using silica as previously described (14), was resuspended in 50 μl of nuclease-free water. DNA was extracted from PBMC by using the Isoquick kit (Orca Research Inc., Bothell, Wash.) or the Puregene Cell and Tissue kit (Gentra Systems, Inc., Minneapolis, Minn.) according to the manufacturers' instructions and were resuspended in up to 50 μl.

RT-PCR and PCR. Ten microliters of RNA extracted from plasma was reverse transcribed by using the GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, Calif.). The first-round PCR of cDNA or of DNA extracted from PBMC was carried out in a 50-μl reaction mixture containing 10 μl of cDNA or 1 μg DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 20 pmol of the primers PRL (GGGACCAGCGGCTACACTAGAAG AAATGATGACAGCATGTCAGG) and RT2 (14), and 2.5 U of *Taq* DNA polymerase (Sigma-Aldrich Corp., St. Louis, Mo.). Cycling conditions were as described previously (14) with a final extension at 72°C for 7 min. Second-round PCR contained 2 μl of first-round product and 20 pmol of primers PRC (CTC CCCCTCAGAAGCAGGAGCCGATAGACAAGGAAACTGTATCC) and RT3 (14), with identical cycling conditions. The amplicon, a 1,033-bp DNA fragment extending from nucleotide 1438 of HIV-1 *gag* to nucleotide 711 of the RT gene according to the HXB-2 numbering system, was visualized in a 1.5% agarose gel with ethidium bromide staining. The amount of DNA resulting from nested PCR was estimated based on the band intensity relative to a DNA Mass Ladder on the same gel. DNA concentrations estimated to be between 12 to 20 ng/μl were subjected to direct sequencing.

Sequencing. The PCR amplicon was purified of residual PCR primers and deoxynucleoside triphosphates by treatment with ExoSAP-IT (Amersham Biosciences, Piscataway, N.J.). Four primers were used in bidirectional sequencing of the PCR products, including protease primers PRC and PR2 (GGAGTATT GTATGGATTTTCAGGCC), covering 459 bp beginning 9 bases upstream of codon 1 and extending to codon 51 of RT, and RT primers RT4 (GGATGGC CCAAAAGTTAAAC) and RT3, spanning 625 bp from RT codons 23 to 230. Two microliters of PCR amplicon was added to the sequencing reaction by using fluorescence-labeled dideoxynucleotide chain terminators (AB PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 1.0; Applied Biosystems) for a final reaction volume of 10 μl. Cycling conditions were 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min for 25 cycles. Excess dye terminators were removed from sequencing reactions by ethanol precipitation or DyeEx Spin columns (QIAGEN, Valencia, Calif.) and were dried. Samples were resuspended according to the manufacturer's instructions based on the sequencer used and were submitted to an AB PRISM 310, 373, or 377 automated sequencer (Applied Biosystems). Sequences were analyzed by Sequencher, version 3.0 (Gene Codes Corp, Ann Arbor, Mich.), with the presence of major and minor peaks recognized when visible in each of the bidirectional strands, without regard to peak height. The Stanford HIVseq Sequence Analysis Program (version 3.2; Stanford Medical School, Stanford University [http://hivdb.stanford.edu]) identified mutations within the sequences. As part of routine quality assurance, genotypes generated were compared on a weekly basis to all of those produced in our laboratory during the previous 6 months by aligning them in ClustalX and reviewing the neighbor-joining phylogenetic tree to monitor for cross-contamination.

OLA. The OLA was performed on the same PCR amplicons that were sequenced, as described previously (14), except for the following modifications. The concentrations of ligase and oligonucleotides used were decreased to 0.167 U and 0.333 pmol per reaction mixture, respectively. Samples were analyzed for mutations coding for amino acids 30, 82, 84, and 90 of PR and 70, 74, 103, 181, 184, and 215 of RT. Briefly, 2 μl of the amplicon was added to a ligation reaction mixture containing oligonucleotide probes labeled at the 5′ end with digoxigenin or fluorescein, specific for wild-type and mutant codons, respectively, and an oligonucleotide probe common to both genotypes that was biotinylated at the 3′ end. Following the ligation reaction, the products were bound to a streptavidin-coated microtiter plate and an enzyme-linked immunosorbent assay was performed with alkaline phosphatase-labeled anti-fluorescein antibodies and horseradish peroxidase-labeled anti-digoxigenin antibodies. In addition to the previously described oligonucleotide probes (5, 14), the following probes were used for evaluation of sequence encoding RT amino acids 103 and 181: K103 wild type (digoxigenin-ACATCCCGCAGGGTTAAAAAAGAAA); 103N mutant (fluorescein-ACATCCCGCAGGGTTAAAAAAGAAC); 103 common (p-AAATCAGTAACAGTACTGGATGTGGGT-biotin); Y181 wild type (digoxigenin-ACAAAATCCAGACATAGTTATCTA); 181C mutant (fluorescein-A CAAAATCCAGACATAGTTATCTG); and 181 common (p-TCAATACATG GATGATTTGTATGTA-biotin). All patient samples and controls (controls are described in the OLA manual https://depts.washington.edu/idimmweb/newver2 /faculty/frenkel.html) were analyzed in duplicate.

Statistical analyses. Logistic regression models were used to estimate the probability and associated 95% confidence intervals (CI) of detecting mutations by sequencing of the PBMC amplicon and by OLA of the plasma and PBMC amplicons. Generalized estimating equations were used to account for repeated observations for some individuals.

Pairwise comparisons of the probability of detecting a mutation among the three methods were made by using a logistic regression model with generalized estimating equations, with covariates indicating which type of genotype assay was employed.

Nucleotide sequence accession numbers. Viral sequences included in this study have GenBank accession numbers AF427169, AF427171 to -5, AF427180 to -1, AF427183 to -6, AF427188, AF427193 to -4, AF427198 to -201, AF427203, AF427205 to -6, AF427209 to -10, AF427214 to -18, AF427221, AF427223 to -5, AF427227 to -8, AF427230, AF427232 to -4, AF427236, AF427238 to -40, AF427244 to -7, AF427250 to -1, AF427254 to -9, AF427262 to -4, AF427266 to -9, AF427271, AF427273, AF427275, AF427278 to -82, AF427286, AF427289, AF427294, AF427296 to -7, AF427299, AF427301, AF427303 to -5, AF427307 to -11, AF427313, AF427316, and AY458676 to -782.

RESULTS

Ninety-one codons of HIV-1 *pol* from 60 individuals were selected for evaluation based on the loss of one or more drug resistance mutations over time as assessed by consensus sequencing of plasma virus. Plasma viral sequences from each individual coded for drug-resistant mutants in the penultimate specimen while the more recent specimen coded for the wild type (84 out of 91) or a different, often non-drug-resistant mutant (7 out of 91). The latter included RT codons (L74I, T215D/V, T215S/Y, and T215L) and protease codons (D30G and V82I). The specimens in which the mutants had receded below the limit of detection by consensus sequencing of plasma were collected a mean of 13.7 (± 6.8 standard deviation) months (range, 3.7 to 32 months) following the penultimate specimens in which the mutant was detected. Phylogenetic analysis of sequences confirmed that the initial and follow-up specimens attributed to each subject were indeed likely to be from the same individual.

Patients' antiretroviral drug histories were submitted for 29 of the 60 patients studied. All but two indicated that the drugs associated with the mutations that became undetectable had been eliminated from the patients' treatment regimens. Adherence to prescribed therapy was not assessed.

The distribution of mutations in the penultimate specimens is shown in Table 1. Most mutations that regressed below the limit of detection by sequencing of plasma were associated with HIV-1 resistance to nucleoside reverse transcriptase inhibitor (NRTI; $n = 58$), although mutants conferring resistance to nonnucleoside reverse transcriptase inhibitor (NNRTI; $n = 18$) and protease inhibitor (PI; $n = 15$) were also studied.

The rates at which the lost mutations were detected by each method are shown in Table 1. The OLA of PBMC detected the mutations most frequently for each class of antiretroviral drugs, with sequencing of the PBMC amplicons detecting a similar fraction of the NNRTI mutants. Analysis of data for all classes of antiretrovirals revealed that sequencing of PBMC and OLA of plasma or PBMC were significantly more likely to detect mutations than was sequencing of plasma. OLA of PBMC was the most sensitive method compared to that of the OLA of plasma and consensus sequencing of plasma or PBMC ($P < 0.001$ for all comparisons).

TABLE 1. Comparison of detection of drug resistance mutations by consensus sequencing and OLA on HIV-1 amplified from plasma or PBMC[c]

Mutation type and HIV-1 drug resistance mutation analyzed	No. of mutants	Consensus sequencing (%)		OLA (%)	
		Plasma RNA[a]	PBMC DNA	Plasma RNA	PBMC DNA
NRTI					
M184V	40	0.0	17.5	27.5	57.5
T215Y	7	0.0	42.9[b]	28.6	85.7
L74V	5	0.0	0.0[b]	20.0	40.0
T215F	3	0.0	0.0[b]	33.3	66.7
K70R	3	0.0	0.0	0.0	33.3
Subtotal	58	0.0	16.9	27.1	59.3
NNRTI					
Y181C	9	0.0	55.6	33.3	56.6
K103N	9	0.0	33.3	44.4	33.3
Subtotal	18	0.0	44.4	38.9	44.4
PI					
L90M	4	0.0	0.0	25.0	25.0
D30N	4	0.0	0.0[b]	0.0	25.0
I84V	3	0.0	66.7	33.3	66.7
V82A	2	0.0	0.0[b]	0.0	50.0
V82S	2	0.0	50[b]	50.0	100.0
Subtotal	15	0.0	25.0	25.0	50.0
Total[c]	91	0.0	23.1	27.5	53.8

[a] Samples were selected for this study when consensus sequencing of plasma revealed wild-type or non-drug-resistant virus in an individual whose earlier specimen had encoded one or more drug-resistance mutations.

[b] Mutations not conferring drug resistance were excluded when calculating the percentage.

[c] The 95% CI values for PBMC DNA from consensus sequencing were 14 to 34; for plasma RNA from OLA were 19 to 39; for PBMC DNA from OLA were 44 to 64.

When the plasma consensus sequence detected non-drug-resistant mutants, the OLA was generally indeterminate. The specificity of the OLA relies on the discriminatory properties of the ligase enzyme. For ligation to occur the two bases of each probe at the ligation site must complement the PCR amplicon (5, 14); thus, mismatches preclude ligation of the probes and color is not produced. Indeterminate results in the OLA occurred for six plasma and two PBMC codons. In a case where consensus sequencing indicated the mixture 215S/Y after 215F in the penultimate specimen, the OLA detected genotypes for three codons, 215Y/F and T215.

DISCUSSION

OLA of PBMC was the most sensitive of the four approaches evaluated for detection of HIV-1 drug-resistant minority genotypes. In these specimens, selected due to the apparent loss of one or more drug resistance mutations from the plasma when evaluated by dideoxynucleotide chain terminator sequencing, mutants were detected at greater rates in PBMC than in plasma by both OLA and sequencing. The higher prevalence of mutants in PBMC than in plasma could have been due to a selection bias, as specimens were chosen for this study based on the loss of plasma mutations as assessed by sequencing. However, the persistence of mutants in resting PBMC in others studies (16) suggests that virus selected by past therapy could persist at a greater frequency in PBMC than in plasma virus due to a relatively slower decay of PBMC-associated virus. These data suggest that routine testing of PBMC DNA may yield a more accurate picture of a patient's antiretroviral resistance profile than the present standard of plasma consensus sequencing.

The OLA detected mutants at a greater rate than did sequencing of either plasma or PBMC amplicons. This was predictable given that the OLA detects mutants comprising 5% of the viral population (5), whereas dideoxynucleotide sequencing reliably detects genotypes comprising the majority (>50%) of the population (24).

The use of sequencing primers that flank drug resistance mutations could increase the detection of mutations. However, these regions of HIV-1 *pol* often include genetic polymorphisms and primary and compensatory mutations associated with drug resistance. Thus, use of primers complementary to these regions could have the undesired effect of sequencing only wild-type viral variants, decreasing the detection of mutant genotypes. The sequencing primers utilized for genotyping of specimens in this study were chosen from conserved regions of the HIV-1 genome, increasing the likelihood of successful PCR and DNA sequencing, and are consistent with methods most widely used for HIV-1 drug resistance testing, including commercially available kits. Also, our primers analyzed fragments limited to 300 to 621 bp, facilitating the generation of unambiguous bidirectional nucleic acid sequences. Furthermore, the rate at which minority mutant genotypes have been detected by dideoxy sequencing in our laboratory has been similar to that of other laboratories when evaluated as part of the ENVA-2 and ENVA-3 panel studies (24) and the Viral Quality Assurance Laboratory genotyping proficiency panels 1 to 3. Therefore, it is unlikely that the OLA was more sensitive due to unusually poor sequencing.

Alternative methods utilizing mutation-specific primers in real-time PCR appear more sensitive in detecting low levels of mutants than consensus sequencing (S. Palmer, V. Boltz, F. Maldarelli, E. Halvas, J. Mellors, and J. Coffin, Abstr. 10th Conf. Retrovir. Opportunistic Infect., abstr. 583, 2003) and the OLA (E. Halvas, G. Androvandi, P. Balfe, I. Beck, V. Boltz, L. Frenkel, M. Kearney, A. Kovacs, K. Metzner, D. Nissley, M. Nowicki, R. Ziermann, Y. Zhao, C. Jennings, and J. Mellors, Abstr. 10th Conf. Retrovir. Opportunistic Infect., abstr. 591, 2003). However, primers may need to be adapted to the variable region of each individual's virus adjacent to the mutation of interest, which limits the practical application of the assay.

The utilization of sensitive assays, such as the OLA, the hybridization line probe assay (LiPA), or the amplification refractory mutation system could improve detection of HIV-1 drug-resistant genotypes at low concentrations (23, 26–29). The OLA, available for research studies from the National Institutes of Health AIDS Reagent Program, detects mutations associated with drug resistance to NRTI (3, 13, 14), including multidrug-resistant mutants with Q151M (30), NNRTI (I. A. Beck and L. M. Frenkel, unpublished data), and PI (5).

Assays that detect point mutations associated with drug-resistant virus have both advantages and disadvantages compared to consensus sequencing. In our laboratory, OLA is more rapid, sensitive, and economical than consensus sequenc-

ing. LiPA and the amplification refractory mutation system have sensitivities similar to that of OLA (25, 27–29). Like sequencing, OLA and LiPA are suitable for the high-throughput evaluation of multiple drug resistance mutations per assay (25, 27, 29). A limitation common to all three point mutation assays is that these assays examine selected nucleic acids and cannot reveal new patterns of mutations. However, oligonucleotide primers to discriminate additional point mutations are relatively simple to develop for the OLA. Another limitation of point mutation assays is that hybridization or probe annealing can fail when genetic polymorphisms occur near the target codon.

Infections with drug-resistant HIV-1, originating from primary infection or previous therapy (2, 8, 15, 16, 19, 20, 21), may persist at low levels not detected by routine tests. While some studies reported little improvement in virologic outcome in association with HIV-1 drug resistance testing (1, 22), other studies have shown improved, although short-lived, suppression of viral replication (4, 9, 10, 12, 22). Investigation is needed to determine if testing of PBMC with more sensitive assays, including the OLA (5, 14), proves advantageous to individuals that have failed ART. Also, as the incidence of primary infections with drug-resistant mutants increases (7, 15, 19, 20), the utility of highly sensitive assays on PBMC-associated viruses should be evaluated in identifying minor populations of drug-resistant mutants and in selecting effective initial antiretroviral regimens.

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