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Quasispecies variant dynamics during emergence of resistance to raltegravir in HIV-1-infected patients

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***Objectives*: Raltegravir is the first approved inhibitor of HIV-1 integrase (IN). In most patients, raltegravir failure is associated with mutations in the IN gene, through two different genetic pathways: 155 (N155H) or 148 (Q148K/R/H). The objective of this study was to characterize the dynamics of HIV-1 quasispecies variant populations in patients who failed to respond to raltegravir treatment.**

***Patients and methods*: Bulk genotyping and clonal analysis were performed during the follow-up of 10 patients who failed to respond to raltegravir treatment.**

***Results*: Treatment failed through the 155 pathway in six patients and through the 148 pathway in two patients; two further patients switched from the 155 to the 148 pathway. In the two patients switching from the 155 to the 148 pathway, clonal analysis showed that Q148R/H and N155H mutations were present on different strands, suggesting that these two pathways are independent. This was consistent with our finding that each genetic profile was associated with different secondary mutations. We observed a greater variability among quasispecies associated with the 155 pathway, and IC_{50} determinations showed that the fold resistance to raltegravir, relative to wild-type, was 10 for the N155H mutant and 50 for the G140S+Q148H mutant.**

***Conclusions*: Clonal analysis strongly suggests that the two main genetic pathways, 155 and 148, involved in the development of resistance to raltegravir are independent and exclusive. Moreover, the switch of the resistance profile from 155 to 148 may be related to the higher level of resistance to raltegravir conferred by the 148 pathway and also to the higher instability of the 155 pathway.**

Keywords: integrase, clonal analysis, drug resistance

Introduction

Integrase (IN) inhibitors constitute a new class of antiretroviral (ARV) agents, which block HIV-1 IN activity.[1,2] HIV-1 IN is required for the integration of double-stranded blunt-ended DNA into host cell DNA, an essential step for HIV-1 replication. It is a 288 amino acid protein, consisting of three independent structural domains: an N-terminal domain with an HHCC motif and a C-terminal domain with DNA-binding activity flanking a central catalytic core domain containing a D,D(35)E catalytic motif in a highly conserved spatial arrangement.

Integration requires two reactions. The first is the 3′-end processing step, which occurs in the cytoplasm within a nucleoprotein complex called the pre-integration complex (PIC).[3] In this step, the terminal GpT dinucleotide is cleaved from the 3′-end of each long terminal repeat (LTR), generating CpA 3′-hydroxyl

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ends.[4] The second reaction involves strand transfer. This takes place in the nucleus, following the transport of PIC through the nuclear pore. During the second step, IN transfers both newly exposed 3′ extremities of the viral DNA into the target DNA by a one-step transesterification; the viral genome is thereby inserted and covalently linked into the host genome.[5,6]

Several classes of inhibitors, interfering either with the 3′-end processing step in the cytoplasm[7,8] or strand transfer in the nucleus,[9–13] have been developed. All of these inhibitors were proved to be able to block HIV replication in cell culture,[7,12] although only strand transfer inhibitors exhibited ARV activity *in vivo*.[14–17] This novel class of ARV agents is potentially valuable as it is active against viruses resistant to other classes of ARVs such as nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTIs (NNRTIs), protease inhibitors (PIs) and entry inhibitors. Raltegravir (MK-0518) is a member of this new class of HIV-1 inhibitors interfering with the strand transfer step.

Recent studies have shown that virological failure in patients receiving treatment with this compound occurs due to the selection of mutations in the viral IN gene. Virological failure is associated with two main different genetic pathways: N155H, associated with the secondary mutations E92Q, V151I, T97A, G163R or L74M, and Q148K/R/H, associated with the secondary mutations G140S/A or E138K. Another pathway, Y143R/C, has also been described, associated with the secondary mutations L74A/I, E92Q, T97A, I203M and S230R.[18,19] However, there are few data available concerning the dynamics of selection of resistance to raltegravir.[20] We performed clonal analysis of the IN gene sequences from serial plasma samples obtained during the development of raltegravir resistance. The aim of this study was to characterize the quasispecies variant dynamics during emergence and amplification of resistance to raltegravir in 10 patients with virological failure.

Materials and methods

Patients

Ten patients who failed to respond to raltegravir treatment were retrospectively studied. The genotypic analysis of IN resistance and the follow-up of viral load were performed following the French National Guidelines.[21] All patients received at least one NRTI with one boosted PI ± enfuvirtide in their optimized regimen. The optimized regimen associated with raltegravir was selected according to previous ARV exposure and genotypic resistance testing was interpreted using the French ANRS AC11 algorithm v16 (www.hivfrenchresistance.org).

Amplification, cloning and sequencing

RNA was extracted from 500 μL of plasma, and a 1086 bp fragment encompassing the entire IN gene was amplified using primers IN12 and IN13 for RT–PCR and IN1 and BH4 for nested PCR, as described previously.[22] Purified PCR products were directly sequenced for bulk genotyping, some of which were cloned into the plasmid vector pCR®4-TOPO® (TOPO TA Cloning® Kit, Invitrogen, Carlsbad, CA, USA). Recombinant plasmid DNA was transformed into One Shot TOP10 chemically competent *Escherichia coli*; transformants were grown on ampicillin plates. Cloned DNA was re-amplified by PCR for sequencing. Between 29 and 93 clones were sequenced for each sample. The IN gene was sequenced using a cycle sequencing reaction with the BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA). Primers IN1, IN4542S, IN4764AS and BH4 were used, as described previously.[22] Sequences were analysed using Sequence Navigator software.

IC_{50}

HIV-1 IN mutants were constructed, as described below: the fragment encoding the pNL43 virus IN was digested by *Age*I and *Eco*RI. Fragments were subcloned into the bluescript vector, and IN mutants containing N155H or G140S+Q148H mutations were obtained by mutagenesis using the QuickChange XL site-directed mutagenesis kit (Stratagene, Jackson Hole, WY, USA) according to the manufacturer's protocol. Mutant constructs were checked by sequencing and inserted into the pNL43 plasmid. Stocks of mutant HIV-1 virus were prepared by transfecting 293T cells. Transfection was carried out using the calcium phosphate method. Viral supernatants were harvested by centrifugation and stored at −80°C. HeLa P4 cells were infected with viruses containing 3 ng of HIV-1 p24 antigen and grown in the presence of increasing concentrations of raltegravir, from 0.001 to 500 nM. After 48 h, virus infectivity was determined by β-galactosidase production using the chlorophenol red-b-D-galactopyranoside (CPRG) substrate (Roche Molecular Biochemicals, Mannheim, Germany). The 50% inhibitory concentration (IC_{50}) is the raltegravir concentration at which 50% of β-galactosidase production was inhibited, relative to untreated infected cells.

Raltegravir plasma concentration

Raltegravir concentrations were determined by HPLC coupled with fluorimetric detection, as described previously.[23] The lower limit for quantification was 5 ng/mL. On the basis of previous findings, raltegravir plasma concentration was considered to be adequate if it reached 15 ng/mL.[16]

Results

Patients and HIV-1 viral load

Ten HIV-1-infected patients failing to respond to 400 mg of raltegravir, administered twice daily, were studied retrospectively. Their baseline characteristics are given in Table 1. At the start of raltegravir therapy, the median CD4 count was 52 cells/mm^3 (range, 2–370 cells/mm^3) and the median plasma HIV-1 RNA level was 4.7 $\log_{10}$ copies/mL (range, 1.8–5.4 $\log_{10}$ copies/mL). All 10 patients harboured highly mutated viruses with resistance to NRTI, NNRTI and PI; their genotypic sensitivity score (number of active ARV in the background regimen associated with raltegravir) was 0 or 1. Determination of plasma raltegravir concentration, before and during raltegravir failure, showed adequate values for all patients, and no correlation between levels of raltegravir concentration and raltegravir resistance pathways was found.

As shown in Figure 1, plasma HIV-1 RNA decreased from the baseline value to a minimal viral load in 1–12 weeks, depending on the patient. At this point, virus was undetectable (<1.60 $\log_{10}$ copies/mL) for five patients (patients H2–H6). The maximum decrease in the HIV-1 RNA level induced by the raltegravir-based regimen ranged from −1.5 to −3.8 $\log_{10}$ HIV-1 copies/mL, with a median of −2.6 $\log_{10}$ copies/mL. The minimal viral load was maintained for variable periods: fewer than 4 weeks for patients H1, H-SH1 and H-SH2 and up to 32 weeks for patient H4.

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Table 1. Baseline characteristics of patients

Patient	Subtype	Viral load (log_{10} copies/mL)[a]	No. of CD4 cells/mm^3	Antiretroviral treatment at day 0[b]	GSS[c]
H1	B	4.8	9	TDF, FTC, TPV/r, T20, RAL	1
H2	B	5.4	39	3TC, TPV/r, T20, RAL	0
H3	B	4.3	2	3TC, TDF, ABC, fos-APV/r, RAL	0
H4	CRF11	5.2	17	3TC, ABC, TDF, ETV, ATV/r, RAL	1
H5	B	4.6	238	3TC, ddI, TDF, LPV/r, T20, RAL	0
H6	B	3.8	192	TDF, FTC, ETV, DRV/r, RAL	1
SH1	B	4.9	65	3TC, TDF, LPV/r, fos-APV/r, RAL	0
SH2	B	4.5	370	ZDV, 3TC, IDV/r, LPV/r, RAL	0
H-SH1	B	5	6	3TC, ATV/r, DRV/r, T20, RAL	1
H-SH2	B	4.7	134	ZDV, 3TC, ABC, TDF, ATV, DRV/r, RAL	0

[a]Levels of HIV RNA in plasma were determined by using the COBAS AmpliPrep/COBAS TaqMan HIV-1 test.
[b]ZDV, zidovudine; ddI, didanosine; TDF, tenofovir; 3TC, lamivudine; LPV, lopinavir; fos-APV, fos-amprenavir; ABC, abacavir; T20, enfuvirtide; DRV, darunavir; FTC, emtricitabine; TPV, tipranavir; ETV, etravirine; IDV, indinavir; ATV, atazanavir; RAL, raltegravir; r, ritonavir.
[c]Genotypic sensitivity score of the optimized background regimen associated with raltegravir, according to genotypic resistance testing interpreted with the French ANRS AC11 algorithm, version 16 (www.hivfrenchresistance.org). Drugs with no resistance were assigned a score of 1, and drugs with intermediate or full resistance were assigned a score of 0.

Genotypic resistance profiles during raltegravir failure

We did not find IN resistance mutations in the bulk PCR products amplified from plasma HIV-1 RNA before the introduction of raltegravir for any patients. However, when the viral load increased towards the initial viral load, N155H or G140S+Q148H substitutions were detected in all patients. Thus, N155H was detected in six patients (H1–H6) and G140S+Q148H in two patients (SH1 and SH2); patients H-SH1 and H-SH2 showed a succession of two different resistant profiles: first N155H and then G140S+Q148H without N155H (Figure 1). For clonal analysis, a total of 1002 sequences were then generated and analysed. The first timepoint of the clonal analysis was between 0 and 12 weeks (w12) after the minimal viral load timepoint, depending on the patient. We evaluated the quasispecies variant dynamics for each timepoint, taking only the major mutations at positions 140, 148 and 155 into account in Figure 1; the numbers of wild-type, N155H, G140S+Q148H, Q148R/H and N155H+G140S/Q148R clones being represented by the bars. All changes in the IN gene are listed and summarized in Table 2. Overall, we identified seven positions (92, 97, 140, 148, 151, 155 and 163) mutated during raltegravir failure. The GenBank (http://www.ncbi.nlm.nih.gov/GenBank) accession numbers for the IN sequences are FJ538973–FJ538994.

Quasispecies variant distribution and dynamics of HIV-1 IN during raltegravir failure through the 155 pathway

Patients H1–H6 had a 155 profile at failure, as determined by bulk sequencing. Clonal analysis revealed substantial variability among the viral quasispecies for these patients, with the exception of patient H6. From 4 weeks after the minimal viral load, the N155H variant was found in all viral quasispecies in patients H1, H4, H5 and H6. The quasispecies variants in patient H3 were initially all wild-type (w16), but then nearly all (98%) switched to an N155H genotype (w24). In parallel, HIV-1 RNA load increased from 2.4 to 4.3 log_{10} copies/mL in this patient. In patient H2, N155H was found in 95% of the viral quasispecies (w28), corresponding to an increase in viral load from 2.2 to 4.2 log_{10} copies/mL (Figure 1). When all changes were considered (Table 2), a mixture of two clonal populations was detected in patients H1, H2 and H3. These populations harboured either the N155H substitution only (representing 74% of the analysed clones in H1, 51% in H2 and 89% in H3) or N155H+G163R (26% of the analysed clones in patient H1), E92Q+N155H (44% in patient H2) and V151I+N155H (9% in patient H3) (Table 2). In patient H1, two additional populations appeared at w28: V151I+N155H (10%) and V151I+N155H+G163R (59%), this population thus predominated over the N155H quasispecies (31%) (Table 2). In patient H4, clonal analysis at w48 showed all clones bearing E92A+N155H. The secondary E92A substitution has not been previously described, but appeared at the same time as N155H during treatment failure. This population declined during the follow-up (72% at w56 and 61% at w60), but continued to represent the majority of clones (Table 2). Other quasispecies bearing N155H alone were observed (27% at w56 and 24% at w60), as well as minor quasispecies populations N155H+G163R (2% at w56), E92Q+N155H (7% at w60), E92A+N155H+G163R (4% at w60) and N155H+G163R (4% at w60) (Table 2). In patient H5, the same four quasispecies N155H, N155H+G163R, V151I+N155H and V151I+N155H+G163R co-existed during the follow-up; the first circulating quasispecies variant to predominate over the other populations was N155H+G163R (48% at w18 and 64% at w20), which then became a minority (2%) at the expense of V151I+N155H, representing 74% of the clones at w30 (Table 2). We identified only one quasispecies in patient H6: T97A+N155H+G163R, representing 100% of the clones and remaining stable throughout follow-up.

Quasispecies variant distribution and dynamics of HIV-1 IN during raltegravir failure through the 148 pathway

Patients SH1 and SH2 displayed a 148 profile during failure, as determined by bulk sequencing. Clonal analysis in these patients revealed substantial homogeneity and stability of the viral quasispecies. Indeed, the levels of the resistant HIV-1 variant bearing G140S+Q148H increased until it represented

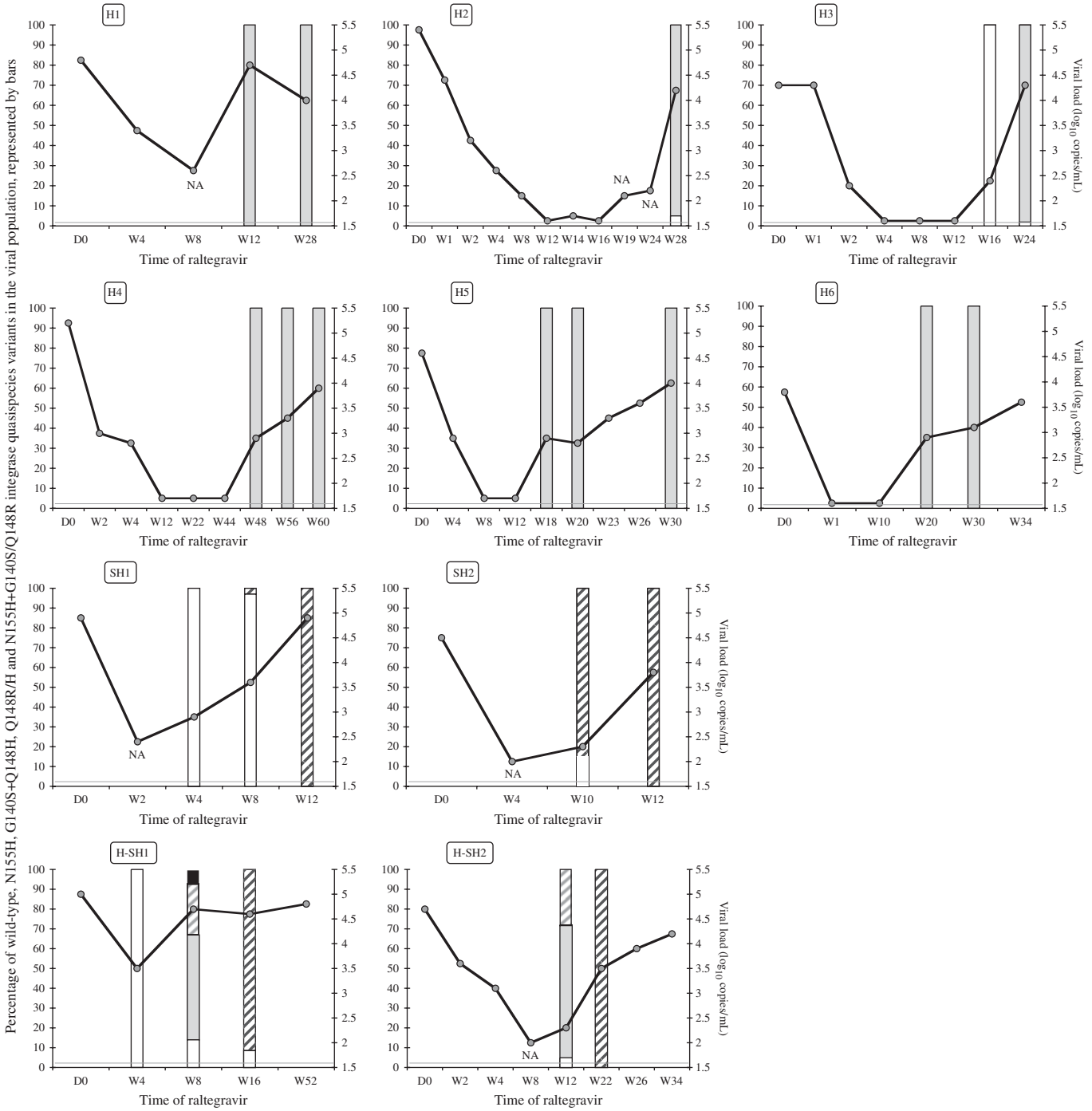

Figure 1. Plasma HIV RNA copy numbers for 10 patients (H1–H6, SH1, SH2, H-SH1 and H-SH2) failing to respond to raltegravir therapy. For each patient, the proportion of clones representing wild-type (white), N155H (grey), G140S+Q148H (dark grey diagonal stripes), Q148R/H (light grey diagonal stripes) and N155H+G140S/Q148R (dark grey) HIV-1 IN quasispecies variants at different timepoints is indicated by the bars. The line at 1.60 $\log_{10}$ copies/mL (40 copies/mL) shows the detection limit of the viral load assay. NA, non-amplifiable; D, day; W, week.

100% of the viral quasispecies in these patients (3% at w8 to 100% at w12 for patient SH1 and 85% at w10 to 100% at w12 for patient SH2); at this point, the HIV-1 viral load had nearly returned to baseline. The G140S+Q148H resistant variant was stable over time, the only other mutated population detected in these patients being a transitory quasispecies population bearing the mutation N155S (13% at w10) in patient SH2 (Table 2).

Quasispecies variant distribution and dynamics of HIV-1 IN during raltegravir failure in patients switching from the 155 to the 148 pathway

Patients H-SH1 and H-SH2 displayed a switch from the 155 to the 148 profile. Clonal analysis of these patients before the switch revealed the presence of similar proportions of wild-type,

Table 2. Clonal analysis of IN sequences during raltegravir therapy

Patient	Time of therapy	HIV-1 load (log_{10} copies/mL)	Bulk genotyping	Cloning	Clone numbers	IN sequence relative to the HxB2 reference sequence						
						92 E	97 T	140 G	148 Q	151 V	155 N	163 G
H1	D0	4.8	x			–	–	–	–	–	–	–
	W4	3.4	x			–	–	–	–	–	–	–
	W12	4.7	x			–	–	–	–	–	**H**	–
				x	29/39 (74%)	–	–	–	–	–	H	–
					10/39 (26%)	–	–	–	–	–	H	R
	W28	4	x			–	–	–	–	**V/I**	**H**	**G/R**
				x	23/39 (59%)	–	–	–	–	I	H	R
					12/39 (31%)	–	–	–	–	–	H	–
					4/39 (10%)	–	–	–	–	I	H	–
H2	D0	5.4	x			–	–	–	–	–	–	–
	W4	2.6	x			–	–	–	–	–	–	–
	W28	4.2	x			**E/Q**	–	–	–	–	**H**	–
				x	23/45 (51%)	–	–	–	–	–	H	–
					20/45 (44%)	Q	–	–	–	–	H	–
					2/45 (5%)	–	–	–	–	–	–	–
H3	D0	4.3	x			–	–	–	–	–	–	–
	W16	2.4	x			–	–	–	–	–	–	–
				x	43/43 (100%)	–	–	–	–	–	–	–
	W24	4.3	x			–	–	–	–	–	**H**	–
				x	40/45 (89%)	–	–	–	–	–	H	–
					4/45 (9%)	–	–	–	–	I	H	–
					1/45 (2%)	–	–	–	–	–	Y	–
H4	D0	5.2	x			–	–	–	–	–	–	–
	W48	2.9	x			**A**	–	–	–	–	**H**	–
				x	37/37 (100%)	A		–	–	–	H	
	W56	3.3	x			**A**	–	–	–	–	**H**	–
				x	31/44 (72%)	A	–	–	–	–	H	
					12/44 (27%)	–	–	–	–	–	H	
					1/44 (2%)	–	–	–	–	–	H	R
	W60	3.9	x			**A**	–	–	–	–	**H**	–
				x	28/46 (61%)	A	–	–	–	–	H	–
					11/46 (24%)	–	–	–	–	–	H	–
					3/46 (7%)	Q	–	–	–	–	H	–

Continued

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Table 2. *Continued*

Patient	Time of therapy	HIV-1 load (log_{10} copies/mL)	Bulk genotyping	Cloning	Clone numbers	IN sequence relative to the HxB2 reference sequence						
						92 E	97 T	140 G	148 Q	151 V	155 N	163 G
					2/46 (4%)	A	–	–	–	–	H	R
					2/46 (4%)	–	–	–	–	–	H	R
H5	D0	4.6	x			–	–	–	–	–	–	–
	W18	2.9	x			–	–	–	–	–	**H**	**R**
				x	23/48 (48%)	–	–	–	–	–	H	R
					19/48 (40%)	–	–	–	–	–	H	–
					4/48 (8%)	–	–	–	–	I	H	–
					2/48 (4%)	–	–	–	–	I	H	R
	W20	2.8	x			–	–	–	–	–	**H**	–
				x	27/42 (64%)	–	–	–	–	–	H	R
					6/42 (14%)	–	–	–	–	–	H	–
					6/42 (14%)	–	–	–	–	I	H	–
					3/42 (7%)	–	–	–	–	I	H	R
	W30	4	x			–	–	–	–	**I**	**H**	–
				x	34/46 (74%)	–	–	–	–	I	H	–
					10/46 (22%)	–	–	–	–	I	H	R
					1/46 (2%)	–	–	–	–	–	H	–
					1/46 (2%)	–	–	–	–	–	H	R
H6	D0	3.8	x			–	–	–	–	–	–	–
	W20	2.9	x			–	**A**	–	–	**I**	**H**	–
				x	48/48 (100%)	–	A	–	–	I	H	–
	W30	3.1	x			–	**A**	–	–	**I**	**H**	–
				x	47/47 (100%)	–	A	–	–	I	H	–
	W34	3.6	x			–	**A**	–	–	**I**	**H**	–
SH1	D0	4.9	x			–	–	–	–	–	–	–
	W4	2.9	x			–	–	–	–	–	–	–
				x	46/46 (100%)	–	–	–	–	–	–	–
	W8	3.6	x			–	–	–	–	–	–	–
				x	37/38 (97%)	–	–	–	–	–	–	–
					1/38 (3%)	–	–	S	H	–	–	–
	W12	4.9	x			–	–	**S**	**H**	–	–	–
				x	34/34 (100%)	–	–	S	H	–	–	–
SH2	D0	4.5	x			–	–	–	–	–	–	–

	W10	2.3	x			–	–	**S**	**H**	–	–	–
				x	41/48 (85%)	–	–	S	H	–	–	–
					6/48 (13%)	–	–	–	–	–	S	–
					1/48 (2%)	–	–	–	–	–	–	–
	W12	3.8	x			–	–	**S**	**H**	–	–	–
				x	36/36 (100%)	–	–	S	H	–	–	–
H-SH1	D0	5	x			–	–	–	–	–	–	–
	W4	3.5	x			–	–	–	–	–	–	–
				x	29/29 (100%)	–	–	–	–	–	–	–
	W8	4.7	x			–	–	–	**Q/R**	–	**N/H**	–
				x	15/28 (53%)	–	–	–	–	–	H	–
					7/28 (25%)	–	–	–	R	–	–	–
					4/28 (14%)	–	–	–	–	–	–	–
					1/28 (4%)	–	–	–	R	–	H	–
					1/28 (4%)	–	–	S	–	–	H	–
	W16	4.6	x			–	–	**S**	**H**	–	–	–
				x	86/94 (91%)	–	–	S	H	–	–	–
					8/94 (9%)	–	–	–	–	–	–	–
	W52	4.8	x			–	–	**S**	**H**	–	–	–
H-SH2	D0	4.7	x			–	–	–	–	–	–	–
	W12	2.3	x			–	–	–	–	–	**H**	–
				x	27/40 (67%)	–	–	–	–	–	H	–
					11/40 (28%)	–	–	–	H	–	–	–
					2/40 (5%)	–	–	–	–	–	–	–
	W22	3.5	x			–	–	**S**	**H**	–	–	**R**
				x	40/40 (100%)	–	–	S	H	–	–	R
	W26	3.9	x			–	–	**S**	**H**	–	–	**R**
	W34	4.2	x			–	–	**S**	**H**	–	–	**R**

D, day; W, week.
All shaded text concerns cloning analysis.
Bold amino acids concern bulk analysis.

Q148R/H or N155H clones: 14% (wild-type), 53% (N155H) and 25% (Q148R) for H-SH1 and 5% (wild-type), 67% (N155H) and 28% (Q148H) for H-SH2. Clonal analysis also showed the presence of one clone bearing Q148R+N155H (4%) and another bearing G140S+N155H (4%) in patient H-SH1. Interestingly, at week 16 for patient H-SH1 or at week 22 for patient H-SH2, these patient profiles switched towards G140S+Q148H; this variant represented 91% and 100% of the total viral quasispecies in patients H-SH1 and H-SH2, respectively (Figure 1 and Table 2). The remaining 9% of clones in patient H-SH1 corresponded to wild-type clones, with no 155 variant clones remaining. Additionally, the G163R substitution was associated with mutations G140S+Q148H in all clones in patient H-SH2 (Table 2).

IC_{50} values

The plasmid pNL43, bearing IN variants N155H and G140S+Q148H, was used to produce viruses and calculate the corresponding IC_{50} values. We obtained the following IC_{50} values: 10 nM for wild-type, 100 nM for N155H and 500 nM for G140S+Q148H. The fold resistance to raltegravir, relative to wild-type, was 10 for the N155H mutant and 50 for the G140S+Q148H mutant.

Discussion

Raltegravir belongs to a new class of ARV compounds that targets HIV-1 IN. Raltegravir failure is generally associated with one of the two pathways: 155 (N155H) or 148 (Q148K/R/H). We carried out clonal analysis of the IN gene during viral escape in 10 individuals infected with multidrug-resistant HIV-1 to better understand the development of resistance and HIV-1 quasispecies dynamics during raltegravir failure.

Sequencing of bulk PCR amplification products before raltegravir treatment and during viral load rebound was performed. Subsequent genotyping analysis identified resistance mutations in the IN gene. As shown in other recent studies,[20,24,25] we observed raltegravir failure through different pathways: the 155 pathway in six patients, the 148 pathway in two patients and a switch from the 155 to the 148 pathway in two patients.

Our clonal analysis showed that the two pathways associated with raltegravir failure seemed to be independent and even exclusive. In patients showing a switch from the 155 to the 148 pathway, clonal analysis showed the presence of Q148R/H and N155H mutations on different strands in the same sample before the switch, demonstrating that these are independent variants. However, in one patient, Q148R/H mutations were associated with N155H in only two clones. Thus, their association may be possible, but seems to be rare. These clones were no longer detected in samples analysed 8 weeks later; the association of these variants probably incurs a high cost for the virus, without conferring any advantages. The independence of the two pathways may be based on two different mechanisms of resistance; however, these mechanisms remain to be determined. The binding of raltegravir to IN is mediated both by interactions with metal ions in the active site and through direct contact with the enzyme. Recent findings suggest that the presence of the N155H substitution disrupts the arrangement of the active site magnesium ions and that the Q148K/R/H mutation affects direct contact with the enzyme.[26] Alternatively, our results suggest that both residues N155 and Q148 may play comparable roles in DNA recognition; mutations at these positions could thus be involved in two independent resistance pathways.[27] We previously showed that biochemical activity was more severely impaired in the Q148H+G140S mutant than in the N155H mutant, suggesting that the switch from the 155 to the 148 pathway is unlikely to be related to biochemical effects of 155 or 148 mutations on enzyme function. The switch of a resistance profile from 155 to 148 may be related to the higher resistance level, determined by the IC_{50} value, of the G140S+Q148H mutant than that of the N155H mutant virus.

Additional mutations were observed in all variant clones analysed during the early phase of failure, for both pathways. This suggests a potential role of such mutations in establishing resistance. Indeed, a recent study showed that impairment of viral fitness induced by mutations N155H and Q148R/H/K can be partially restored by the presence of additional secondary mutations.[28] In summary, the G140S substitution was associated with the 148 pathway, and either one or two of the mutations E92Q/A, T97A, V151I or G163R were associated with the 155 pathway in all clones analysed; this is consistent with the existence of two independent pathways.

G140S was associated with Q148H in all clones studied. G140S+Q148H mutant viruses progressively replaced the wild-type population and seemed to be stable over time with no other mutated populations appearing. The association of these two mutations seemed to be necessary and sufficient to confer a replication advantage to the virus.

In contrast, the N155H virus population displayed a high level of instability; indeed, new quasispecies appeared or frequencies of quasispecies varied over time, whereas the viral load still progressively increased towards the baseline value. Therefore, several quasispecies may be present from the start of treatment failure; some of these—N155H+G163R, V151I+N155H, V151I+N155H+G163R or E92Q/A+N155H—have been described previously.[25,28,29] However, we identified a quasispecies with T97A+V151I+N155H mutations which has not been previously described and appeared to be very stable during the follow-up. The greater variability of quasispecies associated with the 155 pathway suggests that the N155H substitution does not confer a stable state of resistance to raltegravir, regardless of the secondary mutations present.

No secondary mutations associated with either the N155H or Q148H/R substitutions were detected before the switch of pathways. The secondary G140S mutation only appeared to be associated with Q148H at the time of establishment of the 148 pathway and the concomitant disappearance of the N155H quasispecies. Thus, the selection of the 148 pathway does not seem to be driven by the presence of the G140S mutation; rather, this mutation may be selected to compensate its effect.

Our findings have implications for virological follow-up and diagnosis of raltegravir resistance. Indeed, bulk sequencing results showed only wild-type sequences in a patient who had an increase of viral load from 2.9 to 3.6 $\log_{10}$ copies/mL, whereas the more sensitive method involving the analysis of 38 clones detected the early selection of a G140S+Q148H resistant minority variant. The G140S+Q148H mutations were detected by bulk sequencing only after 4 months; all clones were positive for these two mutations. However, this cloning method is not suitable for use in clinical practice. A sensitive selective

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PCR, based on real-time PCR technology, could therefore be developed to detect early selection of raltegravir-resistant variants.

In conclusion, this study strongly suggests that the two main genetic pathways 155 and 148 involved in the development of resistance to raltegravir are independent and exclusive. Moreover, the switch of the resistance profile from 155 to 148 may be related to the higher level of resistance to raltegravir conferred by the 148 pathway and also to the higher instability of the 155 pathway. However, the mechanisms underlying the selection of one pathway over the other remain unknown. All patients harboured adequate raltegravir plasma concentration at the time of failure, and no correlation between levels of raltegravir concentration and raltegravir resistance pathways was found. However, due to the low number of patients analysed in this study, this should be taken into careful consideration and further explored in larger studies. It is likely that other factors, such as host or virus genetic background or viral fitness, are involved in determining the selection of one particular pathway.

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