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Significance of Amino Acid Variation at Human Immunodeficiency Virus Type 1 Reverse Transcriptase Residue 210 for Zidovudine Susceptibility

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Amino acid variation at reverse transcriptase (RT) codon 210 (generally Leu-210 to Trp [L210W], TTG→TGG) is occasionally detected after the initiation of azidothymidine (AZT) therapy. The impact of this variation on AZT resistance and viral replication was addressed by four different approaches. The frequency and genetic background of the L210W mutation in vivo were assessed by analyzing sera of AZT-naive and AZT-experienced patients by RT-PCR and DNA sequencing. The degree of AZT resistance (50% infective concentration [IC_{50}]) of recombinant viruses constructed by using the RT of 21 clinical isolates was stratified by the presence or absence of the 210 mutation. The AZT IC_{50}s of a panel of mutant viruses (with or without W-210) constructed by site-directed mutagenesis in an HXB2 background were assayed by using a HeLa CD4 plaque reduction assay. Finally, the effect of the 210 mutation on viral replication was assessed by replication competition of an AZT-resistant virus, RTMN (L-41/Y-215), and RTMN with the W-210 mutation in the presence and in the absence of AZT. In AZT-naive patients, tryptophan at RT residue 210 was rare. After AZT exposure, W-210 appeared in a minority of those patients, most commonly in association with L-41 and Y-215. The presence of W-210 increased the AZT IC_{50} by two- to fourfold, as determined by both the recombinant virus assay and site-directed mutagenesis. A significant replication advantage in favor of the wild-type L-210 over W-210 was observed, although the selection against the 210 mutant was two- to threefold lower when the viruses were grown in the presence of 5 μM AZT. In summary, the L210W mutation appears to be of marginal significance, conferring approximately two- to fourfold-reduced sensitivity to AZT compared with similar AZT-resistant genomes with L-210. The selection pressure against W-210 may account for the modest proportion of patients in which W-210 appears in vivo.

Zidovudine (AZT, Retrovir) has commonly been used in human immunodeficiency virus (HIV) infection to delay the development of AIDS and to increase survival of patients with AIDS (4, 9, 25). More recently, two major clinical endpoint trials have shown a benefit of AZT-dideoxycytidine or AZT-dideoxyinosine combinations over AZT monotherapy (6, 26). Long-term treatment with AZT monotherapy is associated with the development of viral isolates with reduced susceptibility to AZT (16). Site-directed mutagenesis experiments have confirmed that at least five amino acid changes (at residues 41, 67, 70, 215, and 219) in the HIV type 1 (HIV-1) reverse transcriptase (RT) can contribute to AZT resistance (11, 17; for a review, see reference 14). During AZT therapy, the mutation at position 70 tends to be the first to arise, followed by the mutation at position 215 and then other combinations of the mutations listed above (2, 12). Because of the inherent variability of HIV, amino acid changes other than at these five established positions can also be detected both before and after the initiation of AZT therapy. These sequence changes could occur for a number of reasons. For example, they might actually confer AZT resistance, act as compensatory changes secondary to other mutations which do confer drug resistance, or be a result of other selection pressures acting upon this inherently variable virus. Variation at RT codon 210 (Leu to Trp) was noted in 12 of 38 patients who had been taking AZT for at least 2 years (22) and has also been noted by others (20). Hooker et al. (8) found evidence of W-210 in 7 of 22 patients on long-term AZT therapy and reported that the addition of W-210 in the context of mutations at codons 41 and 215 resulted in virus with 370-fold AZT resistance. On the basis of similar experiments, however, Fitzgibbon et al. (5) concluded that W-210 did not play a significant role in contributing to AZT resistance. The aim of this study was to gain additional insight into the prevalence and significance of sequence variation in HIV-1 RT at residue 210 for AZT resistance.

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MATERIALS AND METHODS

Sequence analysis of HIV-1 RT. The 5′ end of the HIV-1 RT coding region was amplified by a nested RT-PCR procedure starting from patient serum samples previously described (18). Single-stranded product was obtained by using a magnetic bead separation system (Dynabeads M-280 streptavidin; Dynal); 80 μl of Dynabeads (5 μg/μl) was used per PCR product. These were washed twice (10 mM Tris-HCl [pH 8], 1 mM EDTA, 2 M NaCl), mixed with 80 μl of PCR product, and incubated at 40°C for 15 min. The bound DNA was denatured by addition of 25 μl of 0.1 M NaOH for 15 min at room temperature. The negative strand bound to the beads was used for solid-phase sequencing (19). Reactions were performed by using a PRISM T7 terminator single-stranded DNA sequencing kit (Applied Biosystems) and resolved on an ABI 373 DNA sequencer according to the manufacturer's instructions. Data were imported into the programs FACTURA and Sequence Navigator (19) and scored as mutant mixtures if the minor peak area represented greater than 50% of the major peak area.

Construction of recombinant HIV-1 strains from clinical material: recombinant virus assay (RVA). A DNA fragment of the HIV RT coding region derived from the sera of a number of HIV-positive patients undergoing therapy with AZT or AZT-3TC was obtained by nested RT-PCR as previously described (18). These clinical sample RT coding regions were subsequently incorporated into the otherwise wild-type HXB2-D background by homologous recombination (13). The T-cell line C8166 was cotransfected by electroporation with a mixture of the RT-deleted proviral clone pHIVΔRT*Bst*EII and a PCR-derived RT fragment as described previously (18). Generally, viral replication was first evident in the cultures about 6 days posttransfection. Extracellular virus was harvested from

TABLE 1. Occurrence of W-210 in association with changes at codons 41 and 215

RT genotype of isolates with W-210	Codon 41	Codon 67	Codon 70	Codon 215	Codon 219	No. of patients
At entry to trial NUCB3002	Mutant	wt[a]	wt	Mutant	wt	10
	Mutant	Mutant	Mutant	Mutant	wt	1
	wt	Mutant	Mutant	Mutant	Mutant	1
	wt	wt	wt	Mutant	wt	1
After 24 wk of enrollment	Mutant	wt	wt	Mutant	wt	13
	Mutant	wt	Mutant	Mutant	wt	4
	Mutant	Mutant	Mutant	Mutant	wt	2
	Mutant	Mutant	Mutant	Mutant	Mutant	1
	Mutant	wt	wt	wt	wt	1

[a] wt, wild type.

culture supernatants after about 14 days and stored in aliquots at −70°C. The AZT sensitivity of these isolates was examined by using the HeLa CD4 plaque assay (15) in at least duplicate.

Determination of viral fitness by replication competition of defined mixtures of viruses. MT-2 cells (7) were infected with either 50:50 mixtures (based on HeLa $CD4^+$ cell plaque titer) or with 25:75 or 75:25 mixtures of RTMN (HXB2 virus with AZT resistance mutations L-41 and Y-215) (11) or RTMN/210 at a multiplicity of infection of <0.1 PFU per cell. Infected cells containing these mixtures were incubated at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics, either in the absence of inhibitor or in the presence of 5 μM AZT. Progeny virus was serially passaged in MT-2 cells, and the proportion of 210 wild-type (TTG) and 210 mutant (TGG) at each passage was determined by automated DNA sequence analysis of PCR products obtained from infected cell DNA, using the program Sequence Navigator (19).

Relative fitness of the mutant and wild-type viruses was calculated by the simplest possible model, assuming a constant selection against the less fit variant at each replication cycle such that $p(n)/q(n) = [p(n-1)/q(n-1)] \cdot (1-s)$ where p is the proportion of the less fit variant, q is the proportion of the more fit variant, n is the number of replication cycles, and s is the coefficient of selection. Relative viral fitness is therefore $1-s$. Each viral passage was approximately 6 days (except for the first passage in the presence of 5 μM AZT, which took 12 days), and we assumed two rounds of viral replication during this period. Under these conditions, spontaneous mutation would not be expected to contribute significantly to the observations. The accuracy of the approximations used here affects only the absolute magnitude of the selective advantage calculated, not the direction or trends of the advantage.

RESULTS

We first assessed the prevalence of W-210 in patients who had never been treated with AZT or who had received AZT for only a short time. The nucleic acid sequence of a fragment of the HIV-1 RT (amplified by RT-PCR from serum) from patients enrolled in two clinical trials of previously AZT-naive patients (trial BW 34,225-02 [21] and trial NUCB3001 [10]) was determined both before and after 6 months of AZT monotherapy. W-210 was not detected in virus from any patient examined at study entry ($n = 71$) or following 6 months of AZT monotherapy ($n = 31$). Thus, W-210 appears rarely if at all in untreated or moderately AZT treated populations. The prevalence of W-210 was also examined in patients who had previously undergone prolonged therapy with AZT. Entry into clinical trial NUCB3002 (23), which compared AZT monotherapy with AZT-3TC combination therapy, required that patients have had more than 2 years of previous AZT therapy (median, 680 days). At study entry, W-210 was detected in 13 of 110 patients (12%) examined. After 6 months of further AZT therapy, either alone or in combination with 3TC, the incidence of W-210 increased to 8 of 35 or 13 of 70 patients (23 or 19%), respectively.

Patterns of AZT resistance mutations (codons 41, 67, 70, 215, and 219) in those samples (from AZT-pretreated patients) in which W-210 was detected are indicated in Table 1. W-210 generally occurred in HIV genotypes which also had the two mutations L-41 and Y-215, both at study entry (11 of 13 cases [85%]) and after 6 months of additional AZT treatment (20 of 21 cases [95%]).

The effect of W-210 on AZT resistance in viral backgrounds which contain both the L-41 and Y-215 mutations was therefore examined by using an RVA (13). Briefly, HXB2 clinical isolate recombinants were produced by electroporating an RT-deleted HXB2 clone (13) with PCR products derived from the HIV-1 RT of clinical samples, and then the AZT 50% infective dose (IC_{50}) was determined. The AZT sensitivities of a series of these recombinant viruses, with and without W-210, are indicated in Table 2. The median AZT IC_{50} was approximately twofold higher for those viruses which included W-210 (1.66 μM versus 0.79 μM; $P < 0.05$ by the Wilcoxon ranks sum test).

As may be expected, there was some variation in IC_{50} observed in the RVAs derived from clinical samples, though this variation was not extremely large (coefficient of variation of 56% [range, 0.24 to 1.95] for viruses with 41 and 215 mutations; coefficient of variation of 43% [range, 0.75 to 3.4] for viruses with 41, 215, and 210 mutations), given the inherent imprecision of any plaque reduction assay, the presence of other mutations in HIV RT which could directly or indirectly affect the AZT IC_{50} (see reference 14), and the presence of mixtures of genotypes with different linkage patterns in these clinical samples.

The effect of W-210 on AZT sensitivity in a series of well-defined viral backgrounds, using the HeLa CD4 plaque reduction assay, was also determined (Fig. 1). In all cases, addition of W-210 by site-directed mutagenesis resulted in a small increase in the AZT IC_{50} (generally less than twofold). For

TABLE 2. Slightly higher median AZT IC_{50} in association with W-210

AZT IC_{50} (μM)[a]	
L-41/Y-215	L-41/Y-215 + W-210
1.35	1.27
0.39	3.4
0.86	1.21
1.27	1.7
0.24	2.35
1.95	0.99
0.83	2.12
0.76	2.07
0.61	1.3
0.70	1.66
	0.75

[a] Determined from RVA of clinical isolates with L-41 and Y-215 with and without W-210. Median IC_{50} values were 0.79 μM for RTMN and 1.66 μM for RTMN/210.

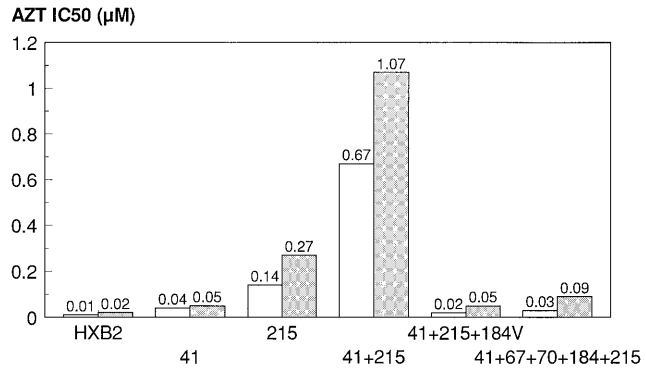

FIG. 1. Site-directed mutagenesis experiments confirm a small decrease in AZT susceptibility caused by W-210. Viruses with the indicated mutations (with and without the W-210 change) were prepared by site-directed mutagenesis in an HXB2 viral background as indicated in Materials and Methods. Viruses which include W-210 are indicated by filled bars.

example, the AZT IC_{50} for virus with 41 and 215 mutations increased from 0.67 to 1.07 μM upon the introduction of W-210. Of interest, the suppression of AZT phenotypic resistance induced by V-184 (1, 18, 24) was not inhibited by W-210, as demonstrated by the low IC_{50} values obtained for viruses containing V-184 even in the presence of the 41 and 215 mutations.

As noted earlier, W-210 occurred relatively rarely in clinical isolates of HIV-1, including those treated with AZT. One hypothesis is that virus with W-210 has a replicative disadvantage relative to virus having a wild-type 210 residue. To quantitate the relative effects of W-210 on viral replication in the presence of the 41 and 215 mutations, RTMN-based viruses (with or without W-210) were mixed at three different initial ratios and grown in MT-2 cells both in the absence of drug and in the presence of 5 μM AZT. This drug concentration is 5- to 7.5-fold higher than the AZT IC_{50} for these viruses. After each passage, population-based sequencing (19) of the viral population was performed to determine directly the relative ratios of viruses with the wild-type and mutant sequences. Three sample sequencing chromatograms are shown in Fig. 2, indicating the gradual replacement of the mainly mutant W-210 population with wild-type L-210 virus. No other sequence changes were observed during the passage series until after passage 7, when a change at codon 209 (CTG to TTG) could be detected in two series in the presence of AZT and one in the absence of AZT. This nucleotide change does not confer an amino acid change and did not appear to be associated with W-210 in vivo (data not shown). Although these experiments were initiated with clonal material, viral mutation is bound to occur during the passage series, and so these experiments do not rule out the possibility of other compensatory changes at one or more other codons in other regions of HIV-1. Regardless, the disappearance of the TGG sequence represents the net reduced fitness of virus(es) based upon RTMN containing W-210 compared with the 210 wild type.

In the absence of AZT, the wild-type L-210 virus rapidly outgrew the virus with W-210, regardless of the initial input ratio of mutant to wild type (Fig. 3). For example, a viral population which was originally nearly an equal mixture of mutant and wild type at codon 210 became nearly 100% wild-type L-210 after eight passages as determined by proviral DNA sequencing. Importantly, the rate of change of all of the curves can be fitted reasonably by using a single parameter, the coefficient of selection, and the assumptions described in Materials and Methods. In this case, the coefficient of selection is about 0.2, indicating that for every 100 viable progeny viruses produced by the wild type, about 80 viable progeny would be expected to be produced by the 210 mutant virus.

In the presence of 5 μM AZT, the W-210 virus is also outgrown by virus with L-210 (Fig. 3B) at all three initial input ratios of virus. The outgrowth of the wild-type virus occurs more slowly than in the absence of drug, as one might expect since W-210 confers a degree of AZT resistance. In the presence of 5 μM AZT, a coefficient of selection of 0.09 allows a reasonable fit to the observed data regardless of the initial ratio of W-210 to L-210, at least for about six passages.

DISCUSSION

The data presented here indicate that W-210 appears to be a relatively rare polymorphism in clinical isolates both before and after 6 months of AZT therapy. These results suggest that W-210 is likely to be an unfavorable mutation compared with the L-210 wild type. The prevalence of W-210 reported here (approximately 20%) in patients who received more than 2 years of AZT therapy is comparable to those reported in trial ACTG 143 after a similar time on AZT therapy (22).

W-210 occurs in vivo mainly in association with mutations at RT codons 41 and 215. Similar results were reported in a group of seven patients having W-210 (8). Both site-directed mutagenesis and RVA experiments indicate that W-210 confers approximately twofold-greater AZT resistance than the Y-41/L-215 combination alone. It is also clear that, by itself, W-210 confers only a negligible degree of AZT resistance. A similar lack of effect of W-210 alone was noted by Fitzgibbon et al. (5). In our hands, the combination of mutations of Y-215 and W-210 did not result in virus with high-level AZT resistance. This is in contrast to the results of Hooker et al. (8), who reported that virus with the W-210/Y-215 combination was more AZT resistant than virus with the L-41/Y-215 combination. The reason for this difference is not clear but may reflect

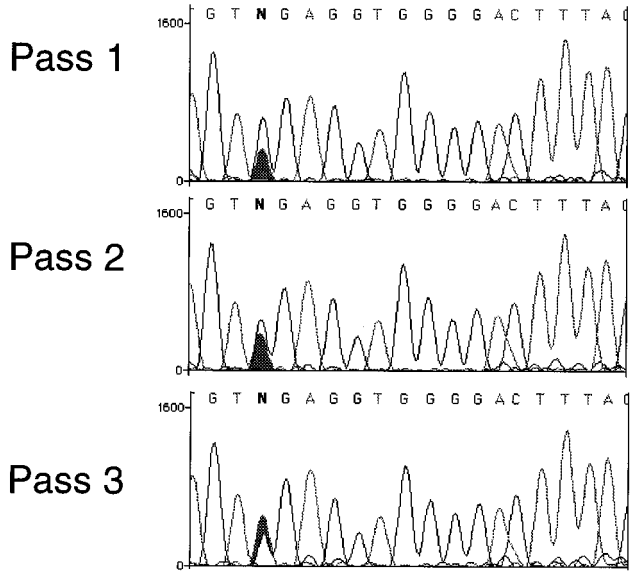

FIG. 2. Sample sequencing chromatograms indicate the outgrowth of wild-type virus. ABI sequence chromatograms indicate the outgrowth of the wild-type L-210 virus over the W-210 virus during the first three viral passages in the absence of AZT, starting from an initial ratio of 25% wild type/75% W-210. The TTG-to-TGG change is indicated by relative increase in the size of the filled peak.

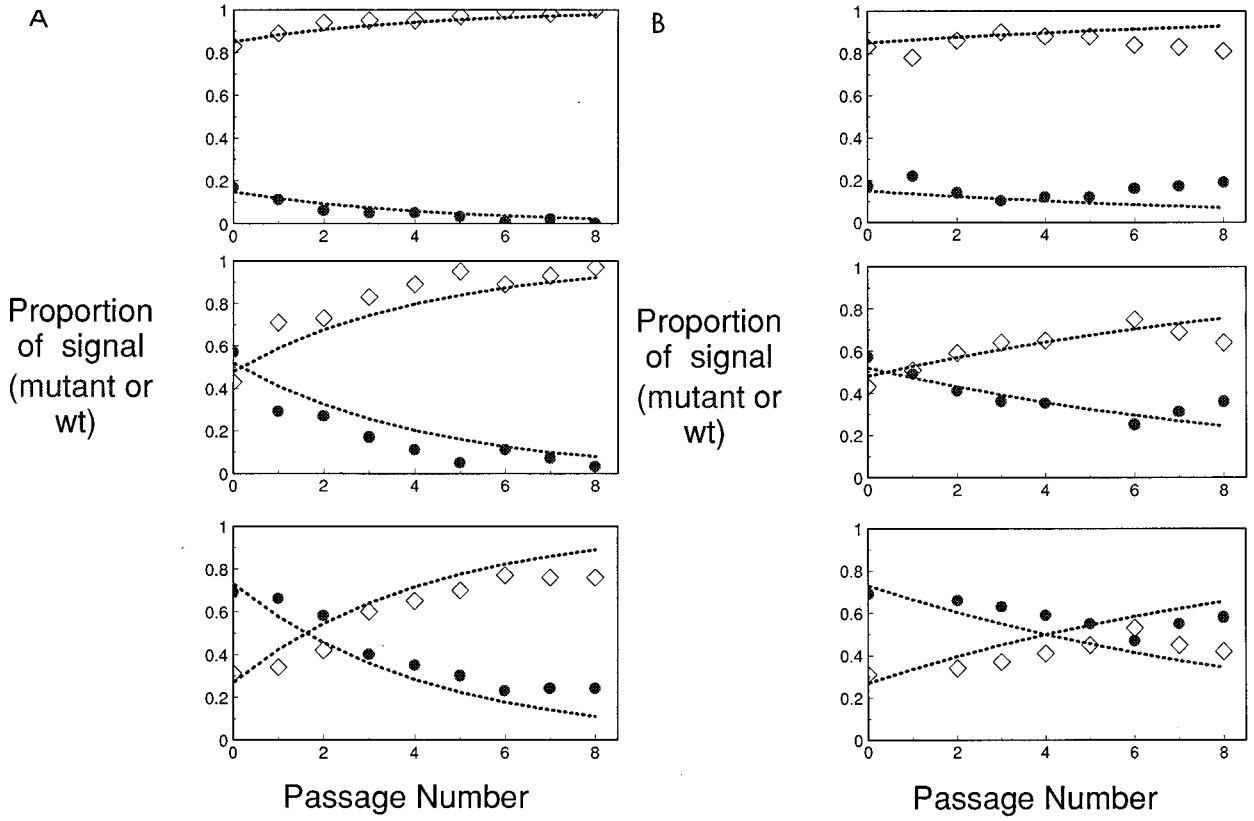

FIG. 3. Determination of relative viral fitness of virus with W-210 in the presence and absence of AZT. (A) Outgrowth of HIV HXB2 mutant RTMN with W-210 by a similar RTMN virus with L-210 (wild type [wt]) at initial W-210/L-210 ratios of 25:75, 50:50, and 75:25. The fraction of the peak chromatogram signal due to L-210 is indicated by diamonds; that due to W-210 is indicated by circles. Observations are indicated by datum points; dotted lines represent fits to the data obtained by using a selection coefficient of 0.21 in favor of the wild type at position 210 and two replication cycles per viral passage. (B) As for panel A except that viruses were cultured in the presence of 5 μM AZT. Shown are fits to the data obtained by using a selection coefficient of 0.09 (in favor of the wild type at position 210) and two replication cycles per viral passage.

differences in assay conditions. The fact that the W-210 change is generally not found with Y-215 alone and that the mutations L-41 and Y-215 precede the change to W-210 (8) are consistent with the W-210/Y-215 virus having only a modest degree of AZT resistance compared with virus with the L-41/Y-215 or L-41/W-210/Y-215 combination. Suppression of phenotypic AZT resistance by V-184 was not inhibited by W-210 (in a limited number of viral backgrounds), suggesting that any contributions of W-210 to drug resistance may not predominate in the context of mutational interactions induced by other antiretroviral agents (1, 15, 24).

The observation that virus containing the 41/215 and W-210 mutations is outgrown in vitro when in direct competition with RTMN virus wild type at codon 210 is intriguing for a number of reasons. The system described provides a rapid, internally controlled method for the direct detection and quantitation of the relative fitness of a given viral mutant, both in the presence and in the absence of a selective drug pressure. The relative replication disadvantage would also partly explain the relative rarity of W-210 in vivo. As noted by Coffin (3), even small disadvantages in viral fitness over the hundreds of HIV replication cycles expected in vivo can be sufficient to account for large differences in the relative frequencies of given viral variants.

In conclusion, the substitution of tryptophan at RT position 210 is quite rare in AZT-naive patients but occurs more commonly in those patients who have been treated with AZT for long periods. In vivo, the appearance of W-210 appears to be associated with L-41 and Y-215. In an HXB2 viral background, W-210 alone does not confer significant AZT resistance. However, in the presence of L-41 and Y-215 (in addition to other backgrounds), W-210 can increase levels of AZT resistance by approximately twofold, as determined both by site-directed mutagenesis and recombinant virus studies. W-210 is rapidly selected against in vitro in growth competition experiments in the absence and (less rapidly) in the presence of AZT. This selective disadvantage may account for the relative rarity of W-210 in vivo.

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