Transmitted Human Immunodeficiency Virus Type 1 Carrying the D67N or K219Q/E Mutation Evolves Rapidly to Zidovudine Resistance In Vitro and Shows a High Replicative Fitness in the Presence of Zidovudine

J. Gerardo García-Lerma,^{1*} Hamish MacInnes,¹ Diane Bennett,² Hillard Weinstock,^{2,3} and Walid Heneine¹

HIV and Retrovirology Branch, Division of AIDS, STD, and TB Laboratory Research,¹ Surveillance Branch, Division of HIV/AIDS Prevention, Surveillance and Epidemiology,² and Epidemiology and Surveillance Branch, Division of STD Prevention,³ National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

Received 7 January 2004/Accepted 19 February 2004

Drug-naïve patients infected with drug-resistant human immunodeficiency virus type 1 (HIV-1) who initiate antiretroviral therapy show a shorter time to virologic failure than patients infected with wild-type (WT) viruses. Resistance-related HIV genotypes not commonly seen in treated patients, which likely result from reversion or loss of primary resistance mutations, have also been recognized in drug-naïve persons. Little work has been done to characterize the patterns of mutations in these viruses and the frequency of occurrence, their association with phenotypic resistance, and their effect on fitness and evolution of resistance. Through the analysis of resistance mutations in 1082 newly diagnosed antiretroviral-naïve persons from the United States, we found that 35 of 48 (72.9%) persons infected with HIV-1 containing thymidine analog mutations (TAMs) had viruses that lacked a primary mutation (T215Y/F, K70R, or Q151M). Of these viruses, 9 (25.7%) had only secondary TAMs (D67N, K219Q, M41L, or F77L), and all were found to be sensitive to zidovudine (AZT) and other drugs. To assess the impact of secondary TAMs on the evolution of AZT resistance, we generated recombinant viruses from cloned plasma-derived reverse transcriptase sequences. Two viruses had D67N, three had D67N and K219Q/E, and three were WT. Four site-directed mutants with D67N, K219Q, K219E, and D67N/K219Q were also made in HIV-1_{HXB2}. In vitro selection of AZT resistance showed that viruses with D67N and/or K219Q/E acquired AZT resistance mutations more rapidly than WT viruses (36 days compared to 54 days; $P = 0.003$). To investigate the factors associated with the rapid selection of AZT mutations in these viruses, we evaluated fitness differences among HXB2_{WT} and HXB2_{D67N} or HXB2_{D67N/K219Q} in the presence of AZT. Both HXB2_{D67N/K219Q} and HXB2_{D67N} were more fit than HXB2_{WT} in the presence of either low or high AZT concentrations, likely reflecting low-level resistance to AZT that is not detectable by phenotypic testing. In the absence of AZT, the fitness cost conferred by D67N or K219Q was modest. Our results demonstrate that viruses with unique patterns of TAMs, including D67N and/or K219Q/E, are commonly found among newly diagnosed persons and illustrate the expanding diversity of revertant viruses in this population. The modest fitness cost conferred by D67N and K219Q supports persistence of these mutants in the untreated population and highlights the potential for secondary transmission. The faster evolution of these mutants toward AZT resistance is consistent with the higher viral fitness in the presence of AZT and shows that these viruses are phenotypically different from WT HIV-1. Our study emphasizes the need for clinical studies to better define the impact of these mutants on treatment responses and evolution of resistance.

Treatment of human immunodeficiency virus type 1 (HIV-1)-infected persons with antiretroviral drugs, including reverse transcriptase (RT) and protease inhibitors, has significantly reduced the rate of HIV and AIDS-related mortality and morbidity. However, the emergence of HIV-1 variants with reduced drug susceptibility is an important cause of treatment failure and is associated with increased mortality (5, 25, 38).

The widespread use of antiretroviral drugs has led to the transmission of drug-resistant HIV-1. Transmission of drug-resistant viruses has been documented through vertical, sexual, and parenteral routes (4, 11, 26, 32). Patients who are infected

with drug-resistant HIV-1 and initiate antiretroviral therapy show poorer treatment responses than patients who are infected with wild-type (WT) viruses (17, 24). A number of studies have shown that the prevalence of viruses with drug resistance mutations in acutely or recently infected persons varies between 10 and 20% (1, 3, 17, 23, 24, 34–36).

The selection of resistance mutations during antiretroviral therapy is associated with a reduction in drug susceptibility and viral fitness (27). Resistance-related mutations have been conventionally classified as primary or secondary based on their effect on drug susceptibility. While primary mutations reduce drug susceptibility, secondary mutations do not confer resistance by themselves but can enhance the replicative fitness and resistance levels of viruses with primary mutations (9). Of the mutations selected by AZT, T215Y/F and K70R are generally considered primary, whereas D67N, L210W, or K219Q/E are

* Corresponding author. Mailing address: HIV and Retrovirology Branch, Centers for Disease Control and Prevention, 1600 Clifton Rd. NE, MS G-19, Atlanta, GA 30333. Phone: (404) 639-4987. Fax: (404) 639-1174. E-mail: GGarcia-lerma@cdc.gov.

considered secondary. Because many AZT resistance mutations can also be selected by stavudine (d4T) in vivo, another thymidine analog, they were more recently referred to as thymidine analog mutations (TAMs).

Despite the accumulation of secondary mutations, drug-resistant viruses generally display a reduced replication capacity compared to WT viruses (7, 9, 27). Therefore, it was not unexpected to observe that transmitted drug-resistant mutants gradually lose resistance mutations that confer high fitness costs as they evolve to more fit viruses (2, 8, 37). The factors affecting the rate of reversion of resistance mutations are not defined although characteristics of the infecting virus, including the number and type of resistance mutations, and the impact of such mutations on viral fitness may play a significant role (2, 8, 14, 16, 37).

The first documented transmissions of drug-resistant HIV-1 involved viruses that were genotypically and phenotypically similar to clinical isolates observed in patients failing antiretroviral treatment. These viruses may contain primary and secondary RT and/or protease resistance mutations and show detectable resistance to one or more classes of drugs in phenotypic assays (4, 11, 18, 20, 34, 36). More recently, a second type of mutants has been identified in drug-naïve persons. These viruses typically carry revertants of primary resistance mutations that are not commonly found in clinical isolates. Such viruses include mutants containing the 215D or 215C mutations (8, 36, 37). We previously found that these isolates are more frequent in recently diagnosed persons than AZT-resistant viruses with the T215Y/F mutations (14). Although phenotypically indistinguishable from WT HIV-1, viruses with 215C or 215D acquire the 215Y mutation in vitro more rapidly than WT viruses, likely reflecting the need for only one nucleotide change, compared to two for WT HIV-1 (13, 14). Clinical studies also suggest that the presence of the 215D/C substitutions may be associated with an increased risk of virologic failure in antiretroviral-naïve adults starting therapy with AZT or d4T (6, 33).

The identification of revertant viruses with unique RT genotypes such as 215D or 215C in treatment-naïve persons and the finding that the virologic and clinical implications of these viruses are different from those of WT viruses emphasize the need to identify viruses with other unusual resistance mutations and to understand their significance. In the present study, we investigated the patterns of TAMs observed in viruses from a large cohort of untreated persons newly diagnosed with HIV-1 infection. We show that a substantial proportion of viruses have uncommon patterns of TAMs with only secondary mutations such as D67N and K219Q/E. We demonstrate that viruses with D67N and/or K219Q/E have no detectable resistance to AZT but select K70R in vitro more rapidly than WT viruses. We relate the rapid selection of resistance to an increased replicative fitness in the presence of AZT. Our findings highlight the phenotypic differences between these viruses and WT HIV-1.

MATERIALS AND METHODS

Study population and mutations associated with resistance to thymidine analogs. The study population comprises 1082 newly diagnosed HIV-1-infected persons consecutively enrolled in 1997 to 2001 from 39 selected HIV care clinics, HIV counseling and testing sites, and other clinical settings in 10 U.S. cities (35). The prevalence of mutations associated with drug resistance was described re-

cently (35). All specimens for which informed consent was provided were also tested by using an HIV enzyme immunoassay (EIA) less sensitive than the standard HIV-1 EIA to identify persons infected within the past 12 months (Vironostika HIV-1 EI; BioMérieux, Inc., Raleigh, N.C.). K70R, Q151M, and T215Y/F were considered primary TAMs, and M41L, A62V, D67N, V75I, F77L, F116Y, L210W, and K219Q/E were considered secondary TAMs.

Cloning and sequence analysis of HIV-1 RT from patient-derived viruses. Full-length HIV-1 RT sequences from plasma of patients RD24, RD25, RD26, RD27, and RD28 were amplified by RT-nested PCR and cloned by using the TA cloning kit (Invitrogen). Briefly, HIV-1 RNA from plasma was extracted by using the NASBA RNA extraction kit (Organon). The RT reaction was done for 1 h at 42°C with primer RT2 as described previously (14). After a first round of PCR amplification with primers AV150 and RT2, 4 µl was subjected to a second round of amplification with primers IN3 and IN5. A 1,703-bp PCR product comprising the complete HIV-1 RT was gel purified (QIAquick Gel Extraction, Qiagen), sequenced, and ligated into the pCR2.1 vector. OneShot competent cells (TOP10F⁺) were then transformed, and RT sequences from single colonies were obtained. Plasmids containing the whole HIV-1 RT were purified from individual colonies (Qiagen plasmid purification kit) and used to generate recombinant viruses. For each patient, 6 to 10 RT clones were sequenced, and a representative RT clone was used to generate the recombinant viruses. For patient RD24, two different RT clones representing the two major species seen in plasma were used: RD24.1 that had the D67N mutation only and RD24.2 that had D67N and a K219E mutation seen in two of the seven RT clones sequenced (data not shown). The D67N and/or K219Q/E mutations were also introduced in the pHXB2RIP7-based infectious clone pSUM9 (kindly provided by H. Mitsuya) by using the QuickChange site-directed mutagenesis kit (Stratagene) as described previously (12).

Generation and characterization of recombinant viruses. Cloned RT sequences from plasma HIV-1 were used to generate recombinant viruses with the RT-deleted HXB2-based proviral molecular clone pHIVΔRTBstEII as previously described (12). Six recombinant viruses were generated by using patient-derived RTs: RD24.1_{D67N}, RD24.2_{D67N/K219E}, RD25_{D67N/K219Q}, RD26_{D67N/K219Q}, RD27_{D67N}, and RD28_{WT}. Five additional recombinant viruses were generated by using RT sequences of HXB2, namely, HXB2_{wt}, HXB2_{D67N}, HXB2_{D67N/K219Q}, HXB2_{K219Q}, and HXB2_{K219E}. The generation and characterization of an HXB2 mutant carrying the T215D mutation has been previously described (14).

The 50% cell culture infectious dose in each virus stock was determined in MT-4 cells by the method of Reed and Muench (29). Replication capacities of recombinant viruses were determined by monitoring p24 antigen production in MT-4 cells infected in duplicate at a multiplicity of infection (MOI) of 0.001 as previously described (14).

Phenotypic analysis. Phenotypic resistance to nucleoside RT inhibitors (NRTIs) was determined by using either the PhenoSense HIV (Virologic, South San Francisco, Calif.) or the Antivirogram assays (Virco, Cambridge, United Kingdom) (19, 28). Phenotypic resistance is determined by measuring fold increase in 50% inhibitory concentration (IC₅₀) values compared to a reference WT HIV-1 isolate (NL4-3 or HXB2). The results were interpreted on the basis of assay cutoff values established for each assay. Assay cutoff values for the PhenoSense HIV assay were 1.7-fold for didanosine (ddI), d4T, and zalcitabine (ddC), 2.2-fold for AZT, and 4.5-fold for abacavir. Assay cutoff values for the Antivirogram assay were 3-fold for d4T and abacavir, 3.5-fold for ddI and ddC, and 4-fold for AZT.

In vitro selection of AZT resistance. Selection of AZT resistance in recombinants HXB2_{D67N}, HXB2_{D67N/K219Q}, HXB2_{K219Q}, HXB2_{K219E}, RD24.1_{D67N}, RD24.2_{D67N/K219E}, RD25_{D67N/K219Q}, RD26_{D67N/K219Q}, RD27_{D67N}, RD23_a, and RD28_{WT} was done as previously reported (14). Briefly, inocula of 1.5×10^6 MT-4 cells were exposed to 1,500 50% cell culture infectious dose (MOI = 0.001) of each virus for 2 h at 37°C. After two washes with phosphate-buffered saline, cells were resuspended in 10 ml of complete medium containing AZT at a concentration close to the IC₅₀ value of HXB2_{wt} determined by the MT-4/MTT assay (0.013 µM). Cultures were then incubated at 37°C, and medium containing AZT was changed every 3 to 4 days as required. Virus production was monitored by microscopic assessment of syncytium formation through all of the culture. Once virus production was evident at a given concentration of drug, 500 µl of clarified supernatant was added to 1.5×10^6 fresh cells and cultured in the presence of a higher concentration of drug (twofold). Genotypic changes in HIV-1 RT were monitored by sequence analysis of the RT from culture supernatant in selected passages. The kinetics of selection of AZT resistance in viruses carrying 215D/C have been detailed elsewhere (14).

Analysis of fitness differences between WT viruses and viruses carrying D67N, K219Q, D67N/K219Q, or T215D. Fitness differences between WT viruses and viruses with D67N, K219Q, D67N/K219Q, or T215D were determined both in the absence and in the presence of AZT by using a growth competition assay as

TABLE 1. Susceptibility to NRTIs in viruses carrying secondary TAMs only

Virus	RT mutation(s)	Fold resistance ^a				
		AZT	ABC	ddI	d4T	ddC
RD24	D67N	1.1	1.3	1.0	1.2	1.0
RD25	D67N/K219Q	1.2	0.9	1.0	1.1	0.9
RD26	D67N/K219Q	1.4	0.4	1.2	0.4	1.0
RD27	D67N	0.9	0.9	1.0	1.0	0.9
RD29	D67N/K219Q	0.8	1.8	1.4	0.5	0.3
RD30	M41L	1.1	1.4	0.7	1.2	1.2
RD31	M41L	0.7	0.2	0.3	0.4	0.2
RD32	M41L	0.6	0.5	1.0	0.3	0.8
RD33	F77L	1.4	0.2	0.8	0.5	0.3

^a Fold increase in IC₅₀ compared to a WT reference HIV-1 isolate. Phenotypic testing was done using the Antivirogram assay in viruses RD26, RD29, RD31, RD32, and RD33 and the PhenoSense HIV assay in viruses RD24, RD25, RD27, and RD30.

previously described (14). Briefly, a mixture of WT and mutant viruses was used to infect MT-4 cells at an MOI of 0.001. After two washes with phosphate-buffered saline, cells were resuspended in complete medium with or without AZT. For experiments done in the absence of AZT, a 100- μ l aliquot of the supernatant collected after 4 to 5 days of culture was used to reinfect a fresh aliquot of MT-4 cells. In experiments done in the presence of AZT, cells were diluted every 3 to 4 days in complete medium with AZT (0.06 or 0.24 μ M) until a full cytopathic effect was observed. Then, a 100- μ l aliquot was collected and used to reinfect a fresh aliquot of MT-4 cells. The relative proportion of the two competing variants was determined both at baseline and at each passage on the basis of the ratios of the specific mutations. Ratios were estimated on the basis of the relative peak heights seen in the electropherograms obtained by dye terminator sequencing. For mixtures of HIV-1_{WT} and HIV-1_{D67N/K219Q}, relative proportions were calculated based on the mean relative peak heights observed at positions 67 and 219 of the RT. Fitness differences were calculated by monitoring the changes in the relative proportion of the less- and the more-fit viruses over time as previously described (13).

Sequence analysis of HIV-1 RT. Sequence analysis of HIV-1 RT (from nucleotides 2529 to 3333 of HXB2; amino acids 7 to 246) was done in an ABI377 automated sequencer with primers AV36, AV44, A35, and NE(1)35 (30). The Vector NTI program (version 7) was used to analyze the data and to determine deduced amino acid sequences.

Statistical analysis. The time for selection of resistance mutations in WT viruses, viruses with D67N and/or K219Q/E, and viruses with 215D/C was compared by using the exact Wilcoxon two-sample test. The level of resistance conferred by K70R and T215F in different groups of viruses was also compared by using the Wilcoxon two-sample test. Statistical significance was defined by an exact one-sided *P* value of <0.05. All statistical analyses were performed by using the SAS system for Windows (version 8.01; SAS Institute, Inc., Cary, N.C.).

Nucleotide sequence accession numbers. The nucleotide sequences of all of the RT clones used to generate recombinant viruses (RD24.1, RD24.2, RD25, RD26, RD27, and RD28) have been deposited in the GenBank database (accession numbers AY461444 to AY461449).

RESULTS

Prevalence of viruses with secondary TAMs only and phenotypic susceptibility to drugs. The prevalence of mutations associated with drug resistance in the study population was described recently (35). The prevalence of all resistance mutations was 8.3%, whereas that of NRTIs was 6.4% (35). Of the 48 isolates that had TAMs, 35 lacked a primary mutation (data not shown). Of these isolates, 9 (25.7%) had only secondary TAMs. Of the nine isolates that had only secondary TAMs, three had K219Q and D67N, two had D67N, three had M41L, and one had F77L. Table 1 shows the drug susceptibility results, indicating that all nine isolates with only secondary TAMs were sensitive to nucleoside analogs. Seven of the nine persons had reactive modified EIA test results, suggesting that the duration of infection in these patients was >12 months.

Impact of D67N and/or K219Q/E on the kinetics of emergence of AZT resistance in vitro. To investigate whether isolates that only have secondary TAMs might evolve rapidly toward AZT resistance, we compared the evolution of AZT resistance in vitro between WT viruses and viruses carrying D67N and/or K219Q/E. The impact of D67N and K219Q/E on the rate of acquisition of resistance was also evaluated in site-directed mutants generated in the HXB2 genetic background.

Selection of AZT resistance mutations in the five WT viruses was seen after a median of 54 days in culture (range, 41 to 81 days) or an increase in the concentration of AZT of 33-fold (range, 16- to 100-fold) (Table 2). Of these viruses, one acquired K70R and T215F (RD22wt), one acquired K70R, D67N, and T215I (RD28wt), one acquired D67N and K70R (isolate RD23wt tested in two separate experiments; RD23a and RD23b), and one acquired D67N only (HXB2wt). Selection of AZT mutations in the nine viruses that had D67N and/or K219Q/E occurred faster than in WT viruses and was seen after a median of 36 days in culture (range, 19 to 60 days) or an increase in the concentration of AZT of 8-fold (range, 4- to 100-fold) (Table 3). Of these viruses, six acquired K70R only, one acquired a K219G mutation followed by K70R (RD24.2), and two acquired K70R followed by D67N (HXB2_{K219Q} and HXB2_{K219E}) (Table 3).

Figure 1 compares the kinetics of selection of AZT resistance mutations in viruses with D67N and/or K219Q with the kinetics of selection of 215Y that we observed previously in viruses with 215D/C (14). Both viruses with D67N and/or K219Q and viruses with 215D/C acquired AZT mutations

TABLE 2. Kinetics of emergence of AZT resistance mutations in WT viruses^a

Passage	AZT concn (μ M)	RD22 _{WT}		RD23a _{WT}		RD23b _{WT}		RD28 _{WT}		HXB2 _{WT}	
		Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)
1	0.03	6	ND	7	ND	6	ND	7	ND	6	ND
2	0.06	12	ND	14	ND	12	ND	18	ND	12	ND
3	0.12	18	ND	21		18	ND	26		18	ND
4	0.24	30		30		28		38		30	
5	0.48	37		41	K70K/R	39		49		44	
6	1	45		52	D67N/D, K70R/K	54	D67N/D	63	K70K/R	57	
7	3	53	K70K/R, T215F	64	D67N, K70R	65	D67N, K70K/R	75	ND	81	D67D/N
8	10	60	K70R, T215F	76	D67N, K70R	74	D67N, K70R	84	D67N, K70R, T215T/I	108	D67N/D

^a Days, cumulative time in culture. Shifts in mutations indicate a mixed genotype. The first amino acid represents the predominant genotype observed in the mixture. ND, not done.

TABLE 3. Kinetics of emergence of AZT resistance mutations in viruses carrying D67N and/or K219Q/E^a

Passage	AZT concn (μM)	RD24.1 _{D67N}		RD24.2 _{D67N/K219E}		RD25 _{D67N/K219Q}		RD26 _{D67N/K219Q}	
		Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)
1	0.03	6	ND	8	ND	6	ND	7	ND
2	0.06	13	ND	15	ND	12	ND	12	ND
3	0.12	21		26		19		19	K70K/R
4	0.24	30		35	K219E/G	28		27	K70R/K
5	0.48	38		43	K219E/G	36	K70K/R	33	K70R
6	1	48		50	K219E/G	43	K70R	40	K70R
7	3	60	K70R	62	K219E/G	51	K70R	49	K70R
8	10	72	K70R	74	K70R, K219G	60	K70R	ND	

^a See Table 2, footnote a.

faster than WT viruses ($P = 0.003$ and $P = 0.001$, respectively; Fig. 1a). Figure 1a also shows that viruses with 215D/C selected resistance mutations faster than viruses with D67N and/or K219Q ($P = 0.029$). We also compared the concentration of AZT at which the first AZT resistance mutation was observed. Figure 1b shows that both viruses with D67N and/or K219Q and viruses with 215D/C acquired AZT resistance mutations at lower AZT concentrations than WT viruses ($P = 0.009$ and $P = 0.001$, respectively). However, such concentrations of AZT

were similar among viruses D67N and/or K219Q/E and viruses with 215D/C (Fig. 1b). Taken together, our results indicate that the comparative order of evolution toward AZT resistance among these three groups of viruses is as follows: HIV-1_{215D/C} $\geq$ HIV-1_{D67N, K219Q/E} $>$ HIV-1_{WT}.

Changes in AZT susceptibility associated with selection of a primary AZT resistance mutation in different RT backgrounds. We next compared changes in AZT susceptibility associated with the selection of a primary mutation in WT viruses (K70R and T215F) and in viruses with D67N and/or K219Q/E (K70R) (Fig. 2). Selection of K70R or T215F in WT viruses resulted in a 2.2-fold reduction (range, 1.2- to 3.2-fold) in AZT susceptibility compared to a 2.6-fold reduction (range, 1.4- to 8.3-fold) associated with the selection of K70R in all of the mutant viruses. This difference was not statistically significant ($P = 0.15$). The levels of AZT resistance were also evaluated in specific groups of mutants. Figure 2 shows that selection of K70R in viruses carrying D67N, K219Q/E, or both D67N and K219Q/E resulted in similar levels of AZT resistance than selection of a primary mutation in WT viruses. However, there was a trend toward higher levels of resistance conferred by K70R in viruses that had both D67N and K219Q/E (median, 5.8-fold; range, 2.2- to 8.3-fold), although this difference was not statistically significant ($P = 0.057$ compared to WT viruses).

The D67N and K219Q mutations confer a selective advantage in the presence of AZT. We next determined whether the rapid selection of AZT resistance mutations seen in isolates with D67N or D67N/K219Q was associated with an increased replication capacity in the presence of AZT. We evaluated the ability of HXB2-based mutants to outgrow WT HXB2 in competition experiments done in the presence of low (0.06 μM) or high (0.24 μM) concentrations of AZT.

Figure 3 shows changes in the relative proportion of these viruses overtime. Viruses with D67N or D67N/K219Q both outgrew WT viruses in the presence of 0.06 and 0.24 μM AZT, indicating that the D67N mutation alone or in combination with K219Q confers a selective advantage in the presence of AZT. The fitness differences between HXB2_{D67N} and HXB2_{WT} were calculated and found to be 19 and 52% for competition assays done with 0.06 and 0.24 μM AZT, respectively. Similarly, fitness differences between HXB2_{D67N/K219Q} and HXB2_{WT} in the presence of 0.06 and 0.24 μM AZT were 28 and 35%, respectively. These findings demonstrate that

FIG. 1. Comparison of the kinetics of selection of AZT resistance mutations in WT viruses, viruses carrying D67N and/or K219Q/E, and viruses carrying 215D or 215C. (A) Cumulative time in culture at which the first AZT resistance mutation was identified; (B) fold increase in AZT concentration at the passage at which the first AZT resistance mutation was identified.

TABLE 3—Continued

RD27 _{D67N}		HXB2 _{D67N}		HXB2 _{K219Q}		HXB2 _{K219E}		HXB2 _{D67N/K219Q}	
Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)	Days	Mutation(s)
6	ND	7	ND	7	ND	7	ND	7	ND
13	ND	15	ND	15	ND	18		14	ND
21		23		26		29	K70K/R	22	
30		38	K70K/R	38	K70K/R	38	K70R	30	K70K/R
38	K70K/R	46	K70R	46	K70R	46	K70R	36	K70R
45	K70R	54	K70R	53	K70R	53	D67N/D, K70R	43	K70R
54	K70R	66	K70R	64	D67N/D, K70R	62	D67N, K70R	52	ND
65	K70R	78	K70R	73	D67N, K70R	74	D67N, K70R	61	K70R

viruses with D67N alone or in combination with K219Q replicate more efficiently than WT viruses in the presence of AZT.

We next evaluated whether the selective advantage conferred by D67N and K219Q in the presence of AZT was associated with low-level resistance to AZT. The IC_{50} values for AZT seen in HXB2_{D67N} (0.009 μ M) and HXB2_{D67N/K219Q} (0.012 μ M) were only 1.3- and 1.7-fold higher than the IC_{50} seen in HXB2_{WT} (0.007 μ M) (data not shown), indicating that the increased replication of HXB2_{D67N} and HXB2_{D67N/K219Q} in the presence of AZT is not associated with significant resistance to this drug.

Impact of D67N and/or K219Q/E on replication capacity and fitness in the absence of drug. We next evaluated the effect that D67N and K219Q/E have on virus replication and fitness in the absence of drug. The analysis is important for the understanding of the stability of these mutations. We first compared replication capacity between WT viruses and viruses carrying D67N, K219Q, K219E, or D67N/K219Q. Figure 4 shows the kinetics of p24 antigen production in acute infections done with HXB2_{WT}, HXB2_{D67N}, HXB2_{K219Q}, HXB2_{K219E}, and HXB2_{D67N/K219Q}. Viruses carrying D67N, K219Q/E, or D67N/K219Q replicated efficiently, with kinetics of p24 antigen production similar to that observed in HXB2_{WT}. These results indicate that, in the HXB2 genetic background, the D67N and/or K219Q/E mutations do not significantly affect replication capacity.

FIG. 2. Fold changes in AZT susceptibility associated with the selection of a primary AZT resistance mutation (K70R or T215Y/F) in WT viruses, viruses with D67N and/or K219Q/E (all mutants), viruses with D67N or K219Q/E, and viruses with both D67N and K219Q/E.

We next evaluated the impact of D67N and K219Q on viral fitness in the absence of drug. We mixed HXB2_{WT} with either HXB2_{D67N} or HXB2_{K219Q} and monitored the relative proportion of the two competing variants overtime. Figure 5 shows the kinetics of viral competitions seen in these mixtures. In mixtures of HXB2_{WT} with HXB2_{D67N} or HXB2_{K219Q}, the relative proportion of the mutant virus gradually decreased overtime, indicating that the D67N and K219Q mutations confer a fitness cost in the absence of drug. The fitness differences between WT viruses and viruses with D67N or K219Q were calculated and found to be 4.6 and 3.5%, respectively.

We next compared the fitness cost associated with D67N and K219Q with that of a mutant carrying the 215D mutation, which is known to persist in vivo for 1 to 3 years (8, 37). Figure 5 shows that HXB2_{WT} was able to outgrow HXB2_{215D} in two competition experiments initiated with different proportions of the two competing variants (28% HXB2_{WT} and 72% HXB2_{215D} or 80% HXB2_{WT} and 20% HXB2_{215D}). The fitness difference among these viruses was calculated in the two competition experiments and found to be 7.2 and 5.2%, respectively. These findings indicate that the fitness cost associated with the 215D mutation is similar to that associated with D67N or K219Q.

DISCUSSION

The identification among treatment-naïve persons of revertant viruses with unusual mutations at codon 215 in the RT, and the finding that the virologic and clinical implications of these viruses are different from those of WT viruses led us to investigate the patterns of all TAMs in HIV-1 from drug-naïve persons. We show in a large cohort of persons newly diagnosed with HIV-1 that the majority of viruses with TAMs lacked a primary mutation. We also found that a substantial proportion of these isolates had only secondary mutations such as D67N or K219Q. Our data demonstrate that new patterns of TAMs that are not commonly seen in clinical isolates from patients who fail treatment can be found in drug-naïve persons. These findings heighten the importance of defining the impact of these mutations on drug susceptibility, fitness, and evolution of AZT resistance.

We show that patient-derived viruses containing D67N and/or K219Q replicate efficiently in the absence of drug, suggesting an ability of these viruses to persist in vivo. This finding is consistent with the detection of these mutations in patients with duration of infection longer than 12 months. The good

FIG. 3. Competitive HIV-1 replication assay between HXB2_{WT} and HXB2_{D67N} or HXB2_{D67N/K219Q} in the presence of two different concentrations of AZT (0.06 and 0.24 μ M). The relative proportion of the two competing variants is shown over time.

replication capacity is further supported by the finding that the D67N and K219Q mutations conferred to HIV-1_{HXB2} a modest fitness cost in the absence of drug. We found that the impact of these mutations on viral fitness was similar to that of 215D, a mutation previously shown to persist in vivo for 1 to 3 years (8, 37). These findings support the stability and persistence of the D67N and K219Q mutations in vivo and highlight the potential for secondary transmission of these mutants, as was noted recently in a transmission chain of viruses containing 215D (31).

Our phenotypic analysis showed that all of the viruses that had only secondary TAMs have no detectable resistance to

AZT and other drugs. However, a key finding of our study came from the in vitro selection with AZT. We showed that despite the absence of AZT resistance, viruses with D67N or D67N and K219Q/E acquired the K70R mutation more rapidly than WT viruses. Interestingly, the presence of D67N or D67N and K219Q/E had no impact on the resistance pathway for AZT since both mutants and WT viruses selected K70R rather than 215Y/F. However, the reduction of AZT susceptibility conferred by K70R was generally higher in the context of D67N and K219Q/E than in WT viruses. The faster evolution toward resistance and the tendency for a higher reduction in AZT susceptibility distinguish these viruses from WT HIV-1 and, therefore, raise clinical implications. However, data on whether D67N and K219Q/E reduce the efficacy of antiretroviral therapy are not available. Therefore, a close monitoring of treatment responses in patients infected with these viruses is prudent.

The preferential selection of K70R and not T215Y seen in all viruses may likely be explained by the number of mutations required for each amino acid change. In contrast to K70R, which requires one nucleotide change (AAA to AGA), acquisition of T215Y requires a two-base transversion (ACC to CAC) and may, therefore, occur at a lower frequency. Since 67N and 219Q/E are more strongly associated in clinical isolates with K70R than with T215Y, it is also possible that the presence of D67N and K219Q/E in our viruses may have played a role in favoring the selection of K70R over T215Y (15). Interestingly, selection of AZT resistance in viruses with 215D/C occurs through acquisition of 215Y and not K70R, although both D/C215Y and K70R require a single nucleotide

FIG. 4. Replication kinetics of HXB2 viruses carrying D67N and/or K219Q/E and comparison with HXB2_{WT}. Mean p24 values from duplicate cultures are shown.

FIG. 5. Competitive HIV-1 replication assay among HXB2_{WT} and HXB2_{D67N} (A), HXB2_{WT} and HXB2_{K219Q} (B), and HXB2_{WT} and HXB2_{T215D} (C and D; two different proportions) in the absence of drug. The relative proportions of the two competing variants are shown over time.

change (14). The preferential selection of 215Y seen in viruses with 215D/C may likely be due to the higher levels of AZT resistance conferred by 215Y compared to K70R (22). It is also likely that the selection of T215Y in some of these viruses may have been influenced by the presence of M41L and L210W, which are both associated clinically with T215Y and not K70R (15). These findings illustrate how newly generated genetic backgrounds in revertant viruses can influence the selection of different drug resistance pathways that might be associated with variable clinical outcomes (10, 12).

Although we found that D67N or D67N/K219Q confer a modest fitness cost in the absence of AZT, the opposite result was seen in the presence of AZT. We demonstrate that despite the absence of detectable AZT resistance D67N and D67N/K219Q confer a fitness gain in the presence of AZT and that such fitness gain increases when the concentration of AZT is also increased. The ability of these mutants to consistently outgrow an isogenic WT virus in two different AZT concentrations suggests that both D67N and K219Q confer low-level resistance to AZT that was not detectable in the phenotypic assay used. The increased replicative fitness of the mutants in the presence of AZT is important because it may explain the rapid evolution of resistance seen in the selection experiments.

Because D67N and K219Q/E are commonly associated with other TAMs in clinical isolates, it is likely that the unique viruses containing these two mutations may have at some point originated from viruses that had additional resistance mutations. The loss or reversion of resistance mutations may have

occurred over time in the absence of AZT in the persons we have identified. The finding that none of these persons was recently infected suggests that opportunities for loss or reversion of TAMs are possible. In these cases, the AZT-resistant mutants that originally infected these persons may remain archived (21). Alternatively, the loss of resistance mutations could have occurred gradually in a different person (s) before these mutants were transmitted to our patients. Both possibilities have the potential to compromise the efficacy of antiretroviral therapy with AZT through either a rapid selection of K70R or by a selection of an archived AZT-resistant virus.

In summary, we show that viruses with secondary TAMs such as D67N and/or K219Q/E are commonly found in the absence of other mutations among newly diagnosed patients. These viruses do not show detectable resistance to AZT but evolve faster than WT viruses toward AZT resistance in the presence of AZT. The faster evolution toward AZT resistance may be related to the higher viral fitness observed in the presence of AZT and may reflect low-level resistance to AZT that is not detectable by phenotypic testing. The identification of these unique mutants highlights the expanding diversity of revertant viruses in the drug-naïve population and emphasizes the need for clinical studies to better define their impact on treatment responses, transmissibility, and evolution of resistance.

ACKNOWLEDGMENTS

H.M. was supported by a fellowship from the Oak Ridge Institute for Science and Education.

The use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention.

REFERENCES

- Boden, D., A. Hurley, L. Zhang, Y. Cao, Y. Guo, E. Jones, J. Tsay, J. Ip, C. Farthing, K. Limoli, N. Parkin, and M. Markowitz. 1999. HIV-1 drug resistance in newly infected individuals. *JAMA* 282:1135–1141.
- Brenner, B. G., J. P. Routy, M. Petrella, D. Moisi, M. Oliveira, M. Dettori, B. Spira, V. Essabag, B. Conway, R. Lalonde, R. P. Sekaly, and M. A. Wainberg. 2002. Persistence and fitness of multidrug-resistant human immunodeficiency virus type 1 acquired in primary infection. *J. Virol.* 76:1753–1761.
- Brodine, S. K., R. A. Shaffer, M. J. Starkey, S. A. Tasker, J. L. Gilcrest, M. K. Louder, A. Barile, T. C. VanCott, M. T. Vahey, F. E. McCutchan, D. L. Birx, D. D. Richman, and J. R. Mascola. 1999. Drug resistance patterns, genetic subtypes, clinical features, and risk factors in military personnel with HIV-1 seroconversion. *Ann. Intern. Med.* 131:502–506.
- Conlon, C. P., P. Klennerman, A. Edwards, B. A. Larder, and R. E. Phillips. 1994. Heterosexual transmission of human immunodeficiency virus type 1 variants associated with zidovudine resistance. *J. Infect. Dis.* 169:411–415.
- D'Aquila, R. T., V. A. Johnson, S. L. Welles, A. J. Japour, D. R. Kuritzkes, V. DeGruttola, P. S. Reichelderfer, R. W. Coombs, C. S. Crumpacker, J. O. Kahn, D. D. Richman, et al. 1995. Zidovudine resistance and HIV-1 disease progression during antiretroviral therapy. *Ann. Intern. Med.* 122:401–408.
- de Ronde, A., M. van Dooren, E. de Rooij, B. van Gemen, J. Lange, and J. Goudsmit. 2000. Infection by zidovudine-resistant HIV-1 compromises the virologic response to stavudine in a drug-naïve patient. *AIDS* 14:2632–2633.
- Deeks, S. G., T. Wrin, T. Liegler, R. Hoh, M. Hayden, J. D. Barbour, N. S. Hellmann, C. J. Petropoulos, J. M. McCune, M. K. Hellerstein, and R. M. Grant. 2001. Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *N. Engl. J. Med.* 344:472–480.
- de Ronde, A., M. van Dooren, L. van Der Hoek, D. Bouwhuis, E. de Rooij, B. van Gemen, R. de Boer, and J. Goudsmit. 2001. Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. *J. Virol.* 75:595–602.
- Devereux, H. L., V. C. Emery, M. A. Johnson, and C. Loveday. 2001. Replicative fitness in vivo of HIV-1 variants with multiple drug resistance-associated mutations. *J. Med. Virol.* 65:218–224.
- Dykes, C., K. Fox, A. Lloyd, M. Chiulli, E. Morse, and L. M. Demeter. 2001. Impact of clinical reverse transcriptase sequences on the replication capacity of HIV-1 drug-resistant mutants. *Virology* 285:193–203.
- Erica, A., D. L. Mayers, D. G. Strike, K. J. Sannerud, F. E. McCutchan, K. Henry, and H. H. Balfour. 1993. Brief report: primary infection with zidovudine-resistant human immunodeficiency virus type 1. *N. Engl. J. Med.* 328:1163–1165.
- García-Lerma, J. G., P. J. Gerrish, A. J. Wright, S. H. Qari, and W. Heneine. 2000. Evidence of a role for the Q151L mutation and the viral background in development of multiple dideoxynucleoside resistant HIV-1. *J. Virol.* 74:9339–9346.
- García-Lerma, J. G., H. MacInnes, D. Bennett, P. Reid, S. Nidtha, H. Weinstock, J. Kaplan, and W. Heneine. 2003. A novel genetic pathway of human immunodeficiency virus type 1 resistance to stavudine mediated by the K65R mutation. *J. Virol.* 77:5685–5693.
- García-Lerma, J. G., S. Nidtha, K. Blumoff, H. Weinstock, and W. Heneine. 2001. Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naïve persons. *Proc. Natl. Acad. Sci. USA* 98:13907–13912.
- Gonzales, M. J., T. D. Wu, J. Taylor, I. Belitskaya, R. Kantor, D. Israelski, S. Chou, A. R. Zolopa, W. J. Fessel, and R. W. Shafer. 2003. Extended spectrum of HIV-1 reverse transcriptase mutations in patients receiving multiple nucleoside analog inhibitors. *AIDS* 17:791–799.
- Goudsmit, J., A. de Ronde, D. D. Ho, and A. S. Perelson. 1996. Human immunodeficiency virus fitness in vivo: calculations based on single zidovudine resistance mutation at codon 215 of reverse transcriptase. *J. Virol.* 70:5662–5664.
- Grant, R. M., F. M. Hecht, M. Warmerdam, L. Liu, T. Liegler, C. J. Petropoulos, N. S. Hellmann, M. Chesney, M. P. Busch, and J. O. Kahn. 2002. Time trends in primary HIV-1 drug resistance among recently infected persons. *JAMA* 288:181–188.
- Hecht, F. M., R. M. Grant, C. J. Petropoulos, B. Dillon, M. A. Chesney, H. Tian, N. S. Hellmann, N. I. Bandrapalli, L. Digilio, B. Branson, and J. O. Kahn. 1998. Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. *N. Engl. J. Med.* 339:307–311.
- Hertogs, K., M. de Bethune, V. Miller, T. Ivens, P. Schel, A. van Cauwenberge, C. Van den Eynde, V. Van Gerwen, H. Azijn, M. Van Houtte, F. Peeters, S. Staszewski, M. Conant, S. Bloor, S. Kemp, B. Larder, and R. Pauwels. 1998. A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. *Antimicrob. Agents Chemother.* 42:269–276.
- Imrie, A., A. Beveridge, W. Genn, J. Vizzard, D. A. Cooper, et al. 1997. Transmission of human immunodeficiency virus type 1 resistant to nevirapine and zidovudine. *J. Infect. Dis.* 175:1502–1506.
- Kuritzkes, D. R. 2001. A fossil record of zidovudine resistance in transmitted isolates of HIV-1. *Proc. Natl. Acad. Sci. USA* 98:13485–13487.
- Larder, B. A., P. Kellam, and S. D. Kemp. 1991. Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. *AIDS* 5:137–144.
- Little, S. J., E. S. Daar, R. T. D'Aquila, P. H. Keiser, E. Connick, J. M. Whitcomb, N. S. Hellman, C. J. Petropoulos, L. Sutton, J. A. Pitt, E. S. Rosenberg, R. A. Koup, B. D. Walker, and D. D. Richman. 1999. Reduced antiretroviral drug susceptibility among patients with primary HIV infection. *JAMA* 282:1142–1149.
- Little, S. J., S. Holte, J. P. Routy, E. S. Daar, M. Markowitz, A. C. Collier, R. A. Koup, J. W. Mellors, E. Connick, B. Conway, M. Kilby, L. Wang, J. M. Whitcomb, N. S. Hellmann, and D. D. Richman. 2002. Antiretroviral-drug resistance among patients recently infected with HIV. *N. Engl. J. Med.* 347:385–394.
- Lorenzi, P., M. Opravil, B. Hirschel, J. P. Chave, H. J. Furrer, H. Sax, T. V. Perneger, L. Perrin, L. Kaiser, and S. Yerly. 1999. Impact of drug resistance mutations on virologic response to salvage therapy. *AIDS* 13:F17–F21.
- Masquelier, B., P. E. Lemoigne, I. Pellegrin, D. Douard, B. Sandler, and H. J. A. Fleury. 1993. Primary infection with zidovudine-resistant HIV-1. *N. Engl. J. Med.* 329:1123–1124.
- Nijhuis, M., S. Deeks, and C. Boucher. 2001. Implications of antiretroviral drug resistance on viral fitness. *Curr. Opin. Infect. Dis.* 14:23–28.
- Petropoulos, C. J., N. T. Parkin, K. L. Limoni, Y. S. Lie, T. Wrin, W. Huang, H. Tian, D. Smith, G. A. Winslow, D. J. Capon, and J. M. Whitcomb. 2000. A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. *Antimicrob. Agents Chemother.* 44:920–928.
- Reed, L. J., and H. Muench. 1938. A simple method for estimating fifty percent end points. *Am. J. Hyg.* 27:493–497.
- Schmit, J. C., J. Cogniaux, P. Hermans, C. Van Vaeck, S. Sprecher, B. Van Remoortel, M. Witvrouw, J. Balzarini, J. Desmyter, E. de Clercq, and A. M. Vandamme. 1996. Multiple drug resistance to nucleoside analogs and non-nucleoside reverse transcriptase inhibitors in an efficiently replicating human immunodeficiency virus type 1 patient strain. *J. Infect. Dis.* 174:962–968.
- Taylor, S., P. Cane, S. Hue, L. Xu, T. Wrin, Y. Lie, N. Hellmann, C. Petropoulos, J. Workman, D. Ratcliffe, B. Choudhury, and D. Pillay. 2003. Identification of a transmission chain of HIV type 1 containing drug resistance-associated mutations. *AIDS Res. Hum. Retrovir.* 19:353–361.
- Veenstra, J., R. Schuurman, M. Cornelissen, A. B. van't Wout, C. A. Boucher, H. Schuitemaker, J. Goudsmit, and R. A. Coutinho. 1995. Transmission of zidovudine-resistant human immunodeficiency virus type 1 variants following deliberate injection of blood from a patient with AIDS: characteristics and natural history of the virus. *Clin. Infect. Dis.* 21:556–560.
- Violin, M., A. Cozzi-Lepri, R. Velleca, A. Vincenti, S. D'Elia, F. Chiodo, F. Ghinelli, A. Bertoli, A. d'Arminio Monforte, C. F. Perno, M. Moroni, and C. Balotta. 2004. Risk of virological failure in patients with 215 HIV-1 revertants starting their first thymidine analog-containing highly active antiretroviral therapy. *AIDS* 18:227–235.
- Weinstock, H., R. Respass, W. Heneine, C. J. Petropoulos, N. S. Hellmann, C. C. Luo, C. P. Pau, T. Woods, M. Gwinn, and J. Kaplan. 2000. Prevalence of mutations associated with reduced antiretroviral drug susceptibility among human immunodeficiency virus type 1 seroconverters in the United States, 1993–1998. *J. Infect. Dis.* 182:330–333.
- Weinstock, H., I. Zaidi, W. Heneine, D. Bennett, J. G. García-Lerma, J. M. Douglas, M. LaLota, G. Dickinson, S. Schwarcz, L. Torian, D. Wendell, S. Paul, G. Goza, J. Ruiz, B. Boyette, and J. E. Kaplan. The epidemiology of antiretroviral drug resistance among drug-naïve HIV-1 infected persons in 10 U.S. cities. *J. Infect. Dis.*, in press.
- Yerly, S., L. Kaiser, E. Race, J. P. Bru, F. Clavel, and L. Perrin. 1999. Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet* 354:729–733.
- Yerly, S., A. Rakik, S. K. De Loes, B. Hirschel, D. Descamps, F. Brun-Vezinet, and L. Perrin. 1998. Switch to unusual amino acids at codon 215 of the human immunodeficiency virus type 1 reverse transcriptase gene in seroconverters infected with zidovudine-resistant variants. *J. Virol.* 72:3520–3523.
- Zolopa, A. R., R. W. Shafer, A. Warford, J. G. Montoya, P. Hsu, D. Katzenstein, T. C. Merigan, and B. Efron. 1999. HIV-1 genotypic resistance patterns predict response to saquinavir-ritonavir therapy in patients in whom previous protease inhibitor therapy had failed. *Ann. Intern. Med.* 131:813–821.