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Recovery and Analysis of Human Immunodeficiency Virus Type 1 (HIV) RNA Sequences from Plasma Samples with Low HIV RNA Levels

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Amplification of human immunodeficiency virus type 1 (HIV) reverse transcriptase (RT) and protease (PT) sequences from plasma is difficult when HIV RNA levels are low, and it usually cannot be accomplished in samples with <1,000 HIV RNA copies/ml. Because the RNA extraction step is critical for the success of subsequent amplifications and sequence analyses, two RNA extraction methods were compared to study plasma samples with low HIV RNA levels. Forty-four plasma samples containing <500 HIV RNA copies/ml in a branched-DNA (bDNA) assay (Quantiplex HIV RNA assay version 2.0 [Chiron Corp., Emeryville, Calif.]) were studied. RNA was extracted by using two commercial kits (QIAamp Viral RNA kit [Qiagen, Hilden, Germany] and NucliSens kit [Organon Teknika, Boxtel, The Netherlands]). Fragments (1,144 bp) encompassing HIV PT and RT sequences were amplified by nested PCRs. Amplified products were sequenced by using a commercial kit (Applied Biosystems). HIV RNA was recovered from a total of 21 plasma samples, including 20 samples after extraction by the NucliSens method, and 8 samples after extraction by the QIAamp method ($P < 0.05$). Mean HIV RNA levels in these samples, measured by an ultrasensitive bDNA assay (Quantiplex HIV RNA assay version 3.0; Chiron Corp., Emeryville, Calif.), were 848 copies/ml (median, 666; range, 154 to 2,606 copies/ml). Analysis of RT and PT sequences in five samples demonstrated an average of 3.8 and 2.4 resistance mutations in these regions, respectively. The NucliSens RNA extraction kit is a valuable method for obtaining HIV RNA for genotypic studies from plasma fractions of individuals with low HIV RNA levels.

Current recommendations for the treatment of human immunodeficiency virus type 1 (HIV)-infected individuals include the use of combinations of antiretroviral agents to achieve suppression of HIV replication. In clinical practice, suppression of HIV replication has been considered equivalent to the presence of HIV RNA levels in plasma below the detection limit of current HIV RNA assays. However, recent studies have demonstrated the occurrence of ongoing residual viral replication in patients who have undetectable levels of HIV RNA in plasma during potent combination antiretroviral therapy (3, 15). In addition, patients receiving antiretroviral therapy frequently have low but detectable levels of plasma HIV RNA (7, 8; S. Deeks, R. Loftus, P. Cohen, S. Chin, and R. Grant, Progr. Addendum Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother. abstr. LB-2, p. 8, 1997). Although these patients might be clinically and immunologically stable, the presence of detectable HIV RNA levels in plasma indicates ongoing viral replication (7, 13). Replication of HIV in the presence of antiretroviral pressure can cause selection of resistant viruses containing mutations in the reverse transcriptase (RT) and/or protease (PT) regions and can ultimately be associated with therapeutic failure (4). Therefore, the ability to analyze HIV RT and PT sequences when plasma HIV RNA levels are low would be of importance in determining whether drug-resistant HIV has emerged in patients receiving antiretroviral therapy, and this knowledge could be helpful in guiding therapeutic decisions.

Genotypic resistance assays are based on the amplification of viral sequences, followed by an analysis of the HIV RT and PT regions of the virus to determine whether mutations associated with antiretroviral resistance are present. However, sequencing of HIV RT and PT is difficult if plasma HIV RNA levels are low, and it usually cannot be accomplished in samples containing fewer than 1,000 HIV RNA copies/ml (6). Genotypic analysis of plasma HIV RNA involves several steps: extraction and purification of total RNA, reverse transcription, amplification of HIV DNA sequences encompassing the RT and PT regions, and sequencing of amplified products for detection of mutations associated with antiretroviral resistance. Because the initial extraction step is critical for the success of subsequent amplification and sequencing (12), we compared the efficiency of two extraction methods for the recovery of sufficient amounts of RNA for genotypic HIV studies from plasma samples containing low HIV RNA levels.

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MATERIALS AND METHODS

Plasma samples. Plasma samples included in the study were selected from those available at the University of Minnesota HIV Laboratory. Only specimens with <500 HIV RNA copies/ml as measured by a branched-DNA (bDNA) assay [Quantiplex HIV RNA assay (version 2.0); Chiron Corp., Emeryville, Calif.] (10) were studied. Plasma fractions were separated from ACD-treated blood by centrifugation within 6 h of collection, were aliquoted, and were stored at −70°C until analyzed.

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RNA extraction from plasma. RNA was extracted from plasma samples by two commercial methods (QIAamp Viral RNA kit [Qiagen, Hilden, Germany] and NucliSens kit [Organon Teknika, Boxtel, The Netherlands]), following the manufacturers' instructions. For the QIAamp method, 1-ml aliquots of plasma were quickly thawed and centrifuged at 20,000 × *g* during 30 min at 4°C. RNA pellets were then resuspended in 140 μl of RNase-free water, lysed in 560 μl of lysis buffer containing carrier RNA, and incubated at room temperature for 10 min. Absolute ethanol (560 μl) was then added, and the solution was passed through silica columns by centrifugation at 6,000 × *g* for 1 min. The columns were then washed twice, centrifuged at 20,000 × *g* for 2 min, and incubated at 80°C during 5 min with 50 μl of preheated RNase-free water. RNA-containing eluates were recovered in RNase-free tubes by centrifugation at 6,000 × *g* for 1 min and were used as templates for amplification of HIV RT and PT sequences.

In the NucliSens method, 1-ml aliquots of plasma were quickly thawed and thoroughly mixed with 9 ml of lysis buffer. Next, 50 μl of a silica bead suspension was added, and the mixture was incubated 10 min at room temperature with the tubes being inverted every 2 min and was centrifuged at 1,500 × *g* for 2 min. After the supernatant was removed, the silica pellets were resuspended in 1 ml of washing buffer, transferred to 1.5-ml tubes, centrifuged at 10,000 × *g* for 1 min, and sequentially washed in washing buffer, 70% ethanol (twice), and acetone. After the acetone was removed by centrifugation, the pellets were dried at 56°C during 10 min, resuspended in 50 μl of elution buffer, and incubated at 56°C during 10 min. After centrifugation at 10,000 × *g* for 2 min, RNA-containing eluates were transferred to fresh tubes, centrifuged again to pellet any remaining silica particles, and used as templates for amplification of HIV RT and PT sequences.

Amplification of HIV RT and PT Sequences. Twenty microliters of freshly obtained RNA-containing eluates was used in one-step reverse transcription-PCRs (RT-PCRs) to amplify sequences encompassing codons 1 to 99 of the HIV PT and codons 1 to 250 of the HIV RT. Primer sequences were 5′-CCAGAA GAGAGCTTCAGGT-3′ (Z2167, forward), and 5′-GTGCTTTGGTTCCTCTA AGG-3′ (Z3429, reverse). RT-PCR mixtures contained 0.4 μM concentrations of each primer, 0.2 mM concentrations of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1× RT-PCR buffer, 5 mM dithiothreitol, 12 U of RNase inhibitor (Promega Corp., Madison, Wis.), and 1 μl of a mixture of the enzymes AMV RT, *Pwo* DNA polymerase, and *Taq* DNA polymerase (Titan One Tube RT-PCR system; Boehringer, Mannheim, Germany) in a final reaction volume of 50 μl. RT-PCR conditions consisted of the following: 30 min at 55°C (for DNA synthesis), followed by 5 min at 94°C (for DNA denaturation); 10 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s; 30 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 50 s (increasing by 5 s in each sequential elongation), and a final elongation period of 7 min at 68°C. Positive and negative controls were included in each run. One-microliter aliquots of HIV RNA that was extracted by the QIAgen method from 1 ml of a plasma sample containing >100,000 HIV RNA copies/ml were used as positive controls. Distilled water was used as a negative control. Contamination with HIV DNA sequences of RNA-containing eluates was ruled out by nested PCR with the primers and reaction conditions described above but with the reverse transcription step omitted.

Five microliters of RT-PCR products was used in a second (nested) PCR, with primers 5′-GACGATAGACAAGGACCTG-3′ (Z2235, forward), and 5′-GCG TAAATCTGACTTGCCC-3′ (Z3379, reverse). PCR mixtures contained 0.2 μM concentrations of each internal primer, 0.1 μM concentrations of each dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer, and 2.5 U of *Taq* DNA polymerase (Perkin Elmer, Branchburg, N.J.) in a final volume of 100 μl. PCR conditions consisted of 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final elongation step of 10 min at 72°C. An extraction and amplification reaction was considered successful when a 1,144-bp band was visualized after electrophoresis of PCR products in 1% agarose gels and ethidium bromide staining.

Analysis of HIV RT and PT sequences. Nested PCR products were filter purified and were sequenced by using a commercial kit (Prism Ready Reaction Dyedeoxy Terminator Cycle Sequencing; Applied Biosystems, Foster City, Calif.) with the above-described PCR primers. Two additional pairs of internal sequencing primers (5′-TGTTGACTCAGCTTGGGTGC-3′ [Z2533, forward], 5′-TTGGGCCATCCATTCCTGGC-3′ [Z2622, reverse], 5′-ACAGTACTGGA TGTGGGTG-3′ [Z2883, forward], and 5′-CTGATATCTAATCCCTGGTG-3′ [Z2999, reverse]) were used to generate complete PT and RT sequences. These were aligned with existing sequence data to detect previously described drug resistance mutations (6). RT and PT sequences were also aligned with those of reference strains HIV MN (GenBank accession no. M17449), and HXB2 (GenBank accession no. K03455) to determine whether mutations were present in the regions analyzed (5, 14).

Plasma HIV RNA levels (bDNA assay version 3.0). Because all study samples had <500 HIV RNA copies/ml as determined by the standard bDNA assay, actual HIV RNA levels were measured by a more sensitive method (Quantiplex HIV RNA assay [version 3.0]; Chiron Corp., Emeryville, Calif.). The analytical sensitivity of this assay is 50 HIV RNA copies/ml (1; A. Erice, W. Li, K. Henry, J. Simpson, W. Paxton, and H. H. Balfour, Jr., Progr. Abstr. Sixth Conf. Retroviruses Opportunistic Infect., abstr. 149, p. 95, 1999; J. L. Perez, P. Perez, J. M. Escribà, D. Podzamczer, and R. Martin, Progr. Abstr. Sixth Conf. Retroviruses Opportunistic Infect., abstr. 146, p. 95, 1999).

Statistical analysis. McNemar's test was used to compare the proportions of plasma samples that yielded HIV RNA for sequencing studies after being processed by each of the two RNA extraction methods. Mean HIV RNA levels obtained by the ultrasensitive bDNA assay for plasma samples that had been grouped according to the results of the two RNA extraction methods were compared by the two-tailed Student test.

TABLE 1. Recovery and quantitation of HIV RNA from plasma samples

Extraction result[a]	No. of samples from which RNA was recovered (n = 44)	No. analyzed (n = 40)	No. of HIV RNA copies/ml (bDNA version 3.0)		
			Mean	Median	Range
N+ or Q+	21	17	848	666	154–2,606
N+	20	16	877	736	154–2,606
Q+	8	6	1,208	1,065	385–2,606
N+ and Q−	13	11	652	639	154–1,125
N− and Q+	1	1	385	385	
N+ and Q+	7	5	1,372	1,326	666–2,606
N− and Q−	23	23	399	84	<50–3,579

[a] RNA extraction method: N, NucliSens; Q, QIAamp.

RESULTS

Amplification of HIV RNA from plasma. A total of 44 samples was included in the study. HIV RNA was recovered from 21 (47.7%) of the samples, including 8 (18.2%) samples after extraction by the QIAAmp method, and 20 (45.4%) samples after extraction by the NucliSens technique ($P = 0.003$). HIV RNA was recovered from 7 (15.9%) samples by both methods, from 13 (29.5%) samples by the NucliSens method only, and from 1 (2.2%) sample by the QIAamp method only (Table 1). None of the samples were contaminated by DNA.

HIV RNA levels determined by ultrasensitive bDNA assay. All 44 samples included in the study had <500 HIV RNA copies/ml as measured by using version 2.0 of the bDNA assay. Additional aliquots for HIV RNA quantitative studies with version 3.0 of the bDNA assay were available from 40 of the 44 samples (Table 1). The mean HIV RNA level in the bDNA version 3.0 assay was 590 copies/ml (median, 299 copies/ml; range, <50 to 3,579 copies/ml). HIV RNA levels were higher among the 17 samples from which HIV RNA was recovered by both extraction methods than among the 23 samples from which HIV RNA was not recovered by either extraction method (848 versus 399 HIV RNA copies/ml; $P = 0.05$). Eighteen (78.2%) of the 23 samples from which HIV RNA was not recovered by either of the two extraction methods had <200 HIV RNA copies/ml in the bDNA version 3.0 assay (9 samples had <50 copies/ml, 5 had 50 to 100 copies/ml, 2 had 100 to 150 copies/ml, and 2 had 150 to 200 copies/ml). HIV RNA levels in the remaining 5 samples were 506, 622, 1,486, 1,598, and 3,579 copies/ml. Overall, HIV RNA was recovered by the NucliSens extraction method from samples with lower HIV RNA levels, although this difference was not statistically significant (877 versus 1,208 HIV RNA copies/ml, respectively; $P = 0.38$).

Analysis of HIV RT and PT. HIV RT and PT sequences in five samples that were randomly selected from the study specimens were analyzed (Table 2). HIV RNA levels in these samples ranged from 155 to 1,083 HIV RNA copies/ml. HIV RNA was recovered by the NucliSens extraction method from three samples, by the QIAamp extraction method from one sample, and by both extraction methods from the remaining specimen. These samples contained an average of 3.8 drug resistance-related mutations in the RT gene and 2.4 resistance mutations in the PT gene (Table 2).

DISCUSSION

In this study, we were able to recover HIV RNA for genotypic studies from 48% of plasma samples with <500 HIV RNA copies/ml as measured by a conventional bDNA assay. This was accomplished more often when the NucliSens kit was used for RNA extraction from the plasma samples than when the QIAamp extraction method was used. Nevertheless, HIV RNA was not recovered by either of the two extraction methods from 52% of the samples we studied. Whereas HIV RNA levels measured by a more sensitive bDNA assay showed that the majority (78%) of these specimens contained <200 HIV RNA copies/ml, HIV RNA could not be recovered from 5 samples containing >500 HIV RNA copies/ml (range, 506 to 3,579 HIV RNA copies/ml). This suggests that the efficiency of HIV RNA extraction methods is variable in plasma samples with low numbers of HIV RNA copies per milliliter.

There are several possible causes for the difference in RNA yield we found between the two RNA extraction methods: (i) the initial centrifugation step (QIAamp) might be less efficient for RNA recovery than mixing the plasma samples with a large volume of guanidinium thiocyanate (NucliSens), (ii) the use of silica columns and short (1-min) column centrifugation (QIAamp) might be less efficient for RNA binding to silica particles than a longer incubation (10 min) of plasma and a silica bead suspension (NucliSens), (iii) the use of a large volume (10 ml) of lysis buffer (NucliSens) might dilute proteins and PCR inhibitors present in plasma, and (iv) a buffer solution is used in the NucliSens method instead of water (QIAamp) to prepare RNA eluates. It is important to note that the NucliSens method is more labor-intensive (2 h for five samples) than the QIAamp method (1.5 h for five samples) and requires using a centrifuge with capacity for 10-ml tubes.

An important finding of our study was the detection of mutations related to antiretroviral resistance in the HIV RT and/or PT regions among five samples selected at random for genotypic studies. Although the number of samples we sequenced was small, the finding of antiretroviral resistance mutations in individuals with low plasma HIV RNA levels is in agreement with recent data suggesting that there is ongoing viral replication in blood leukocytes and lymphoid tissues of individuals with suppressed plasma HIV RNA levels that may result in the selection of a resistant virus (4, 9, 11). Conversely, a recent study has shown that a rebound in plasma HIV RNA levels in patients receiving antiretroviral therapy might be due to the selection by therapy of a susceptible virus with increased fitness (D. Havlir, N. Hellman, C. Petropoulos, J. Whitcomb, A. Collier, M. Hirsch, P. Tebas, and D. Richmond, Progr. Abstr. Sixth Conf. Retroviruses Opportunistic Infect., abstr. LB12, p. 207, 1999). Because the therapeutic implications of these two situations are fairly different (switching versus intensification), the ability to analyze HIV RT and PT sequences when plasma HIV RNA levels are low is very important in assessing whether a resistant virus has emerged in patients receiving antiretroviral therapy who have experienced a rebound in plasma HIV RNA levels. Although the presence of a resistant virus in our patients is of concern, HIV containing multiple RT and PT mutations related to antiretroviral resistance has been found in patients receiving combination antiretroviral therapy who remain clinically and immunologically stable but in whom plasma HIV RNA levels have become detectable after having been suppressed (7). It has been postulated that the presence of multiple RT and/or PT mutations might modify the fitness of the mutant viruses (2, 7). Additional prospective studies with partially suppressed individuals

TABLE 2. Drug resistance mutations observed in HIV protease and reverse transcriptase

Sample	Extraction result[a]	No. of HIV RNA copies/ml[b]	Mutation at indicated position[c] in:																			
			Protease										Reverse transcriptase									
			10 (L/I)	36 (M)	46 (M)	63 (L/T)	64 (I)	71 (A)	77 (V)	82 (V)	90 (L)	41 (M)	67 (D)	69 (T)	70 (K)	74 (L)	103 (K)	184 (M)	214 (F)	215 (T)	219 (K)
93	N+ and Q−	399	V	–	–	–	–	–	–	–	–	–	N	D	R	–	–	–	L	V	Q
151	N and Q+	666	–	L	–	–	–	–	–	–	–	–	N	–	R	–	N	V	–	Y	Q
195	N+ and Q−	1,083	–	–	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
369	Q+ and N−	385	–	–	–	–	–	T	I	–	–	–	N	–	R	–	–	V	–	–	–
455	N+ and Q−	154	–	–	I	P	–	T	I	T	M	L	–	–	–	V	–	V	–	Y	–

[a] RNA extraction method; N, NucliSens; Q, QIAamp.
[b] bDNA version 3.0.
[c] Consensus HIV amino acids of HIV strains MN and HXB2 are shown in parentheses. Dashes indicate homology with the consensus sequence.

are necessary to address the clinical significance of these findings.

Because of the study design, all the specimens we analyzed contained by definition <500 HIV RNA copies/ml as measured by the standard version of the bDNA assay (10). Of note is that 78% of the samples had HIV RNA levels above the limit of detection (50 HIV RNA copies/ml) of a more sensitive version of the bDNA assay (1). A study done in our laboratory comparing the standard and the ultrasensitive bDNA assays showed that HIV RNA levels obtained with the ultrasensitive version were on average 3.9 times higher than those obtained with the standard bDNA assay for the same plasma sample (Erice et al., Progr. Abstr. Sixth Conf. Retroviruses Opportunistic Infect.). In that study, 46 (35%) of 132 plasma samples analyzed had <500 HIV RNA copies/ml in the standard bDNA assay but >500 HIV RNA copies/ml in the ultrasensitive bDNA assay (median, 876 HIV RNA copies/ml; range, 507 to 7,510 HIV RNA copies/ml). Similar results have been reported by other authors (Perez et al., Progr. Abstr. Sixth Conf. Retroviruses Opportunistic Infect.).

In summary, the NucliSens RNA extraction kit is a valuable tool for recovering HIV RNA for genotypic analysis from plasma fractions of individuals with low HIV RNA levels. Although the rate of recovery of HIV RNA from plasma samples with low HIV RNA copies/ml is variable, it is possible to obtain adequate amounts of HIV RNA for genotypic studies in a significant percentage of plasma samples containing <1,000 HIV RNA copies/ml by sensitive RNA extraction methods. HIV sequences containing RT and/or PT mutations that are related to antiretroviral resistance can be found in the plasma of subjects with a low number of HIV RNA copies per milliliter. Characterizations of HIV sequences from additional individuals with low plasma HIV RNA levels will help to define the frequency and clinical significance of these mutations.

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