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Emergence of Drug Resistance Mutations in Human Immunodeficiency Virus Type 2-Infected Subjects Undergoing Antiretroviral Therapy

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The reverse transcriptase (RT) and protease genes from 12 human immunodeficiency virus type 2 (HIV-2)-infected individuals who had been exposed to antiretroviral drugs for longer than 6 months were examined for the presence of mutations which could be involved in drug resistance. Four individuals carried virus genotypes with amino acid substitutions potentially associated with resistance to nucleoside analogues: two at codon 70 (K→R) and two at codon 184 (M→V). Moreover, the latter two patients harbored a codon Q151M mutation which is associated to multidrug resistance in HIV-1, and one of these subjects carried some of the typically linked mutations at codons 65 and 69. With regard to the protease inhibitors, substitutions associated with resistance to protease inhibitors at codon 46 were observed in all individuals. Moreover, minor resistance mutations, as well as new ones of unknown meaning, were often seen in the protease gene. In conclusion, amino acid changes in the HIV-2 RT and protease genes which could be associated with drug resistance seem to occur at positions identical to those for HIV-1.

Antiviral therapy against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), the causative agents of AIDS, has been focused mainly on disrupting virus replication. The main therapeutic targets have been viral enzymes such as the reverse transcriptase (RT) and the protease, both encoded by the *pol* gene, which are essential in the replicative cycle of retroviruses. Several potent drugs have been developed to inhibit either the RT or protease functions. However, incomplete suppression of viral replication often occurs during therapy, and a growing number of drug-resistant variants tend to accumulate over time (19).

Up to now, most studies of drug-resistant mutations have been focused on HIV-1, and critical substitution loci have been found for either nucleoside RT inhibitors (NRTI), non-nucleoside RT inhibitors (NNRTI), and protease inhibitors (PI) (2, 9). Studies with HIV-2, however, have been much more limited, most likely because the number of HIV-2-infected individuals has remained low worldwide compared to HIV-1. Moreover, HIV-2 is mainly found in West Africa, where treatment is often not available. HIV-2 isolates appear to be sensitive to most NRTI (4) and PI (24, 25) but are intrinsically resistant to at least some NNRTI (22). Nevertheless, it is difficult to monitor the effectiveness of treatment in HIV-2 carriers since the plasma viral load cannot be determined by the currently available tests (11).

As with HIV-1, HIV-2 susceptibility to antiretroviral drugs may be affected by structural changes in either the RT or the protease. Although overall there is little overlap sequence identity between HIV-1 and HIV-2 viral genomes, the *pol* genes of both viruses are highly conserved. As a result, HIV-1 and HIV-2 proteases display approximately 50% sequence identity, and their structure is quite similar (8). With respect to the RT, HIV-1 and HIV-2 share a 60% identity in their genomic sequence, and their catalytic properties are also quite similar (20). Both the similarity in the amino acid sequence and in the enzymatic behavior suggest that the structure of HIV-2 RT is likely to be very similar to that of HIV-1.

We have investigated here the presence of genotypic changes in the RT and protease coding regions of the HIV-2 *pol* gene, which could be associated with resistance to antiretroviral drugs. For this purpose, blood samples from HIV-2-infected patients being under antiretroviral therapy were examined.

MATERIALS AND METHODS

Patients. Twelve HIV-2-infected patients who had been under antiretroviral therapy for more than 6 months were recruited into the study in January 1998. All of them were on regular follow-up in two hospitals located in Lisbon, Portugal. The main epidemiological and clinical features of the patients are summarized in Table 1. Four of them (HEM-13, HSM-22, HSM-29, and HSM-30) were treated with two NRTI plus one PI. The remaining patients were receiving only one or two NRTI at the time their blood was drawn. None of these individuals had received NNRTI.

Nucleic acid extraction and PCR amplification. Proviral DNA was extracted either from whole blood using the High Pure Viral Nucleic Acid Kit (Boehringer-Mannheim, Barcelona, Spain) or from peripheral blood mononuclear cells by lysis with nonionic detergents (Tween 20 and NP-40), followed by ethanol precipitation. Protease and RT genomic regions were amplified separately. Briefly, PCR reactions were carried out in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 100 ng of each primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer, Foster City, Calif.). A genomic region of 1,054 bp encoding the 5′ end of the RT (*pol* gene) was amplified by nested PCR (amino acids 32 to 383). Outer (RTC and RT2) and inner (RT3 and RT4) primers have been described elsewhere (5). Cycling temperatures and times were as follows. The first round of PCR had an initial denaturation step at 94°C for 5 min. It was followed by 40 amplification cycles (94°C for 45 s, 41°C for 45 s, and 72°C for 1 min 30 s) and then by an elongation step at 72°C for 7 min. Nested-PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 35 amplification cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 1 min and 30 s). The last step was an incubation at 72°C for 7 min.

The whole protease coding region (303 bp, amino acids 1 through 99) was also amplified by nested PCR. The primers used were as follows. PR1 (5′-GGG AAA GAA GCC CCG CAA CTT C-3′) and PR2 (5′-GGGTATTATAAGGATTAG TTGG-3′) were the outer primers. DP27, described elsewhere (19), and PR3 (5′-GCTGCACCTCAATTCTCTCTT-3′) were the inner primers. The cycling conditions for the first amplification included a denaturation step at 94°C for 5

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TABLE 1. Primary clinical and epidemiological features of the study population

Patient	Age (yr)	Sex	Country of birth	Mode of transmission	Disease stage[a]	$CD4^+$ lymphocyte count (cells/μl)	Antiretroviral therapy[b]
HEM-05	41	Female	Guinea-Bissau	Heterosexual	A2	340	ZDV (49 mo) and DDI (19 mo)
HEM-06	45	Female	Guinea-Bissau	Heterosexual	B3	187	ZDV (25 mo) and 3TC (3 mo)
HEM-13	39	Male	Portugal	Heterosexual	C3	28	ZDV (36 mo), DDI (13 mo), RTV (6 mo), and 3TC (3 mo)
HEM-14	35	Male	Portugal	Heterosexual	A2	282	ZDV (7 mo) and DDI (5 mo)
HEM-16	37	Male	Guinea-Bissau	Heterosexual	A2	566	ZDV (68 mo)
HEM-17	59	Male	Guinea-Bissau	Transfusion	B2	323	ZDV (8 mo) and 3TC (4 mo)
HEM-19	46	Female	Cape Verde	Heterosexual	A3	71	ZDV and 3TC (26 mo)
HEM-20	51	Female	Guinea-Bissau	Heterosexual	C3	170	ZDV (36 mo)
HSM-22	45	Male	Ivory Coast	Heterosexual	C3	165	ZDV, 3TC, and IDV (9 mo)
HSM-23	58	Female	Portugal	Heterosexual	A2	308	ZDV and DDI (12 mo)
HSM-29	44	Male	Guinea-Bissau	Heterosexual	C3	197	ZDV, 3TC, and IDV (12 mo); NFV, SQV, and D4T (1 mo)
HSM-30	15	Female	Guinea-Bissau	Vertical	C3	87	D4T, 3TC, and IDV (13 mo)

[a] Per Centers for Disease Control, Atlanta, Ga.
[b] ZDV, zidovudine; DDI, didanosine; 3TC, lamivudine; RTV, ritonavir; IDV, indinavir; NFV, nelfinavir; SQV, saquinavir; and D4T, stavudine.

min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by an incubation at 72°C for 7 min. Nested PCR was carried out using 3 μl of the first-round PCR product. The conditions were as follows: 94°C for 5 min and then 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by an incubation at 72°C for 7 min.

DNA sequence analysis. Amplified nested-PCR products were purified on columns by the High Pure PCR product purification kit (Boehringer Mannheim) and used for direct sequencing. DNA fragments were sequenced on both strands with sense primers RT3 and RT5-HIV2 (5′-GGATGATATCTTAATAGCTA G-3′) and antisense primers RT4 and RT6-HIV2 (5′-GATGTCATTGACTGT CC-3′) for the RT region and primers PR3 and PR2 for the protease region. Sequencing reactions were carried out with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin-Elmer) according to the manufacturer's instructions. Sequences were run on an automated DNA sequencer (Model 310 ABI Genetic Analyzer; Applied Biosystems, Foster City, Calif.). DNA sequences were analyzed, edited, and translated by using the Sequence Navigator software version 1.0.1 (Applied Biosystems). HIV-2 amino acid sequences were compared to sequences from the reference HIV-1 strains HXB2, JRFL, OYI, and RF (GenBank accession numbers K03455, U63632, M26727, and M17451, respectively). HIV-2 ROD, NIHZ, and ISY sequences (GenBank accession numbers M15390, J03654, and J04498, respectively) were used as the wild-type HIV-2 subtype A reference sequences.

GenBank accession numbers. The HIV-2 sequences generated in this study were given accession numbers AF139042 through AF139054.

RESULTS

The RT gene was sequenced in all of the HIV-2-infected subjects except for the HEM-13 subject, whose sample could not be amplified, while the protease gene was sequenced only for the four patients who had received PI. All sequences belonged to subtype A (data not shown). The deduced protease and RT amino acid sequences were aligned with several HIV-1 sequences, as well as with others belonging to HIV-2-naive patients and simian immunodeficiency virus (SIV) sequences found at the Los Alamos National Laboratory Database (14). The higher degree of divergence between HIV-1 and HIV-2 was found at the 3′ end of the RT sequence, although the homology was highly conserved overall. The protease gene and the amino-terminal region of the RT gene showed 50 and 60% DNA sequence identity, respectively, with the HIV-1 subtype B consensus sequence. The amino acid similarities were 70 and 75%, respectively. Furthermore, there was a 68% identity and an 84% similarity between HIV-1 and HIV-2 at residues within

TABLE 2. Amino acid substitutions arising in HIV-2-infected patients exposed to NRTI

Sample[a]	Amino acid position in RT coding region[b]													
	41 (M→L)	65 (K→R)	67 (D→N)	69 (T→D)	70 (K→R)	74 (L→V)	75 (V→I)	116 (F→Y)	151 (Q→M/L)	184 (M→V)	210 (L→W)	215 (T→Y)	219 (K→Q/E)	333 (G→D/E)
HIV-1 (wt)	M	K	D	T	K	L	V	F	Q	M	L	T	K	G
HIV-2 (wt)	M	K	D	N	K	L	I	F	Q	M	N	S	E	Q
HEM-05		K	D	N	K	L	I	F	Q	M	N	S	E	Q
HEM-06		K	D	N	R	L	I	F	Q	M	N	S	E	Q
HEM-14	M	K	D	N	K	L	M	F	Q	M	N	S	E	Q
HEM-16		K	D	N	K	L	I	F	Q	M	N	S	E	Q
HEM-17		K	D	N	K	L	I	F	Q	M	N	S	E	Q
HEM-19		K	D	N	K	L	I	F	M	V	N	S	E	Q
HEM-20		K	D	N	K	L	I	F	Q	M	N	S	E	Q
HSM-22		K	D	N	K	L	M	F	Q	M	N	S	E	Q
HSM-23		K	D	N	K	L	I	F	Q	M	N	S	D	Q
HSM-29	M	R	D	S	K	L	I	F	M	V	N	S	E	Q
HSM-30	M	K	D	N	R	L	I	F	Q	M	N	S	E	Q

[a] wt, Wild type.
[b] Consensus wild-type amino acid at position indicated followed by its known substitution associated with resistance in HIV-1. The amino acid substitution is indicated in parentheses.

TABLE 3. Amino acid substitutions in HIV-2-infected patients potentially associated with resistance to NNRTI

Sample[a]	Amino acid position in RT coding region[b]										
	98 (A→G)	100 (L→I)	101 (K→E)	103 (K→N/R)	106 (V→A/I)	108 (V→I)	179 (V→D)	181 (Y→C/I)	188 (Y→C/L)	190 (G→A)	236 (P→L)
HIV-1 (wt)	A	L	K	K	V	V	V	Y	Y	G	P
HIV-2 (wt)	A	L	A	K	I	V	I	I	L	A	P
HEM-05	A	L	P	R	I	V	I	I	L	A	P
HEM-06	A	L	A	R	I	V	I	I	L	A	P
HEM-14	A	L	A	K	I	V	I	I	L	A	P
HEM-16	A	L	A	K	I	V	I	I	L	A	P
HEM-17	A	L	A	K	I	V	I	I	L	A	P
HEM-19	A	L	A	K	I	V	I	I	L	A	P
HEM-20	A	L	A	K	I	V	I	I	L	A	P
HSM-22	A	L	A	K	I	V	I	I	L	A	P
HSM-23	A	L	A	K	I	V	I	I	L	A	P
HSM-29	A	L	A	K	I	V	I	I	L	G	P
HSM-30	A	L	A	K	I	V	I	I	L	A	P

[a] wt, Wild type.
[b] Consensus wild-type amino acid at position indicated followed by its known substitution associated with resistance in HIV-1. The amino acid substitution is indicated in parentheses.

the NNRTI binding pocket. This observation is consistent with previously reported comparisons (17, 22).

Identification of drug-resistant genotypes. Several amino acid substitutions were observed along the entire RT analyzed region. Mutations commonly linked to NRTI resistance in HIV-1 group M isolates (9) were recognized in some HIV-2 specimens (Table 2). Two patients exposed to zidovudine (HEM-06 and HSM-30) harbored a codon K70R substitution. Another two individuals (HEM-19 and HSM-29) harbored the codon M184V substitution, which is associated with decreased susceptibility to lamivudine. Both were receiving this drug at the time of blood collection. These two patients also carried the codon Q151M mutation, which is associated with multiple NRTI resistance in HIV-1 infections (19). Moreover, patient HSM-29 carried another change associated with multiple resistance (A62V), as well as substitutions at positions 65 and 69 that confer further resistance to dideoxyinosine ddI and dideoxycytosine ddC. Plasma viral load values measured by Amp-RT (6) and QC-PCR were high in both patients (data not shown) (21), and patient HSM-29 had a $CD4^+$ drop to 68 cells/μl before the treatment was modified. Finally, patient HSM-23 harbored a codon E219D change, which has not been linked yet to any drug resistance.

Regarding NNRTI (Table 3), all 12 sequenced strains harbored an isoleucine at position 181, which is classically linked to nevirapine resistance. Also, all but one subject (HSM-29) harbored an alanine at position 190, which is associated with resistance to these compounds.

Sequence analyses of the protease gene in patients treated with PI showed different patterns of substitutions (Table 4). None harbored major mutations potentially associated with resistance to saquinavir, nelfinavir, and amprenavir. However, primary substitutions that might be linked to indinavir resistance were detected in all patients. Moreover, the codon M46I mutation was the predominant polymorphism found in the HIV-2 consensus sequence. One patient (HSM-29) harbored the codon V82F mutation. Substitutions at other sites were also recognized, some of which have been considered minor (compensatory) substitutions in HIV-1.

DISCUSSION

The activity of current antiretroviral drugs is not well known in HIV-2-infected subjects, and data available on the development of drug resistance in vivo are scarce. Based on the structure similarity between HIV-1 and HIV-2 RT and protease enzymes, it seems reasonable to expect in HIV-2 a similar response to antiviral drugs to that seen in HIV-1. Furthermore, drug-resistant mutations might occur in the HIV-2 genome at similar sites to those of HIV-1. The results from our study in 12 HIV-2-infected individuals who had been on antiretroviral

TABLE 4. Amino acid substitutions in HIV-2-infected patients exposed to protease inhibitors

Sample[a]	Amino acid position in protease coding region[b]																
	10 (L→I/R/V)	20 (K→M/R)	24 (L→I)	30 (D→N)	32 (V→I)	33 (L→F)	36 (M→I)	46 (M→I)	48 (G→V)	50 (I→V)	54 (I→V)	63 (L→P)	71 (A→V)	73 (G→S)	82 (V→A/T/F)	84 (I→V)	90 (L→M)
HIV-1 (wt)	L	K	L	D	V	L	M	M	G	I	I	L	A	G	V	I	L
HIV-2 (wt)	V	V	L	D	I	V	I	I	G	I	I	E	V	A	I	I	L
HEM-13	V	V	L	D	I	V	I	I	G	I	I	E	V	A	I	I	L
HSM-22	V	V	L	D	I	V	I	I	G	I	I	E	V	A	I	I	L
HSM-29	V	V	L	D	I	V	I	I	G	I	M	E	I	A	F	I	L
HSM-30	V	V	L	D	I	V	I	I	G	I	I	E	V	A	I	I	L

[a] wt, Wild type.
[b] Consensus wild type amino acid at position indicated followed by its known substitution associated with resistance in HIV-1. The amino acid substitution is indicated in parentheses.

treatment for more than 6 months seem to confirm this hypothesis.

Experimental studies have proven that mutations in the RT gene of HIV-2 isolates at positions known to produce resistance to NRTI in HIV-1 act in a similar fashion in HIV-2 (14, 15). Moreover, experiments carried out with SIV strains, with which HIV-2 shares a high degree of sequence identity, support the hypothesis of similar mechanisms of resistance to NRTI between HIV-1 and HIV-2 (26, 27; R. F. Schinazi, R. M. Lloyd, Jr., A. McMillan, G. Gosselin, J. L. Imbach, and J. P. Sommadossi, Abstr. 4th Int. Workshop Drug Resist., abstr. 10, 1995).

In our study, substitutions at positions 62, 65, 69, 70, 184, and 151 in the RT gene were identified in some HIV-2-infected patients. All of them had been treated with zidovudine, didanosine, stavudine, and/or lamivudine for longer than 1 year. The K65R mutation was observed in one patient. It confers a fivefold loss of sensitivity to tenofovir (PMPA) in SIV, as well as cross-resistance to 3TC, ddI and ddC. This mutation often develops first and is followed by others, such as N69S and I118V mutations (J. M. Cherrington et al., Abstr. 5th Int. Workshop HIV Drug Resist., abstr. 75, 1996; K. Van Rompey et al., Abstr. 6th Int. Workshop HIV Drug Resist., abstr. 117, 1997). Remarkably, the individual carrying the K65R mutation also harbored substitutions at codons 62 (A→V), 69 (N→S), 184 (M→V), and 151 (Q→M), which cause multinucleoside resistance in HIV-1 (19). There was a second individual harboring a codon Q151M substitution. Both patients had experienced a decline in their $CD4^+$ lymphocyte counts and also had high viral load values, as measured by two different techniques, Amp-RT (6) and QC-PCR (21), supporting the idea that they were experiencing treatment failure. Experiments in SIV have confirmed that the Q151M mutation causes a significant loss of sensitivity to NRTI, including zidovudine (27). To our knowledge, this is the first report of multinucleoside genotypic resistance due to the codon 151 complex in HIV-2. The prevalence of this genotype among pretreated HIV-1-infected patients is ca. 2 to 3%, and its emergence confers a worse prognosis (19).

All tested HIV-2-infected individuals harbored substitutions linked to resistance to NNRTI in HIV-1 group M. All of them harbored a codon Y181I substitution and all but one harbored a G190A change; both of these circumstances confer resistance to nevirapine (18). The K103N substitution classically associated with resistance to efavirenz was not found. To our knowledge, it remains unclear whether the susceptibility to efavirenz is preserved to some extent in HIV-2. However, mutagenesis studies (3, 10) have shown that local differences in the composition of amino acid side chains (residues 101 to 106 and residues 176 to 190) contribute globally rather than separately to account for the difference in sensitivity to NNRTI noticed when HIV-1 and HIV-2 (22) are compared.

Regarding the protease gene, to our knowledge this is the first report on the in vivo appearance of substitutions potentially causing resistance to PI in HIV-2-infected patients. Although minor changes in the protease sequence of HIV-1 and HIV-2 seem to produce some differences in substrate and inhibitor binding (17), the majority of the PI seem to inhibit HIV-2 in vitro (7, 13, 25). In fact, the proteases of both viruses display very similar sequences and structures, and most of the amino acid differences reflect conservative changes. This finding translates into functional similarities. In our study, the protease sequence analysis showed substitutions in all four of the examined specimens. Point mutations that confer resistance to PI in HIV-1 were found at the homologous position in the HIV-2 enzyme. It is noteworthy that the codon M46I mutation was found in all isolates, as well as in the HIV-2 wild-type consensus sequence, suggesting that the presence of an isoleucine at position 46 might produce a reduced sensitivity to indinavir in HIV-2, even in untreated individuals. Other changes were seen at positions considered to be compensatory mutations, such as L10V, V32I, M36I, and A71V. They appeared in all tested subjects. In addition, changes not previously reported to be associated with PI resistance in HIV-1 were noticed at codons 20 (K→V), 63 (L→E), and 82 (V→I). The clinical significance of these substitutions is still unknown, but they could just reflect naturally occurring polymorphisms of the enzyme (1, 19). Of the four tested patients, one harbored a codon V82F mutation, which in HIV-1 produces resistance to either ritonavir or indinavir. This patient showed a progressive $CD4^+$ lymphocyte decline despite being treated with indinavir.

In conclusion, the structural similarity of RT and protease in HIV-1 and HIV-2, overwhelming their genetic heterogeneity, could explain how amino acid substitutions causing resistance to antiretroviral drugs might occur at identical positions and play similar roles in the two enzymes of both viruses. The effect of these mutations in HIV-2 should be further analyzed by phenotypic drug susceptibility assays.

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