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Retroviral Recombination Can Lead to Linkage of Reverse Transcriptase Mutations That Confer Increased Zidovudine Resistance

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Genetic recombination between viral genomes has been shown to contribute to the generation of genetic diversity during retrovirus infections. The role of recombination in the development of human immunodeficiency virus type 1 (HIV-1) zidovudine resistance was investigated as a possible cause of the formation of the linked Leu-41/Tyr-215 resistance genotype. Zidovudine resistance is conferred by the presence of subsets of four or five amino acid substitutions in the HIV-1 reverse transcriptase. Zidovudine therapy of asymptomatic HIV-1-infected individuals results in the selection of drug-resistant variants that possess defined combinations of the five zidovudine resistance mutations. The linked Leu-41/Tyr-215 resistance genotype appears central to the continued development of high-level zidovudine resistance. By using genetically tagged mutant viruses, it was possible readily to select recombinant viruses from mixed infections of Leu-41 and Tyr-215 single mutants in the presence of zidovudine drug pressure. After three passages of a mixed infection in the presence of drug, 38% of clones screened were recombinant double mutants. In the absence of zidovudine selection, little change in the mixed virus populations was noted. No evidence of de novo generation of mutations at codons 41 and 215 was seen during any in vitro passage. This provides the first example of the role of retroviral recombination in the development of HIV-1 variants with increased drug resistance.

Zidovudine treatment of individuals infected with human immunodeficiency virus type 1 (HIV-1) results in the selection of HIV-1 isolates with reduced zidovudine sensitivity in vitro (3, 29). Reduction in zidovudine sensitivity is mediated by the acquisition of five specific amino acid substitutions in the reverse transcriptase (RT) of HIV-1 (Met-41→Leu, Asp-67→Asn, Lys-70→Arg, Thr-215→Tyr or Phe, and Lys-219→Gln) (15, 19). The order of appearance of these substitutions responsible for zidovudine resistance has been studied with an asymptomatic patient population (4, 14). Initially, HIV-1 variants with single mutations at codon 41, 70, or 215 were identified in isolates derived from patient peripheral blood lymphocytes. With continued zidovudine therapy, the zidovudine resistance genotype associated with linked mutations at codons 41 and 215 was seen and became the dominant resistance genotype (4, 14). Eventually, additional mutations at codons 67, 70, and 219 may occur in treated individuals, resulting in virus isolates with high-level phenotypic resistance to zidovudine. However, the mutational events that lead to the accumulation of specific zidovudine resistance mutations in HIV-1 RT during the course of the virus life cycle have been poorly addressed.

Genomic heterogeneity is a hallmark of HIV-1 infection (10, 26). Studies of sequential HIV-1 isolates from infected individuals suggest that HIV-1 evolves at a rate approximately one million times greater than that for eukaryotic genomes (10). These large differences in evolution rates for RNA and DNA genomes have been attributed to the varying accuracy of the replication machinery and the high level of homologous recombination that occurs during the retrovirus life cycle. HIV-1 RT lacks a 3′→5′ exonuclease proofreading activity (27) and is highly error prone during DNA-dependent DNA synthesis in vitro (27). Mutations generated by HIV-1 RT consist of base substitutions, additions, and deletions. The error rate of HIV-1 RT has been estimated to be 1/2,000 to 1/4,000 per nucleotide polymerized in vitro, which would represent 5 to 10 errors per HIV-1 genome per round of replication in vivo (26). It is highly likely that the error-prone nature of HIV-1 RT leads to the production of mutations that confer drug resistance phenotypes.

In addition to polymerase errors, frequent recombination events are thought to distribute mutations in the virus population, increase the diversity of the population, and remove lethal mutations. Genetic recombination between retrovirus genomes was first formally demonstrated for RNA tumor viruses (13, 34). Retroviral genetic recombination occurs at high frequency, involving multiple crossover events, resulting in markers as close as 1 kb segregating independently (7). Studies have shown that it is possible for retroviruses to package two distinct RNA genomes, thus resulting in heterozygote viruses. The formation of heterozygote viruses and subsequent virus replication were shown to be required for efficient retroviral recombination (12, 31, 35, 36). More recently, genetic recombination has been proposed to occur during HIV-1 infection (8, 32, 33).

DNA-sequencing studies have provided limited evidence that genetic exchange between zidovudine-resistant strains of HIV-1 may lead to new linkage relationships between zidovudine resistance mutations, particularly between mutations at RT codons 41 and 215 (14). Here we report that retroviral recombination can lead to the production of zidovudine-resistant variants with linked mutations at codons 41 and 215 in vitro, starting from a mixed infection of single-mutant viruses. Under zidovudine selective pressure, the recombinant double-mutant virus was able to outgrow the single-mutant viruses in cell culture.

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MATERIALS AND METHODS

Cells and viruses. The human T-lymphoblastoid cell line C8166 (30) was used to propagate HIV-1 and for electroporation experiments. The T-cell line MT-2 (11) was used for in vitro drug selection experiments. Both cell lines were routinely maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum plus antibiotics (RPMI10). HT4LacZ-1 cells (28) were used to determine the sensitivity of HIV-1 to zidovudine. This cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum plus antibiotics. To produce site-directed mutant virus stocks, C8166 cells were cotransfected with a mixture of the RT-deleted proviral clone pHIVΔRTBstEII and a functional mutant RT coding sequence derived from M13 clones as described elsewhere (15, 16). Cell-free virus supernatants were harvested 12 to 14 days posttransfection and stored at −70°C.

Cell culture of HIV-1 in the presence of zidovudine. MT-2 cells (2×10^6) were infected with a total of 10^4 PFU, based on titration on HT4LacZ-1 cell monolayers, of HIV-1 mutant virus stocks. Virus was allowed to adsorb at 37°C for 1 h. After adsorption, infected cells were added to 20 ml of RPMI10 either with or without the appropriate concentration of zidovudine. Fresh medium and drug were added as required, and at peak cytopathic effect, cell-free virus supernatants were harvested and stored at −70°C. Infected-cell pellets were also stored at −70°C.

Zidovudine sensitivity assay. Plaque reduction assays were performed by infection of HT4LacZ-1 cell monolayers as described elsewhere (6, 20), except that for virus stocks grown in the presence of zidovudine, the inoculating supernatant was removed after 1 h and replaced with 1 ml of fresh medium in order to minimize the effect of carryover drug in the inoculating supernatant. The 50% inhibitory concentrations (IC_{50}s) were derived from plots of percent inhibition against zidovudine concentration.

Construction of mutant viruses. Mutants containing zidovudine resistance mutations at codon 41 or 215 plus additional specific point mutations were created by site-directed mutagenesis of the M13 *pol* gene containing clone mpRTI/H (19) and called 41[S] and 215[S], respectively. Mutations were verified by nucleotide sequence analysis with a Sequenase sequencing kit (United States Biochemicals). M13 replicative-form DNA was prepared, and the mutant RT coding regions were transferred into the HXB2-D genetic background by homologous recombination as described above.

Genetic analysis of infected-cell DNA. Specific oligonucleotide probes were used to identify cloned RT coding sequences containing mutations at either codon 41 or 215 plus the additional specific point mutations. RT coding sequence was amplified by PCR from approximately 500 ng of HIV-1-infected cell DNA by using the PCR primers 5.1 and 7b as previously described (19). PCR products were purified by agarose gel electrophoresis and cloned via *Eco*RI and *Xba*I restriction enzyme sites present in the PCR primers into the plasmid vector pSP72 (Promega). *Escherichia coli* (strain TG-1) cells were transformed, and individual colonies were inoculated onto Hybond-N nylon membrane grids (Amersham) and incubated until colonies were visible. Bacteria were lysed and plasmid DNA was UV cross-linked onto the nylon membranes according to the manufacturer's instructions. Membranes were prehybridized in a 20-ml solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 0.1% BLOTTO, and 0.025 M sodium phosphate at 42°C for at least 6 h. The membranes were then hybridized to ^{32}P-labelled specific oligonucleotide probes in the same solution at 42°C overnight. The probe 41[P], 5′ TTC CAA TTC TGT ACA TTA CTC CAT 3′, was used to identify clones containing 41[S]-specific RT, and the probe 215[P], 5′ TAA TAC CCC CAT TAT AAC AGA TG 3′, was used to identify 215[S]-specific RT. Membranes were first hybridized with the 215[P] probe. Nonspecific hybridization was removed by washing in 5× SSC–0.1% SDS at 43°C for 3 min twice, followed by a final 2-min wash. After autoradiography, the 215[P] probe was removed and the membranes were rehybridized with the 41[P] probe. Nonspecific hybridization was removed by washing, and the membrane was autoradiographed.

RESULTS

To demonstrate genetic recombination between mutants carrying the zidovudine resistance mutations at codons 41 and 215, genetically tagged single-mutant viruses were constructed and passaged together in escalating concentrations of zidovudine. Changes in phenotypic drug resistance were assessed by using the HT4LacZ-1 plaque reduction assay. In addition, true recombinant viruses could easily be identified by the presence of both linked resistance mutations and specific additional genetic markers in the virus RT, thus removing the possibility of the de novo appearance of mutations.

Construction of genetically tagged mutant viruses. We wished to construct two mutant viruses that contained the desired zidovudine resistance mutation at RT codon 41 or 215 in addition to "fingerprint" mutations. Mutant virus 41[S] was constructed by site-directed mutagenesis of the M13 *pol* gene-containing clone mpRTI/H (19) to contain the zidovudine resistance mutation Met-41→Leu and additional silent mutations at codons 36 (A→G), 37 (T→A), and 40 (G→A) (Fig. 1a). The silent mutations were based on RT sequence from the HIV-1 variant SF-2, whose sequence has been published. A second mutant virus, 215[S], was also constructed by mutagenesis of mpRTI/H. 215[S] contained the zidovudine resistance mutation Thr-215→Tyr and additional mutations at codons 210 (G→A), 211 (AGG Lys→AAA Arg), and 213 (A→G) (Fig. 1b). These mutations were based on DNA sequence obtained from a zidovudine-sensitive clinical isolate, P026A (19). Viruses were recovered by recombination with the RT-deleted proviral clone pHIVΔRTBstEII. The in vitro zidovudine sensitivity of the viruses 41[S] and 215[S] was compared with that of the site-directed mutant viruses HIVRTML (Leu-41) (15) and HIVRTMF (Tyr-215) (21) to determine if the additional mutations introduced into each virus affected zidovudine resistance. HT4LacZ-1 plaque reduction assays showed that the 41[S] and 215[S] genetically tagged viruses exhibited a sensitivity to zidovudine (IC_{50}, 0.08 and 0.13 μM, respectively) similar to that of the mutant viruses HIVRTML (0.05 μM) and HIVRTMF (0.16 μM).

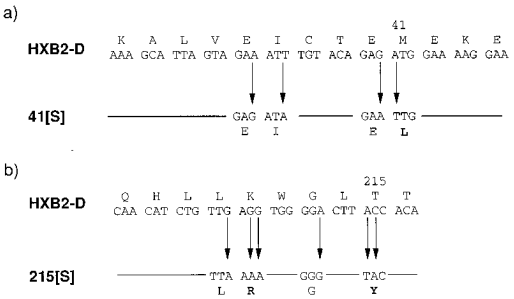

FIG. 1. Mutations introduced into HXB2-D RT to create the viruses 41[S] (a) and 215[S] (b). The amino acid changes conferred by nucleotide substitutions are shown in boldface.

Genetic analysis of 41[S] and 215[S] viruses after in vitro passage. Infected-cell DNA was extracted from each selection culture for genetic analysis of virus populations. However, the methods used for the detection of recombinant viruses required a number of steps that could lead to artifactual recombination. PCR amplification of RT coding region from infected-cell DNA was likely to result in PCR-induced recombination events (24). Also, during cloning of PCR products, the production of concatemers could also lead to apparent background recombination. To assess the level of background recombination, M13 replicative-form DNA from the RT clones 41[S] and 215[S] was mixed in concentrations ranging from 0.1 to 100 ng, PCR amplified, cloned, and analyzed for recombinant RTs by using specific oligonucleotide probes. Analysis showed that as the amount of initial input DNA was increased, so the amount of background recombination also increased (Fig. 2). At 0.1 ng of input DNA, the level of background recombination was 5.5% (14/255). This increased to between 17% (35/206) and 16% (24/146) when the amounts of input DNA were 10 and 100 ng, respectively. Analysis of 15 representative clones showed that the RT genotype, as determined by specific oligonucleotide probes, accurately reflected the genotype as determined by DNA sequence analysis.

To determine the level of background recombination resulting from the analysis of recombinant virus populations, it was therefore necessary to quantify the amount of HIV-1-specific

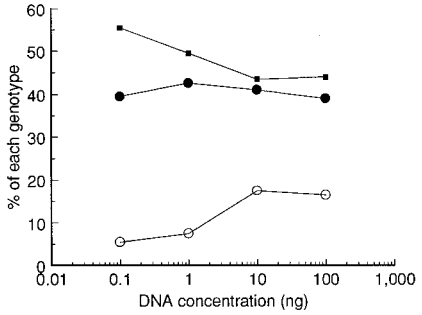

FIG. 2. Genotypic analysis of cloned RT coding sequences amplified by PCR from defined mixtures of 41[S]- and 215[S]-specific mutant DNA. The percentage of each genotype is shown. ●, 41[S]; ■, 215[S]; ○, 41[S]/215[S] recombinants.

DNA present in each infected-cell pellet. Duplicates of infected-cell DNA from each virus passage were dot blotted along with known quantities of an infectious HIV-1 molecular clone and probed with an HIV-1 RT-specific DNA probe. Densitometry analysis enabled the quantitation of each DNA sample with respect to the HIV-1 standards (Table 1). From these data, the background recombination frequency was determined for each sample, and values ranged from 3.7 to 10.6% (Table 1). Virus populations discussed below were corrected by using these background recombination values.

The ability of retroviral recombination events to create new genetic linkage relationships was investigated by serial passage of equal mixtures, based on HT4LacZ-1 cell titer, of the viruses 41[S] and 215[S] in escalating concentrations of zidovudine, or a single passage in the absence of zidovudine followed by continued passage in the presence of zidovudine. The second selection strategy was used in an attempt to allow the formation of significant numbers of heterozygote viruses, which are the proposed progenitors of true genetic recombinants (12, 31, 35, 36). Titers of virus resulting from each passage were determined by using HT4LacZ-1 cells, and 10^4 PFU of virus was used to infect cells for the next passage. The viruses 41[S] and 215[S] were also passaged separately as controls for de novo mutation selection. RT genotype analysis of mixed 41[S] and 215[S] virus infections cultured in the absence of zidovudine selection showed that the populations of 41[S] virus and 215[S] virus remained relatively constant during three passages (Fig. 3a). At passage 1, the 41[S] genotype and 215[S] genotype represented 41% (49/119) and 45% (54/119) of the total population, respectively. By passage 3, these genotypes represented 51% (88/172) and 39% (67/172) of the population, respectively. Interestingly, throughout the selection a small population of apparently recombinant virus was also present. The average proportion of recombinant virus detected during the selection was 6.4% ± 1.7% after correction for background recombination (Table 1).

TABLE 1. Quantitation of HIV-1-specific DNA and background recombination

Sample[a]	HIV-1 DNA concn (ng/μg of cell DNA)	Background recombination (%)
Passage 1, ND	0.13	5.9
Passage 2, ND	0.06	5.1
Passage 3, ND	0.45	7.3
Passage 2, ND-A	0.04	4.8
Passage 3, ND-A	3.4	10.6
Passage 1, A	2.4	9.9
Passage 2, A	0.01	3.7
Passage 3, A	1.3	8.9

[a] ND, no drug; ND-A, no drug and then zidovudine selection; A, zidovudine selection.

Selection of the mixed 41[S] and 215[S] virus infection in the absence of drug, followed by selection in escalating concentrations of zidovudine, resulted in gross changes in the proportion of each virus genotype (Fig. 3b). The concentrations of zidovudine used during the in vitro selections were 0.3 μM for passage 2 and 0.6 μM for passage 3. Population analysis revealed that the 215[S] virus pool remained constant during the selection, with an average of 46% ± 1.8% of viruses of this genotype present during the three passages. However, the proportion of the 41[S] virus population decreased from 41% (49/119) at passage 1 to 23% (41/176) by passage 3. This was mirrored by a commensurate increase in the proportion of recombinant virus present during the selection, namely, an increase from 7.5% (16/119) at passage 1 to 24% (24/176) by passage 3 after correction for background recombination.

Selection of the mixed 41[S] and 215[S] virus population in escalating concentrations of zidovudine resulted in similar but more dramatic changes in the proportion of each virus population in comparison with the previous selection (Fig. 3c). The concentrations of zidovudine used during in vitro selection were 0.2 μM for passage 1, 0.4 μM for passage 2, and 1.0 μM for passage 3. As before, the 41[S] virus population gradually declined during zidovudine selection from 30% (58/192) at passage 1 to 20% (38/191) by passage 3. However, under continuous zidovudine selection the 215[S] virus population also declined from 64% (122/192) at passage 1 to 34% (65/191) by passage 3. At the same time as the decreases in the single-mutant populations occurred, there was a rapid increase in the proportion of 41[S]/215[S] recombinant viruses. At passage 1, no recombinants were detected above background. By passage 3, 38% (88/191) of clones screened appeared to be recombinants after correction for background recombination. In both selection experiments, the outgrowth of the linked 41[S]/215[S] recombinant genotype did not result in the complete turnover of single-mutant virus populations. It is likely that the levels of zidovudine used in the passage experiments were insufficient to completely inhibit replication of the 41[S] or 215[S] single-mutant viruses. This is supported by the presence of significant populations of viruses with each of these mutations, even after selection in 1.0 μM zidovudine.

Another possible route to the formation of Leu-41/Tyr 215 double-mutant viruses was the de novo generation of mutations as a result of RT errors. Analysis of infected-cell DNA from the 41[S] virus passage series and the 215[S] virus passage series was performed by selective PCR (15, 21) to determine the genotype for the remaining zidovudine resistance mutation codons. PCR analysis of the 41[S] virus passage series, under all three selection conditions, confirmed that only mutations corresponding to the 41[S] genotype were present, with all other codons remaining wild type, including codon 215 (data not shown). Similarly, analysis of the 215[S] virus passage series under all three selection conditions showed that only zidovudine resistance mutations at codon 215 were present during selection. Infected-cell DNA from the mixed 41[S] and 215[S] virus infections showed evidence of mixed wild-type and mutant sequences at codons 41 and 215 as expected, with all other codons remaining wild type (data not shown). Overall, this suggests that de novo generation of mutations did not occur at a detectable level under the selection conditions used,

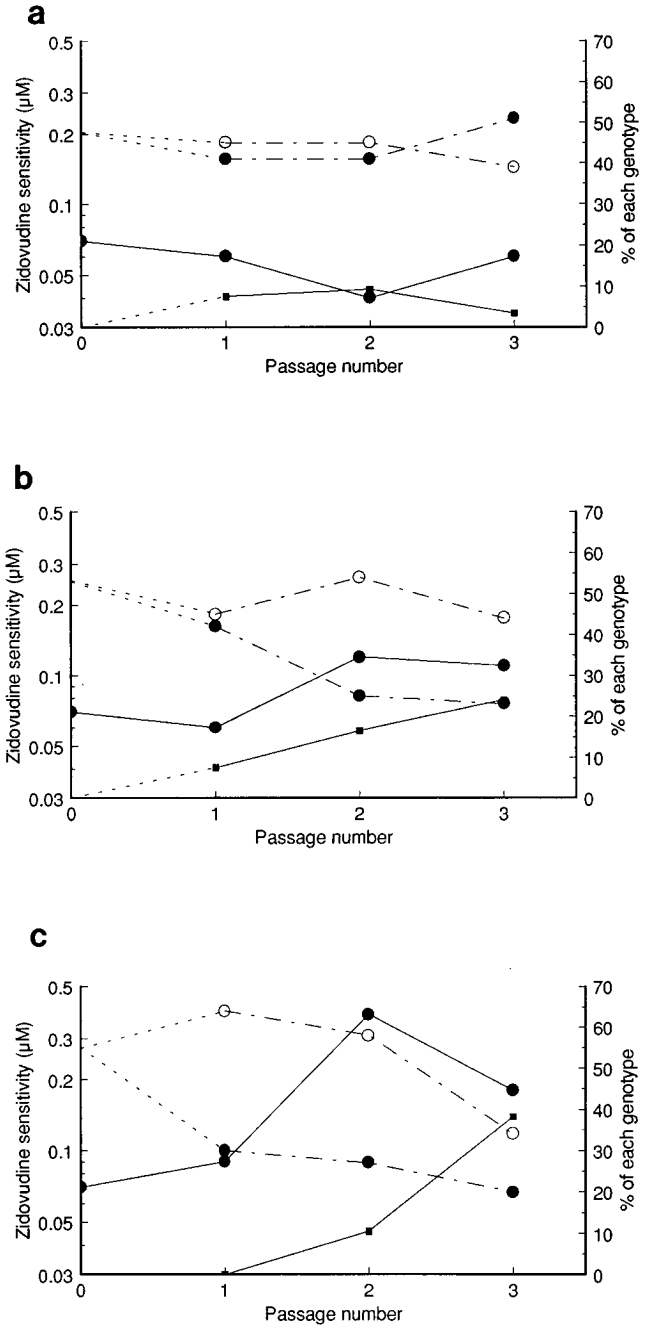

FIG. 3. Genetic analysis of 41[S] (●, broken lines) and 215[S] (○, broken lines) virus populations compared with the amount of recombinant 41[S]/215[S] virus (■), detected during drug-free passage (a), drug-free passage followed by zidovudine passage (b), and continuous zidovudine passage (c) of initially equal mixtures of the viruses 41[S] and 215[S]. The percentages of recombinant virus were corrected for the estimated background recombination frequency. The value for the zidovudine resistance phenotype (zidovudine IC_{50}) (●, solid lines) is shown for each virus passage.

supporting recombination as the cause of the observed changes in virus genotype and phenotype.

Increased zidovudine resistance during in vitro drug selection. Passage of the 41[S] virus either without zidovudine or first without drug and then with escalating concentrations of zidovudine, or serial passage in zidovudine, did not result in a consistent increase in zidovudine resistance over three passages (Fig. 4a). The mean drug sensitivities over three passages were 0.043 μM for virus passaged in the absence of zidovudine, 0.047 μM for virus passaged without drug and then with zidovudine, and 0.06 μM for virus passaged in zidovudine. Virus 215[S] was also passaged by using the same three strategies. Again, no consistent increase in zidovudine resistance was observed over three passages (Fig. 3b). The mean drug sensitivities over the three passages were 0.11 μM for virus passaged in the absence of zidovudine, 0.12 μM for virus passaged without drug and then with zidovudine, and 0.14 μM for virus passaged in zidovudine. In contrast, in vitro passage of mixed 41[S] and 215[S] infections in the presence of zidovudine resulted in an increase in zidovudine resistance in comparison with the drug-free passaged control culture (Fig. 4c). During three passages, the mean sensitivity to zidovudine of the virus passaged without drug was 0.053 μM. This corresponded to a predominantly stable mixed 41[S] and 215[S] virus population with an average of 6.4% ± 1.7% recombinant viruses. For the culture passaged first without drug and then with zidovudine, the level of zidovudine resistance increased from 0.06 μM at passage 1 to 0.11 μM on passage 3. Similarly, for the zidovudine-passaged culture, the level of zidovudine resistance doubled from 0.09 μM at passage 1 to 0.18 μM by passage 3.

The high levels of recombinant virus present in mixed virus populations resulted in little more than a twofold increase in the zidovudine resistance phenotype. Sensitivity testing of defined mixtures of viruses with similar ratios of genotypes confirmed that the proportion of each genotype present during the in vitro drug selection accurately reflected the observed zidovudine sensitivity (Table 2). For example, the zidovudine IC_{50} for 50%/50% mixtures of HIVRTML (Leu-41) and HIVRTMF (Tyr-215) was 0.08 μM, very similar to that for the drug-free-passage virus stocks which had approximately equal mixtures of these genotypes. Comparable zidovudine sensitivities were also observed for more complex mixed genotypes. A 20%–60%–20% mixture of HIVRTML, HIVRTMF, and HIVRTMN, respectively, resulted in a zidovudine sensitivity (IC_{50}, 0.14 μM) similar to that of the 23% Leu-41–44% Tyr-215–24% Leu-41/Tyr-215 virus population present at passage three of the virus stock passaged first without drug and then with zidovudine (IC_{50}, 0.11 μM). In addition, defined mixtures containing high proportions of Leu-41/Tyr-215 virus did not result in dramatically elevated IC_{50}s (Table 2). This indicates that care should be taken in interpreting the drug sensitivity of mixed virus populations in the absence of genotypic information.

DISCUSSION

Previous studies have shown that the acquisition of the five mutations responsible for zidovudine resistance occurs in a defined order in asymptomatic HIV-1-infected individuals (4, 14). Central to the pattern of mutation acquisition is the selection and outgrowth of virus carrying the linked double mutation Leu-41/Tyr-215 (14). To date, a poorly addressed question in HIV-1 drug resistance is how multiple drug resistance genotypes occur during virus replication. Undoubtedly the high error rate of HIV-1 RT results in the appearance of viruses containing RT with single amino acid substitutions. However, in the case of zidovudine resistance and, more recently, dual and multiple drug resistance (22), the possibility of other mechanisms should be addressed. The phenomenon of genetic recombination between retrovirus genomes is well documented (1, 2, 12, 23). It is therefore likely that recombination

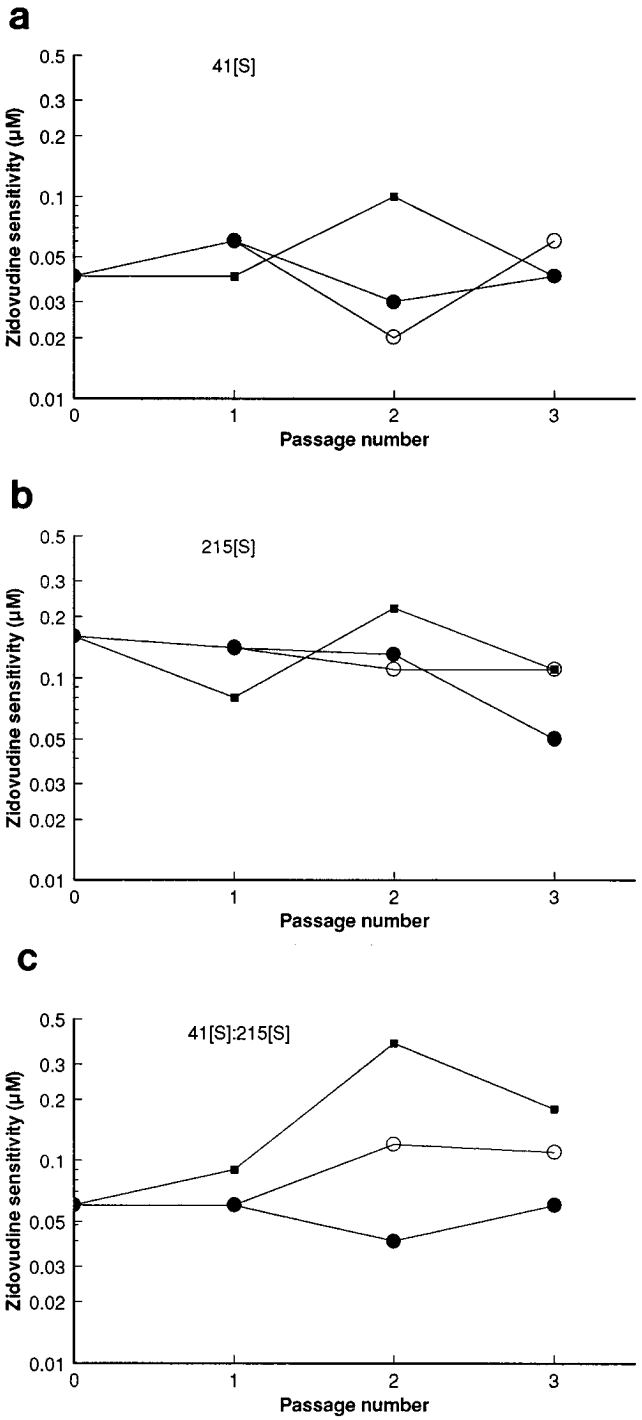

FIG. 4. In vitro zidovudine sensitivities determined for each passaged virus stock by using the HT4LacZ-1 plaque reduction assay. Viruses 41[S] and 215[S] were passaged either separately (panels a and b, respectively) or as an equal mixture of both 41[S] and 215[S] viruses (c). Three selection strategies were used during virus growth: drug-free selection (●), drug-free selection for passage 1 followed by addition of 0.3 μM zidovudine for passage 2 and 0.6 μM zidovudine for passage 3 (○), and selection in escalating concentrations of zidovudine, 0.2 μM (passage 1), 0.4 μM (passage 2), and 1.0 μM (passage 3) (■).

could also be a significant mechanism of generating HIV-1 genetic diversity and drug resistance genotypes.

By using genetically tagged Leu-41 and Tyr-215 mutant viruses, it was possible to demonstrate that recombination

TABLE 2. Zidovudine sensitivities of defined mixtures of viruses

Proportion of indicated virus (%)			Zidovudine sensitivity (IC_{50} [μM])
HIVRTML (Leu-41)	HIVRTMF (Tyr-215)	HIVRTMN (Leu-41/Tyr-215)	
100			0.06
	100		0.14
		100	0.63
50	50		0.08
	50	50	0.24
	75	25	0.19
	25	75	0.4
20	60	20	0.14
10	50	40	0.25

readily occurs between the two virus genomes in vitro, leading to linkage of Leu-41 and Tyr-215. Two recombinant selection strategies were used. One involved passage in escalating concentrations of zidovudine, whilst the other involved an initial drug-free passage followed by zidovudine selection. The latter protocol was designed to enrich for the formation of heterozygote HIV particles containing one genomic RNA molecule from a 41[S] virus and another from a 215[S] virus. The formation of heterozygote virus particles was previously shown to be a prerequisite for the formation of stable genetic recombinants (12, 31, 35, 36). However, this selection procedure actually resulted in slower selection of recombinant virus than did continuous drug passage. It is likely that the initial passage of the continuously drug-selected virus mixture in 0.2 μM zidovudine allowed sufficient virus replication to enable the formation of heterozygotes followed by stable recombinants. However, the two selection procedures differed in the level of zidovudine used, since in the procedure involving an initial drug-free passage followed by zidovudine selection, a maximum zidovudine concentration of 0.6 μM was used, compared with 1.0 μM for the continuous-zidovudine-selection passages. Genetic analysis showed that 1.0 μM zidovudine was able to suppress both the Leu-41 and Tyr-215 populations, whereas 0.6 μM zidovudine was only able to suppress the Leu-41 population. Therefore, it is likely that the higher zidovudine levels used in the continuous-drug-passage series resulted in a more efficient selective amplification of recombinant viruses. It was of interest that recombinant virus could be detected in mixed Leu-41-plus-Tyr-215 infections passaged in the absence of zidovudine, although in the absence of drug selection there was no significant change in the overall mutant virus populations. These data indicate that recombination, even between two loci separated by 522 nucleotides, is common during retrovirus infections in vitro but that the populations of different genotypes remain stable until a selection pressure is applied.

The potential significance of the role of recombination in the generation of resistant variants during drug therapy in vivo remains to be determined. There is some evidence that recombination may occur to a high level in zidovudine-treated patients with late-stage disease, resulting in unusual combinations of mutations, not predicted from the ordered appearance of mutations in asymptomatic patients (21). These could result from an increased level of HIV-1 replication and recombination in infected individuals with AIDS, or alternatively, different mutation acquisition patterns may exist in late-stage patients. It is also interesting to speculate that the syncytium-inducing (SI) phenotype may also be involved in inducing high rates of HIV-1 recombination. Recent studies have suggested that in individuals harboring SI variants, the linked Leu-41 and

Tyr-215 genotype may appear faster than in those harboring non-syncytium-inducing (NSI) variants (5). In addition, it has been suggested that zidovudine therapy might be less effective in prolonging the disease-free period in patients harboring SI virus than in those with NSI variants (17, 18). This suggests that three factors may be linked during zidovudine treatment: presence of SI isolates, the faster appearance of the combined Leu-41 and Tyr-215 amino acid changes, and rapid disease progression. It is possible that these effects are due to a higher recombination rate of SI variants. These variants may facilitate rapid genotypic mixing and heterozygote virus formation in tissues such as lymph node and spleen tissues, where the close proximity of virus-infected cells may result in cell fusion events.

Retroviral recombination should be considered in future clinical trials of combination therapies. Any therapy that allows significant HIV-1 replication to persist is likely to result in drug resistance. Recombination could result in the rapid genetic exchange of resistance mutations leading to the production and selection of multiple resistant variants (22). In addition, the protease and RT inhibitors currently being explored in combination therapy should be monitored for the development of drug resistance. The selection of protease inhibitor-resistant mutants in vitro suggests in vivo resistance is likely to occur (9, 25). Whether dual resistance to RT and protease inhibitors will occur during combination regimens is unknown, although this is likely. The role of retroviral recombination in the development of protease and RT inhibitor dual resistance could also be assessed by in vitro studies using genetically tagged mutant virus and DNA sequencing of patient isolates.

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