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Research Article

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A 6-Basepair Insert in the Reverse Transcriptase Gene of Human Immunodeficiency Virus Type 1 Confers Resistance to Multiple Nucleoside Inhibitors

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Abstract

While many point mutations in the HIV-1 reverse transcriptase (RT) confer resistance to antiretroviral drugs, inserts or deletions in this gene have not been previously characterized. In this report, 14 RT inhibitor–treated patients were found to have HIV-1 strains possessing a 6-basepair insert between codons 69 and 70 of the RT gene. Known drug resistance mutations were also observed in these strains, with T215Y appearing in all strains. Genotypic analysis indicated that the inserts had substantial nucleotide variability that resulted in relatively restricted sets of amino acid sequences. Linkage of patients' treatment histories with longitudinal sequencing data showed that insert strains appeared during drug regimens containing ddI or ddC, with prior or concurrent AZT treatment. Drug susceptibility tests of recombinant patient isolates showed reduced susceptibility to nearly all nucleoside RT inhibitors. Site-directed mutagenesis studies confirmed the role of the inserts alone in conferring reduced susceptibility to most RT inhibitors. The addition of AZT-associated drug resistance mutations further increased the range and magnitude of resistance. These results establish that inserts, like point mutations, are selected in vivo during antiretroviral therapy and provide resistance to multiple nucleoside analogs. (*J. Clin. Invest.* 1998. 102:1769–1775.) Key words: drug resistance • microbial • genotype • phenotype • mutation • human

Introduction

Treatment of HIV-1–infected individuals with antiretroviral drugs has been highly effective in increasing both their duration and quality of life. Currently, combinations of protease and reverse transcriptase (RT)[1] inhibitors are considered the standard of care (1). These combination regimens often reduce the amount of virus circulating in the plasma of patients to below the limits of detection by currently available assays. This impedes the development of resistance to antiretroviral drugs by reducing the number of replicative events in which mutants can be generated. Treatment failure, typically defined as a significant rise in the level of circulating virus, is often associated with the emergence of virus strains resistant to antiretroviral drugs (2).

A number of mutations in the protease and RT gene of HIV-1 are responsible for conferring resistance to antiretroviral drugs. These mutations are selected for and emerge as the predominant HIV-1 strains during drug treatment. In the RT gene, single amino acid changes are associated with resistance to different RT inhibitors. For example, the M184V confers 3TC resistance, L74V is associated with ddI resistance, T69D is associated with ddC resistance, and Y181C is associated with most non-nucleoside RT inhibitor resistance (3). The T215Y mutation is most often associated with AZT resistance, but other point mutations, such as M41L, D67N, L210W, and K219Q, also contribute to AZT resistance (4, 5). While different mutations are typically associated with resistance to specific drugs, there is a variable amount of cross-resistance conferred by each mutation (3).

All of the currently defined RT resistance mutations are single codon changes in the RT gene. These one or two nucleotide substitutions result in the coding for a different amino acid at a given position. The impact of the resulting amino acid substitution affects the structure of the RT enzyme, which can alter the kinetics of enzyme function or change the ability of inhibitors to access the active site (6), providing the mutant virus a competitive advantage under drug pressure.

A recent report described one patient who developed an HIV-1 strain with a unique insert in the RT gene after drug therapy (7), but this strain was not characterized. In work presented here, we characterize HIV-1 strains that possess a 6-basepair insert in their RT gene. Data are presented regarding the nucleotide and amino acid variability among 14 different insert-containing strains, in vitro RT inhibitor susceptibility of recombinant patient isolates, and site-directed mutagenesis data evaluating the impact of the insert alone or in association with other point mutations on drug susceptibility. In addition,

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1. *Abbreviations used in this paper:* PMEA, 9-[2-phosphonyl-methoxyethyl]adenine; RT, reverse transcriptase.

we present an estimate of the prevalence of insert-containing strains in patient populations and define treatment regimens that select for insert-containing strains.

Methods

Patients. Serum or plasma specimens were used from patients either enrolled in clinical trials analyzed at Stanford or from patients submitted for HIV-1 protease and RT genotyping to either Stanford Hospital or Quest Diagnostics. Treatment histories were obtained from either the patients' primary care physician or from clinical trial records.

Genotyping. A 1,300-bp fragment encompassing the protease and the first 300 amino acids of the RT gene was generated by nested RT-PCR. Plasma RNA was isolated using Viral RNA Prep Kits (Qiagen, Chatsworth, CA). Reverse transcription and PCR amplification was performed by adding 18 μl of purified RNA to 32 μl of Superscript One-Step RT-PCR master mix (Life Technologies, Gaithersburg, MD) with 25 pmol of primers MAW-26 (TTG GAA ATG TGG AAA GGA AGG AC) and RT21 (8). Cycling parameters were 45°C for 45 min, 92°C for 2 min, followed by 40 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 2 min, followed by a 10-min hold at 72°C. 5 μl of the first round PCR product were transferred to a second PCR reaction containing 95 μl of master mix (1 × PCR buffer, 2.5 mM $MgCl_2$, 150 μM dNTP, 2.5 U Taq polymerase, and 10 pmol each of primers PRO-1 [CAG AGC CAA CAG CCC CAC CA] and RT20 [8]). Cycling parameters were 35 cycles of 94°C for 15 s, 63° for 20 s, and 72°C for 2 min, followed by a 10-min hold at 72°C. PCR products were typically diluted 1:4 with water, and 10 μl of each were placed into sequencing reactions with dRhodamine dye–labeled dideoxyterminators (Applied Biosystems, Foster City, CA) with 5 pmol of each of the following overlapping primers: RT-a (GTT GAC TCA GAT TGG TTG CAC), RT-b (GTG TCT CAT TGT TTA TAC TAG G), RT-y (GTG TCT CAT TGT TTA TAC TAG G), and HXB2-89 (AAT CTG ACT TGC CCA ATT CAA TTT). The sequencing reactions were then analyzed on an ABI Model 377 instrument (Applied Biosystems). Data were assembled, manually proofread, and edited. Changes in nucleotide and amino acid sequence were determined compared with the consensus clade B sequence (9). Genotypic mixtures were reported when a minor population was found to be at least 30% of the major population from sequencing reactions in both the forward and reverse directions. Nucleotide sequence divergence was calculated for all sequences to rule out laboratory contamination (10) or multiple sampling of the same patient at different testing sites. The nucleotide sequence divergence between patients ranged from 4.7 to 7.4%. Sequences were submitted to Genbank and assigned accession numbers AF096881–AF096894.

Recombinant viruses. Population-based recombinant isolates were prepared as previously described (11). In brief, PCR product derived from patient plasma virus or plasmids was cotransfected with an RT-deleted HXB2 clone into C8166 cells. Virus stocks were expanded into SupT1 cells, and culture supernatants were stored in multiple aliquots at −70°C. The genotype of each isolate was determined by sequencing, as described above, before further testing. In all cases, the genotype of the isolate was identical to the PCR product (from patient plasma or molecular construct) used to generate the isolate.

Site-directed mutagenesis. The Apa I–Eco RI fragment of pNL4-3 was transferred into Bluescript KS(−) plasmid (Stratagene, La Jolla, CA). Mutants were generated using standard *dut-ung* mutagenesis techniques (12). In brief, the pNL4-3(Apa I–Eco RI)–Bluescript plasmid was transformed into XL1-Blue bacteria (Stratagene), then phage was induced by coinfection with VSCM13 helper phage (Stratagene). The resulting phage was then passed through *dut-ung*–CJ236 bacteria (Bio-Rad, Alameda, CA). The resulting phage DNA was purified and used as a template for extention ligation with different phosphorylated mutagenic primers, T4 ligase, and T4 polymerase. The extention–ligation reactions were transformed into XL1-Blue bacteria, and colonies were picked. Miniprep DNA (Qiagen) from overnight cultures of picked colonies underwent restriction digest analysis and then were sequenced. After confirmation of the presence of the mutant, the mutant RT gene was PCR amplified from the plasmid, purified, and used for homologous recombination as described above.

Drug susceptibility tests. The ACTG/DOD consensus method for measuring susceptibility to RT inhibitors was used (13). Virion-associated RT inhibition assay was performed using serial twofold drug concentrations and a commercially available RT assay kit (NEN Life Sciences, Boston, MA). Triphosphorylated drugs were obtained from Pharmacia Biotech (Piscataway, NJ) (ddATP and ddCTP) and from Sierra Bioresearch, (Tucson, AZ) (d4T-TP). PMEA-PP was kindly provided by Julie Cherrington (Gilead Sciences, Foster City, CA). Results presented are a mean of two to four tests.

Results

Genotype of insert-containing strains. The nucleic acid sequence of the RT genes of HIV-1 was obtained from the plasma of HIV-infected individuals and represents the predominant genotype of the virus populations. Table I shows that these patients possessed predominant viral strains that contained a 6-basepair insert that occurred between RT codons 68 and 70. While it is unclear as to whether the insert occurred before or after codon 69, the first amino acid of the insert complex will be referred to henceforth as codon 69. Thus, 13 out of 14 patients had strains that possessed a change from threonine to serine at codon 69 (T69S) in addition to the two–amino acid (6-basepair) insert (termed T69S+XX, where X = single amino acid code). The T69S substitution occurs very rarely in HIV-1 strains and is different than the mutation (T69D) typically associated with ddC resistance (14). The first amino acid of the insert was typically a serine (12 of 14 cases), and three different amino acids were observed as the second amino acid of the insert.

In addition to amino acid changes associated with the insert, all of the patient strains also possessed another well-defined nucleoside inhibitor resistance mutations. AZT-associated resistance mutations were frequently seen, with all strains possessing T215Y or F, 10 of 14 possessing M41L, and 7 of 14 possessing L210W. The D67N mutation was not found in these patients; however, a D67E or G mutation was found in 5 of 14 strains. The 3TC-associated mutation M184V was found in 6 of 14 strains, while the nonnucleoside reverse transcriptase inhibitor associated mutations Y181C or K103N were found in 2 of 14 strains. In only one strain was a ddI-, ddC-, or d4T-resistance mutation found, that being a ddI-resistance–associated L74L/V mixture in patient 14. Sequencing of the protease gene of several patient strains was also performed and showed patterns of mutations consistent with the patients' protease inhibitor treatment history (data not shown).

The nucleotide sequence of the RT gene of the insert-containing strains showed numerous elements of variability (Table II). The T69S amino acid substitution, although consistent among most insert strains, was coded for by four different nucleotide triplets. In contrast, the first codon of the insert, which was typically also a serine, was coded for by only two different nucleotide triplets. The nucleotide sequence of the second codon of the insert was substantially more consistent within each amino acid type. There did not appear to be a consistent linear pattern of nucleotide sequence among all the insert-con-

Table I. Amino Acid Sequence of Insert-containing HIV-1 Reverse Transcriptase Genes Isolated from Patient Plasma Virus

	Codon*																	
Pt.‡	41	62	67	69	INS	INS	70	74	75	103	135	162	181	184	210	211	215	Other changes from consensus B
Con	M	A	D	T	—	—	K	L	V	K	I	S	Y	M	L	R	T	
1	—	—	E	S	S	A	—	—	—	—	—	—	—	—	—	K	Y	S68T, A98S, K173K/E, D177E, I178L
2	L	V	—	S	S	A	—	—	—	—	—	C	—	V	—	K	Y	K20R, K43N, V118V/I, K122E/K, D177E, L228L/H
3	L	V	—	S	S	A	—	—	—	N	—	A	C	—	—	K	Y	E6D, K20R, K43Q, V118I, L228H
4	L	—	E	S	S	G	—	—	—	—	T	A	—	—	—	—	Y	E6D/E, V35E, T39A, W88C, I142T
5	M/L	—	—	S	S	G	K/R	—	—	—	T	—	—	V	—	K	Y	D123E
6	—	V	—	S	S	G	—	—	—	—	T	—	C	—	—	—	Y	K166R
7	L	V	E	S	S	S	R	—	—	—	—	C	—	M/V	W	S	Y	V35I, T39A, V90I, F116Y, V118I, L228H
8	L	—	—	S	S	S	—	—	—	—	—	A	—	—	W	K/R	Y	K43E, S48T, T200T/A, E204E/D
9	L	—	—	S	S	S	—	—	—	—	—	—	—	—	—	—	Y	I142T, T165I
10	—	V	—	S	S	A/S	—	—	—	—	T	—	—	V	W	K	Y	K20R, A98S
11	M/L	V	—	S	S	S	—	—	M	—	—	—	—	V	W	—	Y	G196E, L228H
12	L	—	G	S	E	A	—	—	—	—	T	—	—	—	W	K	Y	K122P, I178M
13	—	—	E	S	T	S	R	—	M	—	—	—	—	—	W	K	F	K43Q, V118V/I, D123E, K166R, G196E
14	L	V	E	A	S	G	—	L/V	—	N	T	Y	—	V	W	K/R	Y	K43E, K64R, L100I, V111I, K122E, L228H

*Reverse transcriptase codons presented in separate columns represent positions where drug resistance mutations occur or where amino acid changes were found in ≥ 40% of the patients. ‡Pt., patient; Con, consensus B; INS, insert amino acids.

taining strains. For the T69S+SS inserts, the 12 base nucleotide sequence between codons 67 and 70 was an AGT-TCT or AGC-TCT repeat. Across the same region, the T69S+SG insert contained three consecutive AGT triplets. There was no repeating nucleotide pattern among the strains possessing the T69S+SA insert.

Treatment history. The treatment histories of patients were obtained to define the drug regimens that were responsible for the evolution and potentiation of the insert-containing strains. Reliable treatment histories were obtained for 10 patients and are shown in Fig. 1. Nearly all patients (9 of 10) had received at least 1 yr of AZT monotherapy as their first treatment regimen (mean = 2.2±1.0 yr). By the time that genotypic analysis revealed the presence of insert-containing strains, patients had been treated for various duration with nearly all available RT inhibitors (average = 3.9 RT inhibitors per patient). Many patients had also received protease inhibitors with some of their treatment regimens (data not shown).

In five patients (patients 6, 7, 8, 9, and 12), retrospective plasma samples were available and sequenced to evaluate the evolution of the insert-containing strains. In all cases, the insert-containing strains appeared during regimens containing ddI or ddC, alone (1 of 5) or in combination with AZT (4 of 5). The insert-containing strains typically appeared within the first year of treatment with these regimens. In one patient, treatment was initiated with ddI, but the insert-containing strains did not develop until the patient was subsequently treated with a combination of AZT+ddI. In the other five patients where sequential plasma specimens were not available, treatment history analysis showed a similar pattern of initial AZT monotherapy followed by ddI or ddC, alone or in combination with AZT.

Table II. Nucleotide Sequence of Insert-containing HIV-1 Patient Strains

		Codon*						
Insert‡	Pt.§	67	68	69	INS	INS	70	71
Consensus	—	GAC	AGT	ACT	—	—	AAA	TGG
T69S+SA	1	GA**A**	A**C**T	A**GC**	**AGC**	**GCT**	AAA	TGG
T69S+SA	2	GAC	AGT	**T**CT	**AGT**	**GCT**	AAA	TGG
T69S+SA	3	GAC	AGT	A**G**T	**AGC**	**GCT**	AAA	TGG
T69S+SG	4	GA**A**	AGT	A**G**T	**AGT**	**GGT**	AAA	TGG
T69S+SG	5	GAC	AGT	A**G**T	**AGT**	**GGT**	A**r**A	TGG
T69S+SG	6	GAC	AGT	A**G**T	**AGT**	**GGT**	AAA	TGG
T69S+SS	7	GA**A**	AG**C**	**T**CT	**AGC**	**TCT**	A**G**A	TGG
T69S+SS	8	GAC	AGT	**T**CT	**AGT**	**TCT**	AAA	TGG
T69S+SS	9	GAC	AGT	**T**CT	**AGT**	**TCT**	AAA	TGG
T69S+SS	10	GAC	AGT	**T**CT	**AGT**	**TCT**	AAA	TGG
T69S+SS/G	11	GAC	AGT	**T**CT	**AGT**	**kCT**	AAA	TGG
T69S+EA	12	GGC	AGT	A**G**T	**GAA**	**GCA**	AAA	TGG
T69S+TS	13	GA**A**	AGT	**T**CT	**ACC**	**TCT**	A**G**A	TGG
T69A+SG	14	GA**A**	AGT	**G**CT	**AGT**	**GGT**	AAA	TGG

*Nucleotides in boldface indicate change from consensus B (wild-type) sequence. ‡Consensus, consensus B; §Pt., patient; INS, insert nucleotides.

Estimation of frequency. Recently, HIV-1 genotyping has become available to patients and their physicians to provide an evaluation of the patients' viral protease and RT drug resistance mutations. In patients who had genotyping tests performed at either Stanford Hospital or Quest Diagnostics from August 1997 to July 1998, insert-containing strains were found at a frequency of ∼ 1% (10 of 1,045). In comparison, the T215Y mutation was found at a rate of 41% (431 of 1,045), the M184V mutation at 50% (522 of 1,045), the multidrug resistant Q151M mutation at 2% (22 of 1,045), and the ddI resistance–associated L74V mutation at 5% (56 of 1,045).

Susceptibility of patient isolates. Population-based recombinant virus isolates were prepared to assess their in vitro susceptibility to RT inhibitors. Patient isolates were selected for testing to represent the majority of the insert genotypes found. Results shown in Table III indicate that the patient isolates

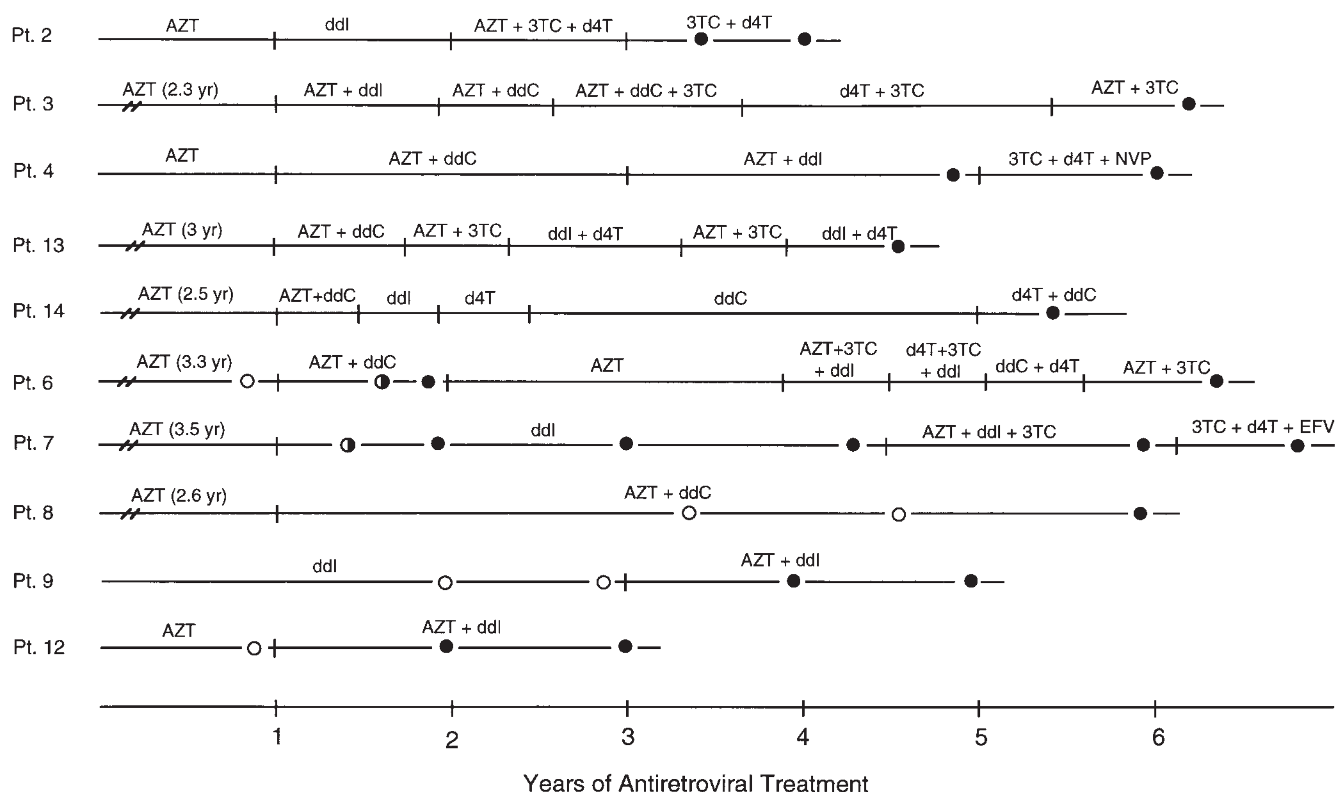

Figure 1. Relationship between patients' reverse transcriptase inhibitor treatment history and the appearance of insert-containing HIV-1 strains. Closed circles indicate the presence of insert-containing strains, half-closed circles indicate mixtures of wild-type and insert-containing strains, and open circles indicate the presence of only wild-type strains. Protease inhibitors that were used in combination with some RT regimens are not shown. NVP, nevirapine; EFV, efavirenz.

had reduced susceptibility of fourfold or greater to nearly all nucleoside RT inhibitors. While four of six strains showed some reduced susceptibility to d4T, in only two strains was the difference fourfold or greater. In addition, all isolates had reduced susceptibility to the nucleotide RT inhibitor PMEA (9-[2-phosphonyl-methoxyethyl]adenine). Reduced susceptibility to AZT could be associated with AZT resistance mutations also present in these strains (T215Y in all strains; M41L in strains from patients 2, 4, 8, and 12; and L210W in strains from patients 8, 12, and 13). Reduced susceptibility to 3TC could be attributed to M184V in patient 2. However, there were no known RT mutations present in the patient-derived strains that could be implicated in conferring the reduced susceptibility to ddI, ddC, d4T, and PMEA, or to 3TC in the absence of the M184V mutation. Only one of five isolates tested against nevirapine showed reduced susceptibility (50-fold); however, this isolate also possessed the Y181C mutation (patient 6, data not shown).

Table III. Susceptibility of Patient HIV-1 Recombinant Isolates to RT Inhibitors

Isolate‡	Insert genotype§	IC$_{50}$ (μM)*					
		AZT	ddI	ddC	3TC	d4T	PMEA
Wild type	—	0.01	0.2	0.02	0.03	0.3	1.2
Patient 1	T69S+SA	**1.5**	**3.0**	**0.65**	**0.99**	**2.5**	**23.0**
Patient 2	T69S+SA	**2.7**	**2.1**	**0.27**	**> 20**	0.5	**5.1**
Patient 4	T69S+SG	**2.2**	**2.3**	**0.41**	**0.25**	1.0	**27.1**
Patient 8	T69S+SS	**0.3**	**0.9**	**0.12**	**0.41**	0.6	**19.6**
Patient 12	T69S+EA	**1.0**	**3.1**	**0.46**	**1.04**	**2.1**	**36.7**
Patient 13	T69S+TS	**0.8**	**1.4**	**0.20**	**0.34**	1.0	**38.4**

*IC$_{50}$ valus represent the mean of two to three tests. Values in boldface are at least fourfold higher than the wild type. ‡Recombinant isolates were prepared and tested as described in Methods. Wild type represents a recombinant virus containing a pNL4-3 RT gene. §All isolates possessed T215Y and other mutations (see Table I).

Site-directed mutagenesis. Viral constructs were created using site-directed mutagenesis to assess the impact of the insert alone on susceptibility to RT inhibitors and the potential interaction of inserts with known RT mutations. Results of cell culture assays show that some but not all recombinant viral constructs containing only the T69S+XX insert showed reduced susceptibility to RT inhibitors. A T69S+EA construct was sensitive to all RT inhibitors, and a T69S+TS construct showed reduced susceptibility only to ddC (ninefold, data not shown). In contrast, both the T69S+SA and T69S+SG constructs showed reduced susceptibility to AZT, ddI, ddC, and 3TC (Table IV). The increase in IC$_{50}$ to ddI and ddC is similar to that conferred by the L74V mutation, but increase in IC$_{50}$ to AZT and 3TC is substantially less than that conferred by the T215Y and M184V mutations, respectively. In this assay, there was no evidence of reduced susceptibility of the insert-containing constructs to d4T, and the T69S+SA and T69S+SG constructs only showed slight increases in IC$_{50}$s to PMEA.

Table IV. Susceptibility of HIV-1 Constructs to RT Inhibitors

Construct[‡]	IC_{50} (μM)*					
	AZT	ddI	ddC	3TC	d4T	PMEA
Wild type	0.01	0.2	0.02	0.03	0.3	1.2
T69S+SA	**0.06**	**1.2**	**0.20**	**0.14**	0.2	2.4
[T69S+SA] + T215Y	**0.14**	**1.9**	**0.36**	**0.29**	**1.2**	**25.0**
T69S+SG	**0.08**	**0.8**	**0.10**	**0.18**	0.2	3.2
[T69S+SG] + T215Y	**0.18**	**1.7**	**0.91**	**0.52**	**1.2**	**13.7**
[T69S+SG] + M41L + T215Y	**2.10**	**4.2**	**1.10**	**1.30**	**2.9**	**> 50**
SG[§]	0.01	**0.8**	**0.26**	**0.26**	0.3	**6.0**
T69S	0.02	0.6	**0.10**	0.06	0.3	3.6
L74V	nt	**1.2**	**0.28**	0.03	nt	nt
M184V	0.01	0.2	0.05	> **20**	nt	nt
T215Y	**0.16**	nt	nt	nt	nt	nt

*IC_{50} values represent the mean of two or three tests. Values in boldface are at least fourfold higher than the wild type. [‡]Constructs contain only the mutations listed. [§]Insert with wild-type codon 69 (genotype not found in vivo).

Table V. Inhibition of Virion-associated Reverse Transcriptase Activity in HIV-1 Constructs by RT Inhibitors

Construct	IC_{50} (μM)*			
	ddATP	ddCTP	d4T-TP	PMEA-DP
Wild type	1.2	1.0	0.7	1.9
T69S+SA	**7.0**	**7.0**	**4.5**	**9.0**
[T69S+SA] + T215Y	**19.7**	**10.3**	**17.3**	**36.7**
T69S+SG	**5.4**	3.8	2.3	**10.0**
[T69S+SG] + T215Y	**10.0**	**5.3**	**6.9**	**18.3**
[T69S+SG] + M41L + T215Y	**17.0**	**5.2**	**7.7**	**27.4**
T69S	1.4	1.4	0.6	3.2
SG[‡]	**10.7**	**5.4**	**4.1**	**20.2**
L74V	**6.7**	**7.6**	nt	nt
T215Y	2.0	1.0	0.9	5.2

*IC_{50} values were obtained using a virion-associated RT inhibition assay and represent the mean of two to four tests. Values in boldface are at least fourfold higher than the wild type. [‡]Genotype not found in vivo.

It has been previously reported that cell-free RT inhibition assays may be more sensitive for measuring reduced susceptibility to ddI (11). A virion-associated RT inhibition assay was used to measure the activity of insert-containing RT enzymes in the presence of the active forms of several RT inhibitors. Results from this assay were, in general, consistent with the cell culture assay results that showed reduced susceptibility to ddI and ddC in the insert-containing constructs tested (Table V). However, this assay also showed reduced susceptibility of the RT from the T69S+SA and T69S+SG constructs to the active forms of d4T and PMEA.

To further evaluate the contribution of the insert to changes in susceptibility, constructs with only partial forms of the inserts were created. While a mutation at codon 69 was usually found in all insert-containing strains, a construct was prepared containing the SG insert but with a wild-type codon 69. Results showed that the IC_{50}s of this construct were equal to or slightly greater than that of the intact insert construct (T69S+SG), except that reduced susceptibility to AZT was lost. In contrast, a viral construct containing only the T69S mutation (with no insert) showed only limited changes in susceptibility compared with wild-type virus. These results suggest that the 6-basepair insert alone was responsible for conferring reduced susceptibility to RT inhibitors except AZT. The association of the T69S mutation with the insert was required for a significant increase in IC_{50} to AZT. The D67E mutation, which was found in 5 of the 15 patient strains, was similarly examined. The IC_{50} values of a viral construct containing only the D67E mutation was identical to the IC_{50}s of wild-type virus against all RT inhibitors tested (data not shown). Addition of the D67E mutation to the T69S+SG construct resulted in no significant change in the IC_{50}s to any RT inhibitors compared with the T69S+SG construct (data not shown).

The impact of the AZT resistance–associated T215Y and M41L mutations on susceptibility of insert-containing constructs was also examined. Viral constructs that contained both the T215Y mutation and an insert showed substantially higher IC_{50}s to the RT inhibitors than the constructs containing only the inserts (Tables IV and V). A construct that possessed the T69S+SG insert and the T215Y and M41L mutations showed even higher IC_{50}s to most RT inhibitors. The fold increase in IC_{50} of this construct to the RT inhibitors tested was consistent with the fold changes seen in the T69S+SG-containing isolate from patient 4.

Discussion

The results presented in this report characterize the evolution and impact of HIV-1 strains possessing 6-basepair inserts in the RT gene. All previously described mutations associated with either protease or RT inhibitor resistance have been one or two base point mutations (3). Selection of virus strains containing an insert in the RT gene has a number of clinical and biochemical implications.

Evidence presented here suggests that the insert-containing strains were selected by ddI or ddC treatment (Fig. 1). A recent report also showed the emergence of an insert-containing strain in one AZT-experienced patient during subsequent treatment with ddI and hydroxyurea (7). Treatment history data further suggests that AZT monotherapy had a strong influence on the emergence of insert-containing strains. Nearly all patients had initial treatment with AZT alone before combinations of AZT+ddI or AZT+ddC. However, none of the patients had developed the insert-containing strains during their extensive (1-3 yr) courses of AZT monotherapy. Other reports have also failed to observe insert-containing strains in patients who had received AZT monotherapy (15, 16). In addition, patients initially treated with 1 yr of AZT+ddI combination did not develop insert-containing strains (n = 105, unpublished data). Genotypic analysis of insert-containing strains showed that all possessed the AZT-associated T215Y. When sequential time points were evaluated, it either preceded or appeared concurrently with the insert. These results

indicate that AZT treatment influences the selection of insert-containing strains but does not independently select for these strains. However, prior or concurrent AZT treatment may be required for the development insert-containing strains. No data has been presented to date showing that RT inhibitors other than ddI or ddC select for insert-containing strains.

Reduced susceptibility of the insert-containing strains to some of the RT inhibitors was often similar in magnitude to that provided by well-characterized point mutations. The average changes in susceptibility of the patient-derived isolates to ddI (11-fold), ddC (17-fold), and PMEA (21-fold) were typically at least as high as that conferred by L74V (17), T69D (14), and K65R (18), respectively. AZT resistance (average of 140-fold) in the patient-derived isolates was consistent with the presence of the T215Y mutation and other AZT-associated changes (4, 5). Reduced susceptibility to 3TC (20-fold) in the isolates without the M184V mutation was lower than that conferred by the M184V mutation alone (19, 20). Reduced susceptibility to d4T (average of threefold) was slightly less than the V75T mutation (21) but variable in magnitude (range 1.7–8.3-fold) between isolates. Three recent abstracts report phenotypic resistance of insert-containing strains to d4T (22–24). The modest changes in susceptibility to 3TC and d4T compared with their benchmark mutations cannot be discounted, as the level of phenotypic resistance (measured by in vitro assays) that is clinically significant has not been defined (2).

Mutagenesis studies showed two constructs containing only T69S+XX complex had reduced susceptibility to nearly all RT inhibitors tested. It is interesting, however, that further reduction in susceptibility to many RT inhibitors was found in the insert-containing constructs that also possessed the T215Y mutation. A further decrease in susceptibility was seen when the M41L mutation was added to the construct containing the insert strain possessing the T215Y mutation (Tables IV and V). The interaction between the AZT resistance–associated mutations and the insert broadened the range of drugs to which the isolates were resistant compared with isolates carrying either genotypic feature alone. In addition, there was a stepwise increase in the magnitude (fold increase) in resistance to all RT inhibitors as AZT-associated mutations were added to the insert strains. The incremental increase in resistance caused by additional mutations in the viral constructs and the average reduction in susceptibility of insert-containing patient strains is similar to that seen with the multidrug resistant–associated Q151M genotype (25–27). HIV-1 strains containing inserts and AZT-resistance mutations (namely T215Y) should be considered to be multinucleoside inhibitor–resistant genotypes.

Two patient isolates (Table III, patients 8 and 12) showed reduced susceptibility to 3TC, d4T, and PMEA even though, by treatment history analysis, the patients had never been treated with these drugs. These results and mutagenesis data (Tables IV and V) indicate that the inserts are likely to have a substantial impact on RT structure and function. The codon 65-74 region (β3-β4 loop) is believed to be responsible for influencing the position and conformation of the primer–template complex as it passes through the active site (6). Mutations in this region likely alter the selection of nucleotides or nucleotide analogs to be incorporated into the growing DNA strand (28). Mutations associated with drug resistance may result in the preferential selection of normal dNTPs and exclusion of nucleotide analogs, allowing reverse transcription in the presence of RT inhibitors. Two amino acid inserts in this region are likely to exert a similar effect. The T215Y mutation is also implicated in modifying the position of the template primer, and both the T215Y and M41L mutations may also indirectly affect active site geometry (6). Additional mutations in this region found in the insert-containing strains, like T69S and D67E, make only a limited contribution to drug susceptibility changes and may primarily serve as compensatory mutations that maintain processivity. Further biochemical and structural analysis of insert-containing enzymes is currently underway and will provide interesting information about factors that influence RT function.

The nucleic acid sequence of the insert has substantial variability in spite of the fact that the resulting amino acid sequence is relatively restricted. While studies have shown that the HIV-1 RT enzyme is substantially error prone (29), exactly how the insert is generated during HIV-1 replication is unclear. The nucleic acid sequence of the T69S+SS inserts appeared to result from a 6-basepair repeat and may be caused by RT pausing (30, 31) and/or primer slippage (32). However, there was no consistent nucleotide pattern found in the other insert types. The restricted diversity of amino acid sequences of the inserts suggests that there may be substantial limitations on the types of amino acid insertions that maintain enzyme function and processivity while still providing a selective advantage under drug pressure. Alternatively, the restricted amino acid diversity may be the result of the relatively uniform conditions under which the insert strains were selected in vivo, that being ddI and ddC therapy. Further evaluation of patients with different, well-characterized treatment histories will provide more information about insert variability.

The prevalence of insert-containing strains among HIV-1–infected, nucleoside-inhibitor–treated patients cannot be estimated from currently available data sets. Results presented here from a population of patients failing antiretroviral treatment indicated a prevalence of $\sim$ 1.0%. This was substantially lower than most other major drug resistance mutations in this patient population. The low prevalence of insert-containing strains suggests that a unique set of host conditions and virus characteristics must exist for the insert-containing strains to emerge under drug selection. Further studies will be needed to accurately assess the prevalence and incidence of insert-containing strains.

The results presented in this report establish that inserts, like point mutations, can be selected by drug therapies and confer reduced susceptibility to nucleoside and nucleotide analogs. While the prevalence of these strains appears to be low, the evidence that genotype confers broad resistance to nucleoside and nucleotide inhibitors poses a difficult problem in formulating treatment strategies for patients with insert-containing strains. Studies that further characterize the factors necessary for selection of these insert-containing strains and the biochemical characteristics of insert-containing RT enzymes may lead to new approaches for the prevention and treatment of these highly resistant HIV-1 viruses.

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