Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naive persons

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Transmission of HIV-1 with reduced susceptibility to antiretroviral drugs raises public health concerns. Through surveillance of drug-resistant HIV-1 in 603 treatment-naïve, recently diagnosed HIV-1-infected persons, we identified a distinct group of viruses that have mutations at codon 215 of the reverse transcriptase (RT) gene that are different from either the wild-type (WT) T or the zidovudine (AZT)-selected T215Y/F. These mutations included 215D/C/S and were found in 20 patients (3.3%). The 215D, 215C, and 215S mutations differ from 215Y by a 1-nt change compared with 2 nt for the WT T215 and likely represent revertants of 215Y. These viruses all were found to have WT susceptibility to AZT, and all replicated efficiently as WT HIV-1$_{T215}$. However, differences in fitness among HIV-1$_{215D}$, HIV-1$_{215C}$, and HIV-1$_{215S}$ were seen when RT backgrounds were changed, demonstrating a role of the RT background in the selection of these revertants. *In vitro* selection with AZT showed that HIV-1$_{215D}$ and HIV-1$_{215C}$ acquired 215Y more rapidly than did WT HIV-1$_{T215}$, likely reflecting the need for only 1-nt change to evolve to 215Y. Our study demonstrates that HIV-1 with unusual mutations at codon 215 replicate efficiently, have WT susceptibility, and are commonly found in treatment-naïve persons. The increased ability for selecting resistance mutations defines this class of WT HIV-1 and highlights the higher potential of these viruses to compromise the efficacy of antiretroviral therapy.

drug resistance | reverse transcriptase inhibitors

Human immunodeficiency virus type 1 (HIV-1) with reduced susceptibility to zidovudine (AZT) has been isolated in patients receiving AZT therapy. Resistance to AZT is conferred by several mutations in the HIV-1 reverse transcriptase (RT) gene, including Thr-215–Tyr (T215Y), K70R, D67N, M41L, and L210W (1). The T215Y mutation is a primary mutation observed after AZT treatment. T215Y alone reduces the susceptibility for AZT ≈16-fold and is the first mutation seen in the majority of patients receiving combination therapy with AZT and other nucleoside analogs such as didanosine (ddI) or zalcitabine (ddC) (2, 3). The 215Y mutation also has been found in some patients treated with stavudine (d4T), and its presence in AZT-experienced patients may compromise the response to subsequent treatments with d4T (4–6).

The widespread use of antiretroviral drugs to treat HIV-1-infected persons has raised concerns regarding transmission of drug-resistant HIV-1. Surveillance of drug-resistant HIV-1 in recently infected persons has documented transmission of HIV-1 carrying several resistance mutations including 215Y (7–12). In addition, a unique set of mutations at codon 215 of HIV-1 RT has been found in treatment-naïve HIV-1-infected persons (11–17). These mutations are mainly 215C$_{(TGC)}$ and 215D$_{(GAC)}$, although other amino acids such as 215N$_{(AAC)}$ and 215S$_{(TCC)}$ also have been seen. Studies of HIV-1 seroconverters infected with viruses carrying the T215Y mutation have shown that 215D, 215C, 215N, and 215S represent revertants of HIV-1$_{215Y}$ (13, 15, 16). HIV-1$_{215D}$, HIV-1$_{215S}$, and HIV-1$_{215N}$ all have been shown to have higher replicative fitness than HIV-1$_{215Y}$ in the absence of AZT *in vitro*, which may explain why the 215Y mutation reverts through these intermediates *in vivo* (15, 18). However, the determinants that influence selection of a particular revertant are not fully understood.

Longitudinal studies of small numbers of patients have shown that 215C, 215D, 215N, and 215S are stable mutations that can persist in the absence of antiretroviral therapy. For instance, Yerly *et al.* (13) reported persistence of 215D and 215C for a period of 1–2 years in four patients, whereas de Ronde *et al.* (15) found persistence of 215D, 215C, 215S, and 215N for 1–3 years in five patients. The stability of these mutations has been explained by small differences in replicative fitness between these viruses and the wild-type (WT) HIV-1$_{T215}$ (15, 19).

Information on the effect of these mutations on susceptibility to nucleoside analogs is limited to four patients who had viruses with 215C or 215D and were found to be sensitive to AZT (13). However, because these mutations differ from 215Y by a 1-nt change they may potentially be prone to evolve rapidly to 215Y under drug-selective pressure. Therefore, it is important to assess the impact of these intermediate mutations on the rate of acquisition of 215Y. A better understanding of the evolution of these viruses in the presence of AZT *in vitro* therefore might shed light on the clinical significance of these mutations and their potential impact on the response to AZT treatment.

In the present study, we determined the prevalence of mutations at codon 215 in a large cohort of treatment-naïve, recently diagnosed, HIV-1-infected persons. We also have examined the impact of these mutations on drug susceptibility, replicative fitness, and rate of acquisition of 215Y.

Materials and Methods

Study Population. The study population consisted of 603 treatment-naïve, recently diagnosed, HIV-1-infected individuals consecutively enrolled in 1997–1999 from selected HIV-1 testing sites and care clinics in 10 cities in the United States. These patients are part of an ongoing sentinel surveillance system

MEDICAL SCIENCES

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Abbreviations: RT, reverse transcriptase; WT, wild type; AZT, zidovudine; ddC, zalcitabine; d4T, stavudine; ddI, didanosine; CCID$_{50}$, 50% cell culture-infectious dose.

Data deposition: The sequences of isolates RD 01, RD 02, RD 03, RD 04, RD 05, RD 22, and RD 23 reported in this paper have been deposited in the GenBank database (accession numbers AF415228–AF415234).

See commentary on page 13485.

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designed to examine the prevalence of mutations associated with decreased susceptibility to antiretroviral drugs among recently diagnosed HIV-1-infected persons.‖ Specimens were tested by using a less sensitive modification of an HIV-1 enzyme immunoassay (3A11, Abbott) to identify persons infected within the past 4–6 months (21).

Phenotypic and Genotypic Analysis. Phenotypic resistance to nucleoside RT inhibitors was determined by using either the PhenoSense HIV (Virologic, South San Francisco) or the Antivirogram (Virco, Mechelen, Belgium and Cambridge, U.K.) assays (22, 23). Phenotypic resistance was determined by measuring fold increase in 50% inhibitory concentration (IC_{50}) values compared with a reference WT HIV-1 isolate (NL4–3 or HXB2). The results were interpreted based on assay cut-off values established for each assay. Assay cut-off values used for the PhenoSense HIV assay were 1.7-fold for ddI and d4T and 2.5-fold for all other drugs, whereas those used for the Antivirogram assay were 3-fold for d4T and abacavir, 3.5-fold for ddI and ddC, and 4-fold for AZT. Genotypic testing was done from the patient-derived PCR products generated for the Antivirogram assay or from resistance test vectors generated for the PhenoSense HIV assay. Selected samples also were genotyped by automated sequencing (Applied Biosystems).

Generation of Recombinant Viruses with Cloned RT Sequences from Patients or HXB2. Full-length RT from plasma HIV-1 was amplified in duplicate by RT-nested PCR and then cloned by using the TA cloning kit (Invitrogen, Promega), as described (24). Cloned RT sequences from patients or HIV-1_{HXB2} were used to generate recombinant viruses with the RT-deleted HXB2-based proviral molecular clone pHIVΔRT*Bst*EII (23, 25). The following recombinant viruses were obtained by using the RT from HIV-1_{HXB2}: $HXB2_{T215}$, $HXB2_{215D}$, $HXB2_{215C}$, and $HXB2_{215S}$. Recombinant viruses generated by using patient-derived RTs were RD 01_{215C}, RD $02_{210W/215C}$, RD $03_{41L/210W/215C}$, RD $03_{41L/210W/215S}$, RD $04_{210W/215D}$, RD $04_{210W/215S}$, RD $05_{41L/215D}$, RD 22_{T215}, and RD 23_{T215}. The 50% cell culture-infectious dose ($CCID_{50}$) in each virus stock was determined in MT-4 cells by using the method of Reed and Muench (26).

Site-Directed Mutagenesis at Codon 215 of HIV-1 RT. Site-directed mutagenesis was done by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) as described (24). Mutations were introduced in the pHXB2RIP7-based infectious clone pSUM9 (kindly provided by H. Mitsuya, Nattional Cancer Institute, Bethesda, MD) or in plasmid preparations containing full-length patient-derived RT sequences (plasmids pRD $03_{41L/210W/215C}$ and pRD $04_{210W/215D}$). Primers T215SF1/T215SR1, T215CF1/T215CR1, and T215DF1/T215DR1 were used to introduce the 215S, 215C, or 215D mutations in the WT infectious clone pSUM9, respectively. Primers $RD03F1_{215S}$/$RD03R1_{215S}$ and $RD04F1_{215S}$/$RD04R1_{215S}$ were used to introduce the 215S mutation in pRD $03_{41L/210W/215C}$ and pRD $04_{210W/215D}$, respectively (Table 1).

***In Vitro* Selection of AZT Resistance.** For *in vitro* selection of AZT resistance, we used an approach similar to that described previously (27, 28). Inocula of 1.5×10^6 MT-4 cells were exposed to 1,500 $CCID_{50}$ (multiplicity of infection = 0.001) of each virus for 2 h at 37°C. After two washes with PBS, cells were resuspended in 10 ml of complete medium containing AZT at a concentration close to the IC_{50} values for each virus (0.03 μM).

‖Zaidi, I., Weinstock, H., Woods, T., Thomas, J., Heneine, W. & Kaplan, J., Fifth International Workshop on HIV Drug Resistance and Treatment Strategies, June 4–8, 2001, Scottsdale, AZ, abstr. 155.

Table 1. Primers used to generate site-directed mutants at codon 215 of HIV-1 RT

Primer	Sequence
T215SF1	5′-GTTGAGGTGGGACTTTCCACACCAGACAAAAAACATCAG-3′
T215SR1	5′-CTGATGTTTTTTGTCTGGTGTGGAAAGTCCCCACCTCAAC-3′
T215CF1	5′-GTTGAGGTGGGACTTTGCACACCAGACAAAAAACATCAG-3′
T215CR1	5′-CTGATGTTTTTTGTCTGGTGTGCAAAGTCCCCACCTCAAC-3′
T215DF1	5′-GTTGAGGTGGGACTTGACACACCAGACAAAAAACATCAG-3′
T215DR1	5′-CTGATGTTTTTTGTCTGGTGTGTCAAGTCCCCACCTCAAC-3′
$RD03F1_{215S}$	5′-GTGGAAGTGGGGATTCTCCACACCAGACAAAAAACATCAG-3′
$RD03R1_{215S}$	5′-CTGATGTTTTTTGTCTGGTGTGGAGAATCCCCACTTCCAC-3′
$RD04F1_{215S}$	5′-GTGGAAGTGGGGATTTTCCACACCAGACAAAAAACATCAG-3′
$RD04R1_{215S}$	5′-CTGATGTTTTTTGTCTGGTGTGGAAAATCCCCACTTCCAC-3′

Cultures were then incubated at 37°C, and media containing AZT were changed every 3–4 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 (generally 2-fold). Genotypic changes at codon 215 and other positions of HIV-1 RT were monitored by sequence analysis of the RT from culture supernatant in selected passages.

Analysis of Replication Kinetics in MT-4 Cells. Inocula of 600 $CCID_{50}$ was used to infect 6×10^5 MT-4 cells (multiplicity of infection = 0.001). After incubation for 2 h at 37°C, cells were washed twice with PBS and resuspended in complete medium at 7.5×10^4 cells/ml. Two-milliliter cultures were done in duplicate both in the absence and the presence of AZT (0.15 μM) by using 24-well tissue culture plates (Costar). Supernatants (200 μl) from each culture were collected at different days, and then an equal volume of culture media was added. Levels of p24 antigen were quantitated in cell-free culture supernatants by using the Coulter HIV-1 p24 antigen assay and were used to monitor replication kinetics. Cultures done in the presence of AZT were diluted every 3–4 days in complete media containing AZT. The concentration of AZT used in these experiments was below the IC_{90} value for AZT determined in WT HIV-1_{HXB2} (1.12 μM).

Analysis of Relative Replicative Fitness in Virus Mixtures. Relative replicative fitness was analyzed in growth competition assays as described (24). Viruses were adjusted according to their $CCID_{50}$ values before mixtures were prepared. Briefly, a 150-ul inoculum of the two competing variants was used to infect 1.5×10^5 MT-4 cells at an multiplicity of infection of 0.001 in duplicate as described above. After 4–6 days in culture, 200 μl of the supernatant from 2-ml cultures was used to reinfect a fresh aliquot of 1.5×10^5 MT-4 cells. The relative proportion of the two competing variants was determined both at baseline and in each passage based on the ratios of the specific mutations. Ratios were estimated based on the relative peak heights in electropherograms obtained by automated sequencing of HIV-1 RT from culture supernatants (24).

Sequence Analysis of HIV-1 RT. Sequence analysis of HIV-1 RT (from nucleotides 2529–3333 of HXB2; amino acids 7–246) was done in an Applied Biosystems 373 automated sequencer by using primers AV36, AV44, A35, and NE(1)35 (29). The DNASIS program (version 2.6; 1998) was used to analyze the data and determine deduced amino acid sequences.

Results

Prevalence of Mutations at Codon 215. Of the 603 patients studied, two (0.3%) had 215Y, one (0.2%) had 215F, and 20 (3.3%) had

Table 2. Susceptibility to nucleoside analogs in viruses carrying unusual mutations at codon 215

Sample ID	RT mutations	Fold resistance*				
		AZT	d4T	ABC	ddC	ddI
RD 01	215C	2.0	1.2	1.0	0.7	0.9
RD 02	210W, 215C	1.7	1.5	1.0	0.8	1.2
RD 03	41L, 210W, 215C	2.6	1.3	1.0	1.2	1.1
RD 04†	210W, 215D	0.7	0.6	0.5	2.8	1.6
RD 05	41L, 215D	0.9	1.0	0.9	1.0	0.9
RD 06	210F, 215D	0.7	1.1	1.5	2.0	1.0
RD 07	41L, 215D	1.6	1.4	1.5	0.9	1.0
RD 08	41L, 215D	2.9	0.2	0.4	1.6	1.8
RD 09	41L, 215D	2.0	0.9	0.6	1.1	0.3
RD 10	41L, 215D	2.5	1.3	1.2	1.3	2.2
RD 11	67N, 215D	1.3	0.7	0.3	1.0	0.9
RD 12	215D	1.0	0.9	0.7	0.8	0.8
RD 13	41L, 215C	3.2	1.7	1.0	2.0	0.4
RD 14	215C	0.7	0.3	0.6	0.7	1.0
RD 15	215C	1.1	1.5	1.4	1.1	1.8
RD 16	41L, 215S	1.3	1.0	0.8	0.9	0.9
RD 17	215S	1.3	1.1	1.1	1.1	1.0
RD 18	215S	0.9	1.2	0.9	0.8	1.0
RD 19	215S	0.6	0.9	0.7	0.6	0.7
RD 20	41L, 215E	1.0	0.9	1.0	1.0	0.9
RD 21	215I, 219E, D67del	6.4	1.1	1.3	1.0	0.7

*Fold increase in IC_{50} compared to a WT HIV-1 reference isolate. Phenotypic testing was done using the Phenosense HIV assay except for samples RD 08, RD 09, RD 10, RD 11, RD 13, RD 14, and RD 15, which were tested by using the Antivirogram assay.

†Not included in the prevalence study.

mutations other than Y, F, or the WT T. Of these patients, eight had 215D, six had 215C, four had 215S, one had 215E, and one had 215I (Table 2). The 215D and 215C mutations were associated with 41L, 67N, and/or 210W/F in 7/8 and 3/6 patients, respectively. The 215I mutation observed in patient RD 21 was associated with 219E and an insertion at position 67, and the 215E mutation observed in patient RD 20 was associated with the 41L mutation. Of the four patients who had the 215S mutation, only one had additional AZT-resistance mutations (Table 2). The 20 persons carrying these mutations had reactive modified enzyme immunoassay tests, suggesting that the duration of infection in all 20 patients was >4–6 months.

Susceptibility to Nucleoside RT Inhibitors of Viruses Carrying Unusual Mutations at Codon 215. To investigate the effect of 215C, 215D, 215S, 215I, and 215E on susceptibility to nucleoside RT inhibitors, IC_{50} values for AZT, ddC, ddI, abacavir, and d4T were determined in viruses from all of the patients who had these mutations. Table 2 shows fold increases in IC_{50} values compared with a reference WT HIV-1 isolate (NL4–3 or HXB2). Drug susceptibility results in a recently infected person carrying the 215D mutation who was identified in a separate study (patient RD 04) are also shown (12). Viruses from 20 of 21 patients had no evidence of decreased susceptibility for AZT, with IC_{50} values similar to that found in the reference WT HIV-1 isolate. Recombinant RD 03 had an IC_{50} value that was 2.6-fold higher than that of NL4–3, which is close to the assay cutoff value (>2.5-fold) for defining AZT resistance in the PhenoSense HIV assay. Similar borderline susceptibility was observed for ddC in recombinant RD 04 (2.8-fold) by using the assay cutoff of 2.5 defined for this drug in the PhenoSense HIV assay (Table 2). Evidence of low-level resistance to AZT (6.4-fold) was observed only in recombinant RD 21, which had the 215I mutation in association with 219E and a deletion at codon 67. Overall, these findings indicate that these mutations do not confer decreased susceptibility to AZT or other nucleoside analogs.

Table 3. Characteristics of recombinant viruses generated by using RT sequences from HXB2 or treatment-naïve, recently diagnosed, HIV-1-infected patients

Recombinant virus	Mutations	$CCID_{50}$/ml
Patient-derived RT		
RD 01	215C	25,281
RD 02	210W, 215C	32,340
RD 03	41L, 210W, 215C	12,383
RD 04	210W, 215D	197,461
RD 05	41L, 215D	15,625
RD 22	—	78,125
RD 23	—	78,736
HXB2 RT		
$HXB2_{T215}$	—	174,825
$HXB2_{215D}$	215D	174,825
$HXB2_{215C}$	215C	174,825

Effect of 215D and 215C on Virus Replication. We next determined the effect of 215D and 215C on virus replication. We focused on 215D and 215C because these were the two mutations more frequently seen in patients. The effects of 215D and 215C on replication were examined in recombinant viruses that had these mutations alone or in combination with other secondary mutations such as 41L and/or 210W. Recombinant viruses were generated by using RT sequences derived from HIV-1_{HXB2} or from patients who had 215C, 215D, or the WT T215. Table 3 shows that all mutant and WT sequences generated recombinant viruses with high infectious virus titers.

To determine whether the presence of these mutations could

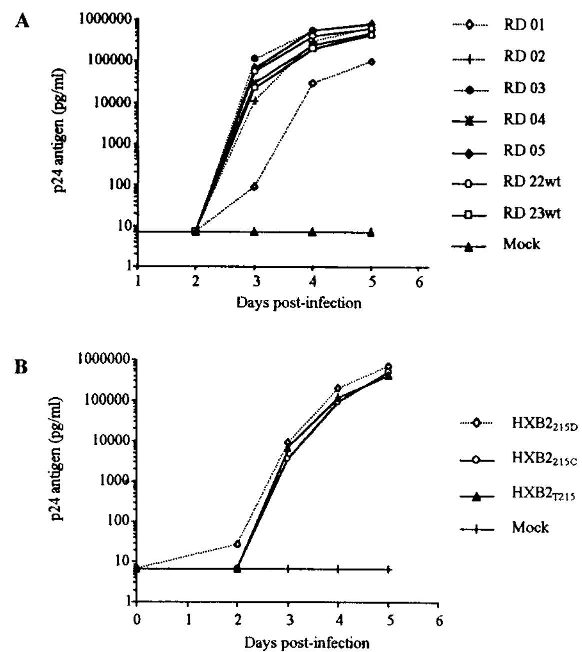

Fig. 1. Replication kinetics of recombinant viruses carrying the 215C or 215D mutations alone or in association with 41L or 210W, and comparison with viruses having the WT T at codon 215. (*A*) Recombinant viruses carrying patient-derived RTs. (*B*) Recombinant viruses generated with the RT of HIV-1_{HXB2}. Mean p24 values from duplicate cultures are shown. Mock, uninfected cells.

MEDICAL SCIENCES

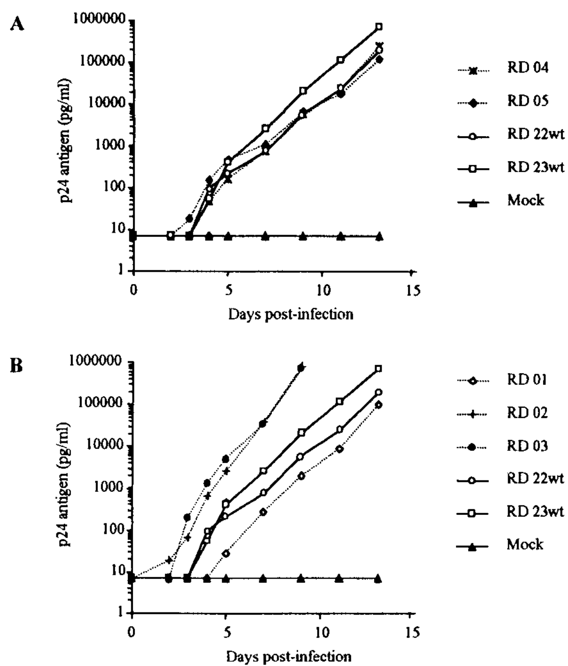

Fig. 2. Replication kinetics in the presence of 0.15 μM AZT of patient-derived recombinant viruses carrying 215D (*A*) or 215C (*B*) and comparison with viruses having the WT T at codon 215. Mean p24 values from duplicate cultures are shown. Mock, uninfected cells.

affect virus replication, we evaluated replication capabilities of recombinant viruses in experiments of acute infection in MT-4 cells. Fig. 1*A* shows the kinetics of p24 antigen production observed with viruses having C, D, or WT T at codon 215. Viruses carrying the 215D mutation in combination with 210W (recombinant RD 04) or 41L (recombinant RD 05) replicated efficiently, with kinetics of p24 antigen production similar to those observed in WT viruses (recombinants RD 22 and RD 23). Similar findings were observed in recombinant viruses carrying the 215C mutation in combination with 210W (recombinant RD 02), or 41L and 210W (recombinant RD 03). Decreased replication capability was observed only in recombinant RD 01, which had the 215C mutation alone (Fig. 1*A*). However, the 215C mutation did not affect virus replication in the RT genetic background of HXB2 when compared with $HXB2_{215D}$ or $HXB2_{T215}$ (Fig. 1*B*), suggesting that the observed effect of 215C in the RT from patient RD 01 may be related to the RT background of this virus. Taken together, these results indicate that the 215C and 215D mutations do not significantly affect replication capacity.

Replication Capability in the Presence of AZT in Patient-Derived Recombinant Viruses Carrying the 215D or 215C Mutation. We next determined the ability of $HIV\text{-}1_{215D}$ and $HIV\text{-}1_{215C}$ to replicate in the presence of AZT. Fig. 2*A* shows that the two recombinants carrying the 215D mutation (RD 04 and RD 05) replicated as efficiently as WT viruses. In contrast, recombinant viruses carrying the 215C mutation in association with other secondary mutations (recombinants RD 02 and RD 03) replicated more efficiently than WT viruses or viruses carrying the 215D mutation (Fig. 2*B*). Similar to that found in the absence of AZT, recombinant RD 01, which had the 215C mutation alone, had the lower replication capability among all viruses tested, suggesting that 215C may have a deleterious effect on virus replication in the RT background of this virus (Fig. 2*B*).

Evolution of 215D and 215C in the Presence of AZT. To investigate the effect of the 215C and 215D mutations on the rate of acquisition of AZT resistance mediated by 215Y, we monitored genotypic changes at codon 215 and other positions of the RT in $HIV\text{-}1_{215C}$ and $HIV\text{-}1_{215D}$ cultured in the presence of AZT. We compared the rate of acquisition of 215Y among $HIV\text{-}1_{215C}$, $HIV\text{-}1_{215D}$, and the WT $HIV\text{-}1_{T215}$. The evolution of $HIV\text{-}1_{215C}$ and $HIV\text{-}1_{215D}$ in the presence of AZT was examined in recombinant viruses that had these mutations alone or in association with other secondary mutations (41L and/or 210W).

Table 4 shows the kinetics of emergence of AZT resistance after sequential passages of the viruses in the presence of AZT. All seven viruses carrying the 215C or 215D mutations evolved to 215Y after a mean of 25 days (range = 18–33) and 31 days (range = 27–37) of culture, respectively. No significant differences in the rate of evolution to 215Y were observed among viruses that had 215D or 215C alone or in combination with other AZT resistance mutations, indicating that the rapid evolution from D or C to Y was caused by these amino acid changes and not by the presence of other secondary mutations associated with AZT resistance such as 41L or 210W.

In contrast, the first evidence of mutations associated with AZT resistance in the three control WT viruses tested (RD 22, RD 23, and $HXB2_{T215}$) was observed after a mean of 63 (range = 53–81) days in culture. Selection of AZT resistance in these three control viruses involved mutations other than 215Y. For instance, the T215F and K70R mutations were the first mutations observed in recombinant RD 22, whereas D67N was the first mutation identified in recombinant RD 23 and $HXB2_{T215}$. Taken together, these results indicate that $HIV\text{-}1_{215C}$ and $HIV\text{-}1_{215D}$ have increased ability to evolve to $HIV\text{-}1_{215Y}$.

The Order of Relative Replicative Fitness Among $HIV\text{-}1_{215D}$, $HIV\text{-}1_{215C}$, and $HIV\text{-}1_{215S}$ Is Influenced by the RT Genetic Background. The analysis of mutations associated with 215D, 215C, and 215S observed in this and other studies indicates that 215C and 215D generally are associated with secondary mutations such as 41L, 67N, or 210W, whereas 215S is frequently seen alone (11, 13–17). We therefore evaluated whether the observed association of 215C/D with 41L or 210W could be explained by a fitness gain conferred by 215D or 215C compared with 215S in these particular RT backgrounds. We mutated 215D or 215C to 215S in the RT from two patients who also had the 41L and/or 210W mutations (RD 03 and RD 04), and then determined the order of relative replicative fitness among these viruses.

Fig. 3 shows the relative proportion of $RD\ 03_{41L/210W/215S}$ and $RD\ 04_{210W/215S}$ over time in virus mixtures with $RD\ 03_{41L/210W/215C}$ and $RD\ 04_{210W/215D}$, respectively. The results show that, in both cases, viruses carrying the 215C or 215D mutation outgrew viruses carrying the 215S mutation, indicating that replicative fitness of $HIV\text{-}1_{215D}$ or $HIV\text{-}1_{215C}$ is higher than that of $HIV\text{-}1_{215S}$ in all of these RT genetic backgrounds. These findings may likely explain the frequent association of 41L and/or 210W with 215C or 215D but not with 215S observed *in vivo*.

We next investigated whether the observed order of fitness could be maintained in a background that lacks 41L and/or 210W. We evaluated relative replicative fitness of viruses carrying the 215D, 215C, or 215S mutations in the context of $HIV\text{-}1_{HXB2}$ to determine the impact of these mutations alone. Fig. 4 illustrates the relative proportion of 215S over time in mixtures of $HXB2_{215S}$ with $HXB2_{215D}$ or $HXB2_{215C}$. The results show that in this RT background $HIV\text{-}1_{215S}$ outgrows both $HXB2_{215C}$ and $HXB2_{215D}$, indicating that fitness of $HXB2_{215S}$ is higher than that of $HXB2_{215C}$ or $HXB2_{215D}$. Taken together, these findings indicate that the RT genetic background contributes to the order of relative replicative fitness among viruses carrying the 215C, 215D, or 215S mutations.

Table 4. Kinetics of emergence of AZT resistance in recombinant viruses carrying Cys (C), Asp (D), or Thr (T) at codon 215 of the RT

Recombinant virus	AZT, uM	Cumulative time, days*	RT mutations	
			Codon 215	Other position
RD 01_{215C}				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	C/Y†	—
	0.24	24	Y	—
RD $02_{210W,215C}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	C	—
	0.24	24	C/Y	—
	0.48	28	Y/C	—
RD $03_{41L,210W,215C}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	C	—
	0.24	24	Y/C	—
	0.48	28	Y	—
$HXB2_{215C}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	n.d.	n.d.
	0.24	33	Y/C	—
	0.48	40	Y	—
RD $04_{210W,215D}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	D	—
	0.24	30	Y	—
RD $05_{41L,215D}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	D	—
	0.24	27	Y	—
$HXB2_{215D}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	22	D	—
	0.24	37	Y	—
RD $22_{wild\ type}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	n.d.	n.d.
	0.24	30	T	—
	0.48	37	T	—
	0.96	45	T	—
	3	53	F	K70K/R
	10	60	F	K70R
RD $23_{wild\ type}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	n.d.	n.d.
	0.24	28	T	—
	0.48	39	T	—
	0.96	54	T	D67N
	3	65	T	D67N, K70K/R
	10	74	T	D67N, K70R
$HXB2_{T215}$				
	0.03	6	n.d.	n.d.
	0.06	12	n.d.	n.d.
	0.12	18	n.d.	n.d.
	0.24	30	T	—
	0.48	44	T	—
	0.96	57	T	—
	3	81	T	D67D/N
	10	108	T	D67N/D

n.d., Not done.

*Total days in culture with sequential passages of increasing AZT concentrations.

†Mixed genotype. The first amino acid represents the predominant genotype observed in the mixture.

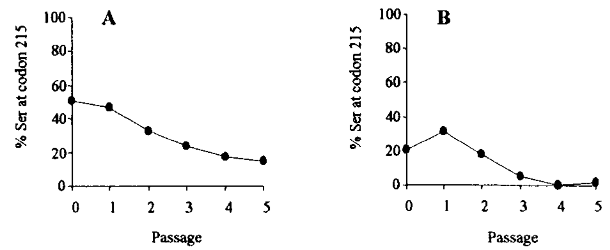

Fig. 3. Competitive HIV-1 replication assay among patient-derived recombinant viruses carrying Asp (D), Cys (C), or Ser (S) at codon 215. HIV-1_{215D} or HIV-1_{215C} were mixed with HIV-1_{215S} at known ratios, and the proportion of S at codon 215 was monitored over time. Day 0 represents proportions in the initial virus mixture. (*A*) Mixture of $RD03_{41L/210W/215S}$ and $RD03_{41L/210W/215C}$. (*B*) Mixtures with $RD04_{210W/215S}$ and $RD04_{210W/215D}$.

Discussion

We have investigated the prevalence of HIV-1 with mutations other than Y/F at codon 215 of the RT in 603 drug-naïve persons with established HIV-1 infection. We show that these mutations are not rare and are more prevalent than any other primary resistance mutations seen in the study population.‖ The substantial prevalence heightens the importance of defining the significance of these mutations.

Our phenotypic results on all 20 HIV-1 isolates carrying 215C, 215D, or 215S or 215E indicate that these mutations do not confer resistance to AZT or other nucleoside analogs. The WT susceptibility to AZT was seen despite the presence of 41L, 67N, and/or 210W in some viruses, suggesting that a single nucleotide change from Y to either D, C, or S is sufficient for the loss of phenotypic resistance to AZT. These findings demonstrate that phenotypic reversion is not always associated with full genotypic reversion at codon 215. The low-level resistance to AZT found in one patient who had the 215I mutation may likely be related to the deletion at position 67, which has been previously associated with AZT resistance (30). The loss of 215Y in viruses containing 41L and/or 210W suggests that reversion at codon 215 is the first to occur, probably reflecting a higher impact of 215Y on fitness compared with other AZT-selected mutations such as 41L or 210W (16, 18).

A key issue was to determine whether these unusual amino acid substitutions at codon 215 could affect the rate of acquisition of 215Y. We found that 215Y was more rapidly selected in viruses with 215D or 215C than in viruses that had the WT T215. The increased capacity to select for 215Y distinguishes these viruses from other WT HIV-1 and may be explained by the requirement of a single nucleotide change to evolve from D/C to Y compared with the two nucleotide changes required for the

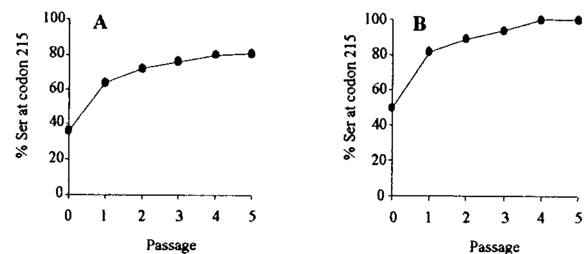

Fig. 4. Competitive HIV-1 replication assay among HXB2 viruses carrying Asp (D), Cys (C), or Ser (S) at codon 215. $HXB2_{215D}$ or $HXB2_{215C}$ were mixed with $HXB2_{215S}$ at known ratios, and the proportion of S at codon 215 was monitored over time. Day 0 represents proportions in the initial virus mixture. (*A*) Mixtures of $HXB2_{215S}$ and $HXB2_{215C}$. (*B*) Mixtures of $HXB2_{215S}$ and $HXB2_{215D}$.

MEDICAL SCIENCES

WT T215. The increased potential of these viruses to select 215Y and become AZT-resistant may imply that patients who are infected with these viruses and are treated with antiretroviral regimens containing AZT may be at increased risk for developing AZT resistance. Therefore, these findings highlight the importance of defining the clinical impact of these mutations by assessing the rate of virologic failures and emergence of AZT resistance in these patients compared with those infected with viruses carrying WT 215 genotypes. In the absence of such clinical data, our study supports reporting these mutations in genotypic test results and indicates that a close monitoring of treatment responses in patients infected with these viruses is prudent.

Our results also may have implications for patients treated with d4T, because 215Y also can be selected in some patients treated with this drug (4). The data on the rapid selection of 215Y in one patient infected with HIV-1_{215D} after receiving combination therapy with ddC and d4T are not reassuring and may be predictive of a poor response to both d4T and AZT (20). Therefore, a better understanding of the ability of these mutations to evolve to 215Y after exposure to d4T both *in vitro* and *in vivo* is needed.

The presence of viruses with revertants at codon 215 in treatment-naïve persons has been associated with two types of primary infections. The first involves infection with HIV-1 carrying revertants of 215Y from patients who had past treatment with AZT, as recently reported for 215C/S/D (refs. 11 and 15).** The second is associated with a reversion of 215Y in persons infected with viruses carrying this mutation (13, 15, 16). The timing of 215Y reversion in our chronically infected patients is not known because information on the genotype of the transmitted virus is not available. However, both types of transmissions have the potential to compromise the efficacy of AZT through either a rapid evolution of 215D/C/S to 215Y, or by a selection of an archived virus with 215Y.

Our results showing five different amino acid substitutions at codon 215 confirm that reversion from 215Y can occur through multiple intermediates. Our data demonstrate that viruses with 215 D/C/or S all can replicate efficiently, which may explain the persistence of these viruses *in vivo* (13, 15). We also show that the RT background may play a role in the selection of specific intermediates because differences in fitness among HIV-1_{215D}, HIV-1_{215C}, and HIV-1_{215S} were seen when RT backgrounds were changed. For instance, we found that the fitness gain conferred by 215S alone was lost when 215S was substituted for either 215D or 215C in patient-derived RT backgrounds containing 41L and/or 210W. The fitness gain conferred by 215D/C compared with 215S observed in these RTs might explain the frequent association of 215D/C but not 215S with 41L and 210W observed in this and other studies (11, 13–17). In contrast, the higher fitness of HIV-1_{215S} compared with HIV-1_{215C} or HIV-1_{215D} seen in RT backgrounds that only had these mutations may explain the frequent observation of 215S alone *in vivo* and suggest that 215S may be favored over 215D/C in the reversion of viruses carrying 215Y alone.

In conclusion, we found a substantial prevalence of HIV-1 with mutations other than Y/F at codon 215 in treatment-naïve persons. These newly adapted viruses are fit and represent a distinct class of WT HIV-1 that has increased ability for selecting the 215Y mutation *in vitro*, thus raising concerns about its potential to compromise the efficacy of antiretroviral therapy.

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