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Multidrug Resistance Genotypes (Insertions in the β3–β4 Finger Subdomain and MDR Mutations) of HIV-1 Reverse Transcriptase from Extensively Treated Patients: Incidence and Association with Other Resistance Mutations

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Multiple nucleoside resistance involves specific mutational patterns of the HIV-1 *pol* gene that are independent of the classic mutations conferring resistance to individual dideoxynucleosides. These include a cluster of five mutations in the reverse-transcriptase (RT) coding region (A62V, V75I, F77L, F116Y, and Q151M) generally referred to as multidrug resistance (MDR) mutations, and insertions of one or several amino acid residues between codons 67 and 70 of RT, a flexible region joining two antiparrallel β sheets (β3–β4 insertions). The objectives of this study were (i) to determine the prevalence of multidrug resistance genotypes (MDR mutations and β3–β4 insertions) in a cohort of 632 patients who were extensively pretreated with anti-HIV drugs and not responding to their current antiretroviral therapy, and (ii) to analyze the association of multidrug resistance genotypes with other resistance mutations in the RT and protease genes. Among viruses sequenced from these patients, 15 (2.4%) of them contained an insertion and 2 (0.3%) contained a deletion in the β3–β4 finger subdomain of RT. In 9 cases, the insertion was associated with a D67S, G, or E mutation. In addition, we identified 13 (2.1%) viruses harboring specific MDR mutations (mainly Q151M and/or A62V, V75I, F116Y). Interestingly, the A62V mutation was found in 6 of the 15 strains with an insertion, whereas the other MDR mutations were not observed in insertion mutant strains. Especially high levels of resistance to zidovudine were observed for viruses with a β3–β4 insertion in the background of A62V, L210W, and T215Y. Otherwise, MDR mutations and β3–β4 insertions were found in association with the classic mutations conferring resistance to zidovudine, lamivudine, nonnucleoside RT inhibitors, and protease inhibitors, according to treatment history. Finally, we observed a genome with a deletion of codon 70 associated with a Q151M MDR mutation. These data suggest that the emergence of HIV-1 multidrug resistance, which may occur in various genetic contexts, poses a challenging problem in formulating treatment strategies.

INTRODUCTION

The infection cycle of HIV-1 is dependent on the functions of reverse transcriptase (RT), a virally encoded polymerase which converts the viral RNA genome into proviral DNA prior to its integration into the host genomic DNA. It is a heterodimer of a 66- and 51-kDa subunit, termed p66 and p51. Crystallographic data have revealed that both subunits form a right-hand-like structure consisting of several subdomains designated palm, fingers, and thumb subdomains that cooperate to form the template-binding cleft (Kohlstaedt *et al.,* 1992; Jacobo-Molina *et al.,* 1993; Huang *et al.,* 1998; Sarafanios *et al.,* 1999). The high variability of HIV-1 is due to the high mutation rate of RT which is a poorly processive enzyme (Kew *et al.,* 1998). The processivity of polymerase refers to the average number of deoxynucleotides incorporated per template-primer encounter and reflects the accuracy of the RNA-dependent DNA polymerization. It has been proposed that the low processivity of retroviral RT has been evolutionarily conserved to facilitate the strand transfer reaction, which is a key step in retroviral DNA synthesis (Pathak and Temin, 1990). Considering the lack of 3′ exonuclease proofreading activity of RT, the poor processivity of RT may be the principal cause for the high rate of nucleotide substitutions, deletions, insertions, and recombination events observed for HIV-1 RT. Upon treatment with nucleoside analog RT inhibitors, HIV-1 variants exhibiting resistance-associated mutations in the RT gene are selected and the replication of these strains is a major cause of treatment failure (Hirsch *et al.,* 1998). Some of these mutations, often referred to as MDR (multidrug resistance) mutations, may induce a broad resistance phenotype to various nucleoside analogs. MDR mutations include A62V, V75I, F77L, F116Y, and Q151M (Maeda *et al.,* 1998). Recently, it has been shown that resistance to multiple nucleoside analogs could also result from the insertion of a short sequence in the

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TABLE 1

Amino Acid Changes in the RT Protein of Insertion, Deletion, and MDR Mutant HIV-1 Compared to HXB2

	codon	41	50	62	65	66	67	68	69	70	74	75	77	98	100	103	106	108	116	151	181	184	188	190	210	215	219
	Wild-type	M	I	A	K	K	D	S	T	K	L	V	F	A	L	K	V	V	F	Q	Y	M	Y	G	L	T	K
insertion mutants	BS 02/97	-	-	-	-	-	E gaa	**S tcc**	**S tcc**	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	W	Y	-
	BS 12/97	-	-	-	-	-	E gaa	**S tcc**	**S tcc**	-	-	-	-	-	-	-	-	VI	-	-	YC	I	-	-	W	Y	-
	JY 01/97	L	-	V	-	-	-	**S tcc**	**S agt**	S agt	-	I	-	-	-	-	-	-	-	-	-	V	-	-	W	Y	-
	JY 01/98	L	-	V	-	-	-	**S tcc**	**S agt**	G ggt	-	I	-	-	-	N	-	•	-	-	-	V	-	A	W	Y	-
	JY 08/99	L	-	V	-	-	E gaa	**G ggt**	**S tcc**	G	-	I	-	-	G	-	-	-	I	-	-	-	V	-	A	W	Y	-
	JY 11/99	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	
	AR 11/97	-	-	-	-	-	G	-	-	**T act**	R aga	-	VA	-	S	-	-	-	-	-	-	I	-	-	-	F	Q
	AR 01/98	-	-	-	-	-	G	-	-	**T act**	R aga	-	VA	-	S	-	-	-	-	-	-	I	-	-	-	F	Q
	AR 04/99	-	-	-	-	-	G	-	-	**T act**	R aga	-	VA	-	S	-	-	-	-	-	-	MIV	-	-	-	TFIS	Q
	GM 10/98	L	-	-	-	-	E	-	S agc	**V gtg**	**T act**	-	-	-	S	-	KT	-	-	-	-	-	-	-	W	YF	-
	GM 06/99	L	-	-	-	-	E	-	S agc	**V gtg**	**T act**	-	-	-	S	LI	-	-	-	-	-	-	L	-	W	Y	-
	GM 11/99	L	-	-	-	-	E	-	S agc	**V gtg**	**T act**	-	-	-	S	-	-	-	-	-	-	-	L	-	W	YF	-
	BP 07/98	L	-	V	-	-	-	**S agc**	**S agt**	S tct	-	LV	-	-	G	-	-	-	VI	-	-	V	-	-	W	Y	-
	BP 07/99	L	-	V	-	-	-	**S agc**	**S agt**	S tct	-	V	-	-	G	-	KN	-	I	-	-	V	-	GA	W	Y	-
	BP 08/99	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W	Y	-
	BP 10/99	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W	Y	-
	BP 11/99	L	-	V	-	-	-	**S agc**	**S agt**	S tct	-	V	-	-	G	-	-	-	I	-	-	C	V	-	A	W	Y	-
	AC 01/95	-	-	-	-	-	-	-	S agt	**T act**	**G ggt**	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AC 04/98	-	-	-	-	-	-	-	S agt	**V gtt**	**G ggt**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-
	AC 06/99	-	-	-	-	-	-	-	S agt	**V gtt**	**G ggt**	-	-	-	-	N	-	-	-	-	C	-	-	GA	-	-	-
	DD 08/98	-	-	-	-	-	G	-	S agt	**S agt**	**G ggt**	-	-	-	-	-	-	-	-	-	-	V	-	-	-	Y	-
	DD 02/99	-	-	-	-	-	DGE	-	S agt	**S agt**	**G ggt**	-	-	-	-	-	-	-	-	-	-	V	-	-	-	Y	-
	DD 09/99	-	-	-	-	-	G	-	S agt	**V gtt**	**G ggt**	Q	-	-	-	-	-	-	-	-	-	V	-	-	-	Y	-
	GR 09/97	ML	-	AV	-	-	-	-	S agc	**S agt**	**A gct**	-	-	-	-	-	-	-	-	-	-	-	-	-	LW	Y	-
	GR 08/98	L	-	V	-	-	-	-	S agc	**S agt**	**T act**	-	-	-	-	-	-	-	-	-	-	-	-	-	W	Y	-
	NR 04/97	L	-	-	-	-	-	-	A gct	**S agt**	**G ggt**	-	-	-	-	-	-	-	-	-	-	-	-	-	W	Y	-
	BR 03/99	L	-	-	-	-	-	**S agc**	**S agt**	T act		-	LV	-	-	-	N	-	-	-	C	-	-	A	W	Y	-
	SR 02/99	-	-	-	-	-	G	**Y tat**	-	D		R	-	-	-	-	-	-	-	-	C	I	-	-	-	-	Q
	GB 02/99	-	-	AV	-	-	E	-	**S agt**	**E gag**	S tct	-	-	-	-	-	-	-	-	-	C	-	-	A	W	Y	-
	MP 03/99	-	-	V	-	-	-	-	**S tcc**	**S agt**	S tct	R	-	-	-	-	-	-	-	-	-	V	-	-	-	F	-
	NO 10/99	L	-	-	-	-	E	-	**S agc**	**V gtt**	T act	-	-	-	-	-	-	-	-	-	-	-	-	-	W	Y	-
	JS 12/99	-	-	V	-	-	E gaa	**S agt**	**S tcc**	-	S	-	-	-	-	I	N	-	-	FY	-	-	I	-	-	W	Y	-
deletion and MDR mutants	NC 03/97	-	-	-	-	-	S	-	G	**(-)**		-	-	-	-	-	-	-	-	-	-	V	-	-	-	L	E
	NC 10/97	-	-	-	-	-	S	-	G	**(-)**		-	-	-	-	-	-	-	-	-	-	V	-	-	-	F	E
	NC 01/98	-	-	-	-	-	S	-	G	**(-)**		-	-	-	-	-	-	-	-	-	-	V	-	-	-	F	E
	QV 09/99	-	-	-	-	-	-	-	-	**(-)**		-	-	-	-	KN	-	-	-	**M**	-	V	-	S	-	-	-
	BRP 04/99	-	-	V	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	**M**	-	-	-	-	-	-	-
	OK 02/99	-	-	V	-	-	-	-	-	-	-	I	L	-	-	-	-	-	Y	**M**	-	-	-	-	-	-	-
	OK 07/99	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**-**	-	-	-	-	-	-	-
	TK 02/99	L	-	-	-	-	-	-	-	-	LI	-	-	-	-	S	-	-	-	**-**	-	V	-	A	-	Y	-
	TK 09/99	L	-	-	-	-	-	-	-	-	-	VA	-	-	-	S	-	-	-	**M**	-	V	-	A	-	Y	-
	BF 03/99	-	-	-	-	-	-	-	D	-	-	-	-	-	-	N	-	-	-	**LM**	-	-	-	-	-	-	-
	BF 07/99	-	-	-	-	-	-	-	D	-	-	-	-	-	-	N	-	-	-	**M**	-	-	-	-	-	-	-
	PP 09/98	-	-	-	-	-	N	-	D	R	-	-	-	-	-	-	-	-	-	**M**	-	-	-	-	-	F	Q
	PP 02/99	-	-	-	-	-	N	-	D	R	-	-	L	-	-	-	-	-	-	**M**	-	-	-	-	-	F	Q
	BB 04/99	-	-	-	-	-	N	-	D	R	-	-	-	-	-	-	-	I	YF	**QL**	YC	V	-	-	-	IF	Q
	BC 04/99	-	-	-	R	-	-	-	-	R	-	I	L	-	-	-	-	-	Y	**M**	-	V	-	-	-	-	-
	BM 04/99	-	-	-	-	-	NS	-	N	R	-	-	-	-	-	-	-	-	Y	**M**	-	-	-	-	-	F	Q
	FC 03/99	-	-	-	R	-	-	-	-	T	L	I	L	-	-	-	-	-	Y	**M**	C	-	-	-	-	-	-
	PT 11/98	-	-	V	-	-	-	-	I	-	-	I	L	-	-	KN	-	-	Y	**M**	YC	V	-	GA	-	-	-
	VD 06/99	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	Y	**M**	-	-	-	-	-	-	-
	PJ 12/99	-	-	-	-	-	N	-	D	R	-	-	-	-	-	-	-	-	-	**M**	-	MV	-	T	-	F	Q

Note. Insertions are shown in bold type with the nucleotide sequence indicated under the corresponding amino acid. Dashes correspond to wild-type codons. Deletions are figured as (−). The dates when the viruses were recovered from the patients are indicated after the patient's initials (e.g., BS 02/97 means that the virus has been sequenced from a plasma sample obtained from patient BS in February, 1997).

β3–β4 hairpin loop of the finger domain of RT (Tamalet *et al.,* 1998), although it is unclear whether the insertion induces resistance by itself or only when combined to other mutations such as T215Y (Winters *et al.,* 1998; Larder *et al.,* 1999). The β3–β4 subdomain of HIV-1 RT is directly involved in the interaction of the enzyme with its substrates (i.e., the template-primer complex and the incoming dNTP), thereby controlling the processivity of the RNA-dependent DNA polymerization (Arion *et al.,* 1996; Arnold *et al.,* 1995; Kew *et al.,* 1998; Tantillo *et al.,*

1994). The aim of the present study was to document the presence of multidrug resistance genotypes in a cohort of 632 patients under combination therapy and to analyze their association (or lack of association) with other resistance mutations in the RT and protease genes.

RESULTS

Insertions and deletions in the $\beta3$–$\beta4$ hairpin loop of the finger subdomain of HIV-1 RT

The genotypic analysis of plasma HIV-1 RNA from 632 patients revealed rearrangements in the RT coding region (between amino acids 67 and 70 belonging to the $\beta3$–$\beta4$ hairpin loop) in 17 cases (Table 1). These genetic rearrangements resulted in a net deletion of one amino acid residue (i.e., T69 for patient NC, and K70 for patient QV) or in a net gain of one or two residues in the RT protein. The inserted sequences were particularly enriched in serine (S). An additional mutation at codon D67 (D67S, G, or E) was detected in 9 cases. Interestingly, the deletion in patient NC was also associated with a D67S mutation. However, the classical D67N substitution, which is generally associated with resistance to zidovudine, was not detected in the inserted or deleted sequences analyzed in this study. Most importantly, the deletion in patient QV was associated with the major MDR mutation Q151M.

The stability of the rearrangements in the $\beta3$–$\beta4$ hairpin loop was assessed by sequencing the RT gene from 9 patients at different time points over a minimal period of 10 months (patient BS) and a maximal period of 54 months (patient AC). In the case of patient JY, the initial SSS insertion was later replaced by SSG. This amino acid change was due to a single mutation of codon AGT (coding for serine) leading to GGT (coding for glycine). In fact, a minor population with the amino acid SSG sequence could be detected first (Fig. 1), suggesting that the SSG variant was selected from a mixture of SSS and SSG. The virtual absence of fluorescence background in the electrophoregram shown in Fig. 1 allowed the identification of the minor G peak unambiguously. Moreover, the presence of the minor SSG mutant at MO was confirmed by sequencing the complementary strand (not shown). After 18 months, the SSG insertion was shifted and replaced by EGS. Then, the therapy of this patient was stopped and the insertion was no longer detected, whereas M41L and T215Y remained the only resistance mutations observed in the RT gene. A similar disappearence of the insertion was observed for patient BP (08/99) who stopped the therapy in May 1999. Yet the insertion mutant was detected again in November 1999, 1 month after the therapy was started again. Other cases of evolution of the inserted sequence were noted for patients AC (STG → SVG), DD (SSG → SVG) and GR (SSA → SST).

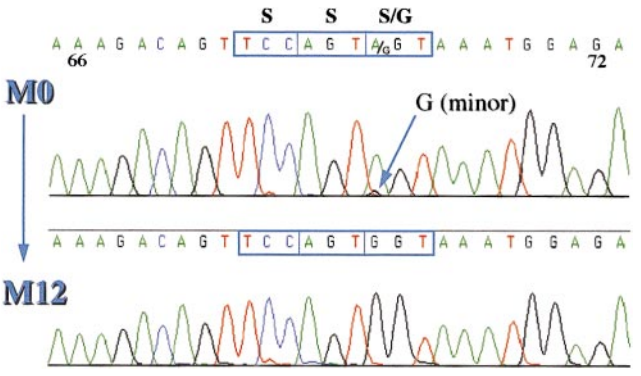

FIG. 1. Emergence of the SSG inserted sequence from a mixture of SSS and SSG sequences. The figures shows the electrophoregram corresponding to the RT coding region (from codons 66 and 72) for patient JY at two sequential time points (M0 and M12).

MDR mutations

MDR mutations were detected in 13 patients: A62V (3 cases), V75I (4 cases), F77L (6 cases), F116Y (7 cases), and Q151M (12 cases). Moreover, substitutions at some of these positions with unusual residues were also observed: V75T (1 case) which occurs upon stavudine selective pressure but rarely *in vivo,* and Q151L (2 cases), which may represent an intermediate genotype which precedes the Q151M MDR mutation. Indeed, the Q → M substitution requires two difficult nucleotide changes (a double transversion) of the codon (CAG → ATG). A single A → T transversion in this codon (CAG → CTG) would facilitate the process. In agreement with this hypothesis, both Q151Q/L and Q151L/M heterozygotes could be detected in patients BB and BF, respectively (Table 1). In this latter case, the Q151L/M mixture evolved toward Q151M. Therefore, it is tempting to speculate that the difficult Q151M substitution can occur upon selective pressure because the intermediate Q151L mutation is not deleterious for the enzyme. Recent data on the comparative fitness of MDR resistant HIV-1 strains support this view (Kosalaraksa *et al.,* 1999).

Resistance mutations associated with inserted and MDR genotypes

As shown in Table 1, the A62V MDR mutation was present in 6 of the 15 strains with an insertion, whereas the other MDR mutations were not observed in insertion mutant strains. However, in one case, the Q151M mutation was found in association with a deletion of codon 70 (patient QV). The major zidovudine resistance mutations, i.e., K70R and T215Y/F, could be detected in association with a $\beta3$–$\beta4$ insertion (4 cases for K70R, 11 cases for T215Y, and 4 cases for T215F). Similarly, these mutations were found in combination with MDR mutations (5 cases for K70R, 1 case for T215Y, and 4 cases for T215F). In contrast, the minor L210W mutation was not observed in association with MDR mutations, whereas D67N was

TABLE 2

Amino Acid Changes in Protease of Insertion, Deletion, and MDR Mutant HIV-1 Compared to HXB2

	codon	10	20	24	30	32	33	36	46	47	48	50	54	63	71	73	77	82	84	88	90	93
	Wild-type	L	K	L	D	V	L	M	M	I	G	I	I	L	A	G	V	V	I	N	L	I
insertion mutants	BS 02/97	I	-	-	-	-	-	I	I	-	-	-	V	PA	-	-	-	A	-	-	-	-
	BS 12/97	I	-	-	-	-	-	I	I	-	-	-	V	A	V	-	-	A	V	-	-	-
	JY 01/97	I	-	-	-	-	-	-	I	-	-	-	-	P	V	S	I	-	-	-	M	L
	JY 01/98	I	-	-	-	-	-	-	I	-	-	-	-	P	V	S	I	VA	-	-	M	L
	JY 08/99	I	-	-	-	-	-	-	MI	-	-	-	-	P	V	S	I	-	-	-	M	L
	JY 11/99	I	-	-	-	-	-	-	I	-	-	-	-	P	V	S	I	-	-	-	M	L
	AR 11/97	I	R	-	-	-	-	I	-	-	-	-	V	P	-	-	-	A	-	-	-	-
	AR 04/99	I	R	-	-	-	-	I	-	-	-	-	V	P	AV	-	-	SA	-	-	-	-
	GM 10/98	I	MI	-	-	-	-	MI	-	-	-	-	-	P	V	S	-	VA	-	-	M	-
	GM 06/99	I	I	-	-	-	-	-	-	-	-	-	-	P	V	S	-	VA	-	-	M	-
	GM 11/99	I	I	-	-	-	-	-	-	-	-	-	-	P	V	S	-	A	-	-	M	-
	BP 07/98	I	KR	-	-	-	-	I	-	-	V	-	-	P	V	-	-	A	-	-	M	-
	BP 08/99	-	-	-	-	-	-	MI	-	-	-	-	-	P	-	-	-	-	-	-	-	-
	BP 11/99	I	R	-	-	-	-	I	-	-	GV	-	-	P	V	-	-	A	-	-	M	-
	AC 01/95	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	I	-	-	-	-	-
	AC 05/96	-	-	-	-	-	-	-	-	-	-	-	-	TPS	-	-	IV	-	-	-	-	-
	AC 04/98	I	-	-	-	-	-	-	-	-	-	-	V	P	TI	-	V	A	-	-	-	-
	AC 06/99	I	-	-	-	-	-	-	-	-	-	-	V	P	T	-	-	A	-	-	-	-
	DD 08/98	LI	KMI	-	-	-	-	-	I	-	-	-	-	LP	-	-	-	TA	-	-	M	IL
	DD 02/99	LI	I	-	-	-	-	-	I	-	-	-	-	LP	-	-	VI	-	-	-	M	IL
	DD 09/99	I	I	-	-	-	-	MI	I	-	-	-	-	LP	-	-	-	-	-	-	M	L
	GR 06/97	-	-	-	-	VA	-	-	-	-	-	-	-	LP	-	-	-	-	-	-	-	-
	GR 09/97	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-
	GR 08/98	-	-	-	-	I	-	V	I	V	-	-	-	P	-	-	-	VA	-	-	-	-
	BR 03/99	I	-	-	-	-	-	-	I	-	V	-	T	P	V	-	I	A	-	-	-	L
	SR 02/99	LI	-	LI	-	-	F	-	-	-	-	-	V	-	-	-	I	A	-	-	-	-
	GB 02/99	-	I	-	-	-	-	I	I	-	-	-	V	P	V	S	-	-	V	-	-	-
	MP 03/99	-	-	-	-	-	-	-	-	-	-	-	-	P	V	S	-	-	-	-	-	-
	NO 10/99	I	-	-	-	-	-	-	-	-	-	-	L	P	T	T	I	-	V	-	-	L
	JS 03/99	I	R	-	-	-	-	-	I	-	V	-	T	S	V	-	-	A	-	-	-	-
deletion and MDR mutants	NC 03/97	V	-	-	-	-	-	-	-	-	-	-	-	P	I	-	-	-	-	-	-	-
	NC 10/97	V	-	-	-	-	-	-	-	-	-	-	-	P	I	-	-	-	-	-	-	-
	QV 09/99	-	-	-	-	-	LV	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-
	BRP 04/99	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-
	BF 03/99	IV	-	-	-	-	-	-	MI	-	-	-	IV	S	AV	-	I	VA	-	-	M	L
	PP 09/98	-	R	-	-	-	-	I	-	-	-	-	IV	P	V	-	-	S	-	-	-	-
	BB 04/99	I	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	M	-
	BC 04/99	-	KT	-	-	-	-	MIV	-	-	-	-	-	LP	-	-	-	VI	-	-	LM	L
	BM 04/99	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	I	-	-	-	-	-
	FC 03/99	-	T	-	N	-	-	-	-	-	-	-	-	P	V	-	I	-	-	ND	M	-
	PT 11/98	-	R	-	N	-	-	I	-	-	-	-	-	P	-	-	-	-	-	-	-	L
	VD 06/99	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-
	PJ 12/99	I	I	-	-	-	-	I	-	-	-	-	V	P	V	S	-	A	-	-	M	-
	TK 02/99	Y	I	-	-	-	-	I	-	-	-	-	V	P	V	-	-	-	V	-	M	-
	TK 09/99	Y	I	-	-	-	-	I	-	-	-	-	V	P	V	-	-	-	V	-	M	-
	OK 02/99	R	KT	-	-	-	-	-	MIL	-	-	-	IV	P	AVTI	-	-	-	-	-	-	-
	OK 07/99	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-

detected only in those strains bearing the Q151M MDR mutation. Finally, mutations conferring resistance to non-nucleoside RT inhibitors (K103N, V108/I, Y181C, Y188L, and/or G190A) were found in association with MDR as well as insertion mutations.

The pattern of resistance-associated mutations in the protease gene (Table 2) was consistent with the treatment history of the patients. Patients FC and PT, for instance, show a D30N mutation consistent with the use of nelfinavir in their combination therapy. Curiously, the L10I mutation was more frequently observed in genomes with an insertion in the RT gene than in genomes with MDR mutations. Further studies will help to clarify whether mutation patterns of HIV-1 RT may affect the polymorphism of the protease gene, as recently discussed by Berkhout (1999). Finally, a rare L10Y substitution was observed in one case (patient TK).

DISCUSSION

In this study, we have analyzed the incidence of MDR genotypes (including insertions/deletions in the $\beta3$–$\beta4$ finger subdomain of RT and MDR mutations) in a homogenous cohort of 632 patients with therapeutic failure. We detected 15 (2.4%) genotypes with an insertion, 2 (0.3%) with a deletion, and 13 (2.1%) with MDR mutations (mainly Q151M and/or A62V, V75I, F116Y). Overall, multiresistant genotypes (i.e., either MDR mutations or insertions) were found in 29 (4.6%) of the viruses sequenced from the 632 patients studied. These data are in agreement with previous population studies showing an incidence of 3% for $\beta3$–$\beta4$ insertions (De Jong *et al.,* 1999; Ross *et al.,* 1999; Tamalet *et al.,* 1998), and 1–2% for MDR mutations (Shirazaka *et al.,* 1995; Iversen *et al.,* 1996). Moreover, neither MDR nor $\beta3$–$\beta4$ insertions were detected in therapy-naive patients with primary HIV infection, supporting

the concept that multidrug resistant viruses are selected under antiretroviral treatment. In this respect, various combinations of zidovudine, didanosine, zalcitabine, and stavudine seem to precipitate the emergence of those viruses (Larder *et al.,* 1999; Ross *et al.,* 1999).

The presence of a rare amino acid change at codon D67 (D67S, G, or E) associated with a β3–β4 insertion confirms and extends previous data reported by three laboratories (Winters *et al.,* 1998; De Jong *et al.,* 1999; Larder *et al.,* 1999). In addition, one major outcome of the present study is the finding of a statistically significant combination of the MDR mutation A62V and a β3–β4 insertion ($P < 0.01$). This mutation, when associated with Q151M, modifies the hydrophobic core of the finger subdomain of HIV-1 RT in order to adjust the orientation of methionine 151 (Huang *et al.,* 1998). In the same way, insertions and/or deletions in the β3–β4 hairpin loop are likely to affect the dNTP-binding pocket through subtle conformationnal changes. Therefore, our data raise the interesting hypothesis that the A62V mutation may help the enzyme to accommodate the insertion in the β3–β4 hairpin loop. This would lead to a reorganization of the dNTP-binding pocket exhibiting a decreased affinity for some nucleoside analogs. In agreement with this hypothesis, phenotypic assays with recombinant mutant RT (Table 3) showed that mutation A62V, in the background of T215Y, L210W, and a SSS insert, increased the level of zidovudine resistance by more than 10-fold (Larder *et al.,* 1999). A high level of resistance to zidovudine was also reported by De Jong *et al.* (1999) for a virus with both a β3–β4 insertion (SSG) and mutation A62V in the RT gene (Table 3). In order to determine whether mutation A62V could act in synergy with a β3–β4 insertion to increase the level of zidovudine resistance, population-based recombinant isolates were prepared from patient plasma virus and assayed for susceptibility to zidovudine. As shown in Table 3, high levels of resistance (>1500-fold) were observed for the viruses obtained from patients BP and GB, with a β3–β4 insertion in the background of A62V, L210W, and T215Y. Interestingly, a lower level of resistance was observed when the insertion was combined with L210W and T215Y, but not A62V (patient BR). Moderate levels of resistance were found when the β3–β4 insertion was associated with T215F, with or without A62V (patients MP and AR, respectively). Taken together, these data suggest that the presence of a β3–β4 insertion associated with mutations A62V, L210W, and T215Y confers a particularly high level of resistance to zidovudine.

Insertion mutants generally appear in the background of zidovudine resistance mutations, especially T215Y. Indeed, viral constructs that contained both the T215Y mutation and an insertion showed higher IC_{50} to the RT inhibitors than the constructs containing only the insertions (Winters *et al.,* 1998; Larder *et al.,* 1999). Previous modeling studies did not allow an explanation of how the zidovudine resistance mutations act in concert with the insertion to increase the level of resistance (Larder *et al.,* 1999). It is possible that the additional effect of the T215Y mutation on the magnitude of resistance induced by the insertion may be due to a hydrophobic-mediated reorganization of residues lining the dNTP-binding pocket, especially Asp 113. However, it is interesting to note that in two patients of the present study, the insertion was found in association with a wild genotype at codon 215 (patients AC and SR). In one of these cases (patient AC), the insertion was exclusively associated with three mutations that confer resistance to nonnucleoside inhibitors of RT (i.e., K103N, Y181C, and G190G/A). Therefore, the appearance of β3–β4 insertions can, in rare cases, occur in a genome devoid of zidovudine resistance mutations, suggesting that zidovudine therapy is not a prerequisite for subsequent development of insertions. This result is in agreement with a recent study by Ross *et al.* (1999). Finally, it should be emphasized that the MDR mutation Q151M is never observed in association with a β3–β4 insertion ($P < 0.001$). In only one case, this mutation was present in combination with a deletion of codon 70. To our knowledge, this is the first time that such an association is described. Overall, these data further illustrate the complexity of genetic rearrangements that may potentially affect the conformation of the dNTP-binding pocket in the context of multidrug therapy.

From an evolution point of view, the emergence of the SSG variant from a mixed population enriched in genomes with the SSS insertion (patient JY, Fig. 1 and Table 1) suggests a selective advantage for SSG. Indeed, phenotypic assays from Winters *et al.* (1999) have shown that a virus with a SSG insert is significantly more resistant to zidovudine than a virus with a SSS insert (Table 3). In addition, biochemical studies have shown that the SSS variant has a lower RT activity than the SSG variant, suggesting that the SSS/SSG switch may represent a compensatory mechanism allowing a gain of fitness (Boyer *et al.,* 1999). Thus, a fine "tuning" of the inserted sequence may be necessary to optimize the orientation of the β3–β4 hairpin loop, and this may be achieved by the appearance of additional mutations within the insert or nearby the dNTP-binding pocket (see Huang *et al.,* 1998, for a discussion of "tuning" effects in HIV-1 RT). From an enzymatic point of view, insertions in the β3–β4 finger subdomain have been shown to increase the processivity of the polymerase (Kew *et al.,* 1998). Namely, the enrichment of the loop in G and S residues may respectively contribute to enhance the mobility of the peptide chain and its capacity to hydrogen bond to the substrates. By strengthening its interactions with the template and the dNTP, the mutant RT may become more processive (Kew *et al.,* 1998) and may have a selectively decreased affinity for the triphosphorylated analogs. That most mutations conferring resistance to zidovudine increase the processivity of RT strongly supports this

hypothesis (Arion *et al.,* 1996, 1998). Alternatively, it has been proposed that resistance to zidovudine could also partially result from an increased pyrophosphorolytic cleavage of chain-terminated viral DNA by the mutant RT, a molecular mechanism which may be interpreted as a type of proofreading activity expressed by the mutant RT (Arion *et al.,* 1998). In this context, the increased processivity of the mutant RT may represent a compensatory mechanism for the increased reverse reaction rate.

CONCLUSIONS

Genetic rearrangements in the $\beta3$–$\beta4$ hairpin loop of the finger subdomain of RT and MDR mutations are still uncommon changes of HIV-1 RT (prevalence <5%) emerging in extensively treated patients. These genotypes, which appear in various genetic contexts and are generally associated with therapeutic failure, pose a challenging problem in formulating treatment strategies. The location of these mutations in the three-dimensional structure of RT is consistent with a conformationnal effect on the dNTP-binding pocket leading to cross-resistance to various nucleoside inhibitors of RT. From a diagnostic point of view, one should be aware that alternative genotyping technologies based on hybridization may fail to detect $\beta3$–$\beta4$ insertions in clinical samples (Vahey *et al.,* 1999). Considering that the polymorphism of RT nearby resistance codons is also a major shortcoming of these techniques (Kozal *et al.,* 1998; Koch *et al.,* 1999), dye terminator cycle sequencing is probably the more versatile and reliable technology for the detection and characterization of resistant HIV-1 strains.

MATERIALS AND METHODS

Patients

A cohort of 1109 patients under combination therapy including various associations of zidovudine, lamivudine, didanosine, d4T, nevirapine, indinavir, ritonavir, nelfinavir, or saquinavir was followed in the reference hospitals for HIV/AIDS located in Marseille, France. A total of 3064 HIV-1 *pol* DNA and/or RNA sequences has been obtained since January 1997 and stored in a specifically designed database (Yahi *et al.,* 1999). In the present study mutant genotypes were examined in plasma HIV-1 RNA from a subpopulation of 632 extensively treated subjects considered to be failing therapy (viremia greater than 1000 HIV-1 copies/ml), and with at least three resistance mutations (as defined by Hirsch *et al.,* 1998) in the RT gene.

Genotypic analysis

Genotypic resistance was evaluated by sequencing of the RT and protease genes with the ABI PRISM dye terminator cycle sequencing kit using Amplitaq Polymerase FS (Applied Biosystem) as described previously (Tamalet *et al.,* 1998). The direct sequencing of PCR products gives the composition of the nucleotides most frequently observed at each position and excludes the current artifacts associated with cell culture and cloning. Sequence products were analyzed with the Applied Biosystem 377 automatic sequencing system. The sequences were aligned on the HXB2 RT and protease genes with the Sequence Navigator software (Applied Biosystem). A χ^2 test was applied to assess the significance of specific associations (or lack of association) of resistance mutations.

TABLE 3

Susceptibility of Insertion Mutants to Zidovudine

Genotype	Fold increase in IC_{50} of zidovudine
69-[S-S-S], 210W, 215Y[a]	220
62V, 69-[S-S-S], 210W, 215Y[a]	>2500
67S, 69-[S-S-G], 215Y[b]	1346
62V, 67E, 69-[S-S-G], 210W, 215Y[b]	1880
41L, 69-[S-S-S], 210W, 215Y[c]	30
41L, 67E, 69-[S-S-G], 215Y[c]	220
Patient AR (11/97): 67G, 69-[T-T], 70R, 215F, 219Q	9
Patient BP (07/98): 41L, 62V, 69-[S-S-S], 210W, 215Y	3745
Patient BR (03/99): 41L, 69-[S-S-T], 210W, 215Y	169
Patient GB (02/99): 62A/V, 67E, 69-[S-E-S], 210W, 215Y	1999
Patient MP (03/99): 62V, 69-[S-S-S], 70R, 215F	36

Note. The nucleoside susceptibility of recombinant patient or mutant viruses were obtained from [a] Larder *et al.* (1999), [b] De Jong *et al.* (1999), and [c] Winters *et al.* (1998). The results are expressed as a fold increase in IC_{50} relative to wild-type HXB2 virus control. The RT and protease genotypes of recombinant viruses derived from the patients of the present study are indicated in Table 1 and Table 2, respectively.

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