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Infectious potential of human immunodeficiency virus type 1 reverse transcriptase mutants with altered inhibitor sensitivity

(acquired immunodeficiency syndrome/site-directed mutagenesis/RNA-dependent DNA polymerase/replication/azidothymidine)

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ABSTRACT There is considerable interest in the potential of human immunodeficiency virus type 1 (HIV-1) to develop drug resistance, especially as 3′-azido-3′-deoxythymidine (Retrovir) is now in widespread clinical use to treat people with AIDS and AIDS-related complex (ARC). To address this possibility, mutations in the HIV reverse transcriptase [deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (RNA-directed), EC 2.7.7.49] gene have been introduced by site-directed mutagenesis of cloned constructs in *Escherichia coli*. Analysis of the recombinant mutant reverse transcriptase from a number of these constructs revealed enzymes that maintained enzyme activity but had a reduced ability to recognize inhibitors such as azidothymidine triphosphate. To assess the infectivity of these mutants, several constructs of proviral HIV clones with mutant reverse transcriptase genes have been made and used to transfect T cells. All five mutants tested have lower infectious potential, suggesting considerable levels of reverse transcriptase activity are required for efficient virus replication. Viable virus recovered from two clones showed decreased sensitivity to the antiviral compound phosphonoformate, thus demonstrating the potential for drug-resistant HIV to replicate. However, although the reverse transcriptase from these mutant viruses showed decreased sensitivity to azidothymidine triphosphate, paradoxically these viruses were hypersensitive to azidothymidine when tested in culture.

Reverse transcriptase [RT; deoxynucleoside-triphosphate: DNA deoxynucleotidyltranscriptase (RNA-directed), EC 2.7.7.49] plays a key role in the replication of the human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immune deficiency syndrome (AIDS) (refs. 1–3), by converting the virus RNA genome to a double-stranded DNA intermediate which integrates into host cell DNA (4). The clinical experience with 3′-azido-3′-thymidine (AZT, zidovudine, or Retrovir), whose triphosphate form effectively inhibits HIV RT activity (5), confirms RT as a viable chemotherapeutic target (6, 7). Efficient expression of the enzyme in *Escherichia coli* and the creation of mutant enzymes by site-directed mutagenesis is facilitating a detailed functional analysis of the enzyme (8, 9). We have made mutants with single amino acid changes in specific highly conserved regions of the enzyme and these mutants have allowed us to identify residues essential for enzyme function (9). Interestingly, a number of mutants show reduced RT activity and altered inhibitor binding. Although these mutant enzymes were active as determined by *in vitro* assay, it was not known if such mutations introduced into the HIV-1 genome would allow efficient replication of the virus. In addition, should mutant viruses of this nature replicate, it would be of considerable interest to assess susceptibility to antiviral compounds that were recognized less well by the mutant RT polypeptides.

To establish the infectious potential of virus harboring mutations in the RT gene, we have constructed proviral HIV clones with mutant RT genes and used these to transfect T cells. Results of these experiments indicate considerable levels of RT activity are required for efficient virus replication, as all five mutants examined have a lower infectious potential than wild type (wt). Indeed, the two examples of viable virus produced in this manner were from mutants that had the least reduction in overall RT activity but that had increased resistance to both AZT triphosphate (AZTTP) and phosphonoformate (PFA). Interestingly, the resulting mutant viruses both showed a dichotomy between AZT and PFA sensitivity in cell culture, remaining sensitive to AZT but gaining resistance to PFA. The significance of these results to the potential development of drug resistance of HIV *in vivo* are discussed.

MATERIALS AND METHODS

Construction of RT Mutants. Mutations in the HIV-1 RT gene were created by site-directed mutagenesis using synthetic oligonucleotides as described (9–11). The parental M13 clone mpRT4 was used to construct the "RTM" series of mutants. This clone contains only the 1.7-kilobase (kb) RT gene coding region and expresses an active 66-kDa RT polypeptide on infection of *E. coli* (8). The parental M13 clone mpRT1/H was used to construct the "RTM/H" series of mutants. This clone comprises a 2.55-kb *Bgl* II–*Eco*RI fragment from the HIV-1 *pol* gene (derived from clone HXB2-D) inserted into M13mp19. Substantial amounts of RT activity are induced on infection of susceptible *E. coli* strains with bacteriophage mpRT1/H.

Preparation and Assay of HIV RT. Recombinant HIV RT was prepared by infecting *E. coli* (strain 5KCPolA^{ts}F′) at high multiplicity with bacteriophage clones. After induction with isopropyl β-D-thiogalactopyranoside, cells were lysed and RT was obtained by high-salt fractionation as described (8). Virion-associated RT in cell culture supernatants was prepared by two methods. For small-scale preparations, virus from clarified culture supernatants was pelleted in 1-ml aliquots using a Beckman TL-100 ultracentrifuge (40,000 rpm, 10 min at 4°C) and solubilized in buffer containing 0.5% Triton X-100, 500 mM KCl, 50 mM Tris·HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, and 5 mM 2-mercaptoethanol. For larger scale preparation of virion-associated RT, virus was precipitated from clarified infected cell supernatants at 4°C with 10% (wt/vol) PEG/0.1 M NaCl. Virus was solubilized in the buffer described above.

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTTP, AZT triphosphate; CPE, cytopathic effect; HIV-1, human immunodeficiency virus type 1; PFA, phosphonoformate; RT, reverse transcriptase; wt, wild type.

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RT activity was assayed using poly(rA)·oligo(dT) as primer template and [^{3}H]TTP (5 μM and 10 μCi/ml; 1 Ci = 37 GBq) as described (8).

Construction of Mutant Proviral HIV Clones. Mutants created by site-directed mutagenesis in clone mpRT1/H were used to prepare replicative form DNA and the 1.9-kb *Bal* I fragment from each (which contained the RT mutation) was isolated and transferred into the infectious proviral HIV clone HXB2-D (12) previously digested with *Bal* I to remove the wt *Bal* I fragment. Mutant proviral clones were verified by digestion with various restriction enzymes, including *Bal* I, *Hin*dIII, and *Kpn* I. Clones pHIVRTM6, pHIVRTM16, and pHIVRTM18 were also digested with *Nsi* I to confirm loss of an *Nsi* I site and clone pHIVRTM2 shown to have gained an *Nco* I site due to the initial nucleotide substitutions introduced by mutagenesis of the RT gene. DNA manipulation and cloning procedures were by standard techniques (13).

Transfection of MT-4 Cells. Approximately 2 × 10^{6} MT-4 human lymphoid cells (14) were transfected with 10 μg of DNA from each proviral HIV clone by electroporation (15, 16) using a Bio-Rad Gene Pulser and maintained in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum plus antibiotics. Complete cytopathic effect (CPE) was observed with the wt clone HXB2-D around 6 days after transfection. At the time maximum CPE appeared, cultures were expanded with fresh cells and used to establish virus pools.

HIV Drug-Sensitivity Assays. Sensitivity of HIV isolates to AZT and PFA was assessed using a CPE inhibition assay and an assay based on reduction of plaque number. Inhibition of virus CPE was determined in MT-4 cells using a modified dye-uptake technique (17, 18). Cells were infected with equivalent amounts of culture supernatant virus, as assessed by terminal dilution in MT-4 cells (sufficient to cause complete CPE in control cultures in 5 days) and incubated with various inhibitor concentrations in 96-well microtiter plates (8 × 10^{4} cells in 100 μl of culture medium). Assays were developed after a 5-day incubation by adding 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) at 5 mg/ml and incubating for 2 hr at 37°C; dye was extracted by treating with 20 μl of 20% (vol/vol) Nonidet P-40 and 150 μl of acid isopropanol (0.04 M HCl in isopropanol). The color change due to reduction of MTT in viable cells was quantified by measuring absorbance at 570 nm, and ID_{50} values were derived from plots of cell viability versus inhibitor concentrations ($\log_{10}$). Inhibition of plaque formation (foci of multinucleated giant cells) was assessed using a HeLa cell line (HT4-6C) expressing the human CD4 gene (19). Cell-free virus from MT-4 cultures was used to infect monolayers of cells in 24-well multiwell plates and various concentrations of inhibitor were added to the culture medium [Dulbecco's modified Eagle's medium containing 5% (vol/vol) fetal calf serum plus antibiotics]. Three days after infection, monolayers were fixed with 10% (vol/vol) formaldehyde solution and stained with 0.25% crystal violet to visualize virus plaques. Such foci were obvious using this staining procedure as relatively dense individual multinucleated giant cells. The input virus inoculum was adjusted to give 100–300 plaques per well in the no-drug control monolayer cultures. ID_{50} values were derived from plots of percent plaque reduction versus inhibitor concentration ($\log_{10}$).

RESULTS

Mutational Analysis of the HIV-1 RT. RT mutants had been constructed by site-directed mutagenesis from an M13 clone (mpRT4) expressing active wt 66-kDa RT (9). We have extended this mutational analysis in previously defined conserved regions B and E and in addition have made mutations in region C (9). Alterations in region E which contains a sequence motif common to many RNA polymerases (consisting of two aspartate residues in a region of predominantly nonpolar amino acids), resulted in severe loss of enzyme activity (see Table 1), although mutant polypeptides are expressed to similar levels as wt (ref. 9 and unpublished data). This analysis confirms the importance of this region for enzyme activity. A number of mutations introduced into region B, however, resulted in enzymes with reduced but significant activity. Analysis of the enzyme from these mutants revealed alterations in apparent binding of RT inhibitors AZTTP and PFA (Table 1).

Changes at Tyr-115 were interesting as nonconservative mutations Tyr → Asn and Tyr → His resulted in enzymes highly resistant to AZTTP and PFA, but a conservative mutation (Tyr → Phe) gave an apparently normal enzyme (Table 1). Mutations introduced in region C also resulted in enzymes with altered RT activity and reduced inhibitor binding affinity (Table 1).

Construction of Proviral Clones with Mutations in the RT Gene. We wished to determine whether mutant RT genes were compatible with HIV replication when introduced into the virus, a question that has obvious implications for the development of drug resistance during AZT therapy. Therefore, five mutants with single amino acid changes in conserved regions of the enzyme were chosen that expressed wt levels of a 66-kDa polypeptide but lower enzyme activities, from 1–2% (RTM2) to 80% (RTM6), relative to wt RT (Table 2). The variant enzymes were less sensitive to AZTTP and PFA presumably due to altered ability to bind these inhibitors. To test the ability of the RT mutants to support HIV replication, we decided to transfer the mutant genes into an infectious HIV DNA clone (HXB-2D) and introduced these

Table 1. RT activity induced by mutants obtained by site-directed mutagenesis

Mutated residues	Substitution	RT activity, % wt	Fold increase in ID_{50}	
			AZTTP	PFA
Region B				
110	Asp → Gln*	<0.1	—	—
113	Asp → Gly*	59	17	5
	Asp → Glu	73	7	4
114	Ala → Ser*	80	3.8	4.2
	Ala → Gly	22	17	44
115	Tyr → Asn	12	670	>600
	Tyr → His	8	230	140
	Tyr → Phe	100	1	1
Region C				
151	Gln → His	35	3.5	4
154	Lys → Glu	21	55	132
Region E				
181	Tyr → Asp	1.3	—	—
183	Tyr → Ser*	1.2	—	—
184	Met → Leu	5	—	—
185	Asp → His*	<0.1	—	—
186	Asp → Asn	<0.1	—	—
190	Gly → Arg*	23	3	2.2

Mutants in HIV-1 RT were created by site-directed mutagenesis and activity was assayed using poly(rA)·oligo(dT) as primer template in bacterial extracts. The apparent sensitivity of each mutant RT to AZTTP and PFA is expressed as fold increase in the ID_{50} value compared with the respective ID_{50} value of each compound determined for wt enzyme. The ID_{50} of AZTTP for wt RT was 0.05 μM and for PFA was 0.5 μM. The regions of conserved amino acid residues (B, C, and E) have been defined (9). Region B, Leu-**Asp**^{110}-Val-Gly-**Asp**-**Ala**-**Tyr**^{115}-Phe; region C, Val-Leu-Pro-**Gln**^{151}-Gly-Trp-**Lys**^{154}-Gly-Ser-Pro; region E, Ile-**Tyr**^{181}-Gln-**Tyr**-**Met**-**Asp**-**Asp**-Leu-Tyr-Leu-**Gly**^{190}.

*Certain mutants were six of eight mutants described (9) and are included here for comparison.

Table 2. Comparison of mutant RT activity expressed in *E. coli* using different expression constructions

Clone	Mutation	RT activity, % wt	ID_{50}, μM	
			AZTTP	PFA
mpRT4	wt (LAV)	100	0.06 (1)	0.5 (1)
mpRT1/H	wt (HXB-2D)	100	0.04 (1)	0.4 (1)
RTM2	Tyr-183 → Ser	1.2	—	—
RTM2/H		2.2	—	—
RTM6	Ala-114 → Ser	80	0.23 (3.8)	2.1 (4.2)
RTM6/H		95	0.15 (3.8)	1.4 (3.5)
RTM16	Tyr-115 → Asn	12	7.6 (127)	>250 (>500)
RTM16/H		12	5.0 (125)	178 (445)
RTM18	Asp-113 → Glu	73	0.42 (7)	1.9 (3.8)
RTM18/H		45	0.66 (13.2)	1.9 (4.7)
RTM22	Gln-151 → His	21	3.3 (55)	66 (132)
RTM22/H		35	1.1 (27)	33 (83)

RT activity was assayed using poly(rA)·oligo(dT) as primer template in bacterial extracts (strain $5KCPolA^{ts}F'$ induced with isopropyl β-D-thiogalactopyranoside). The ID_{50} values were obtained from plots of RT activity against $\log_{10}$ (inhibitor concentration). RTM and RTM/H mutants were constructed by site-directed mutagenesis using oligonucleotides. Numbers in brackets are fold increase in the ID_{50} value of each mutant to inhibitor, compared with the respective wt RT value.

into T cells by transfection. The most convenient way to do this was to utilize a 1.9-kb *Bal* I fragment located within the *pol* gene (see Fig. 1). However, as one of the *Bal* I restriction sites lies in the endonuclease coding region (not present in our wt RT clone, mpRT4), it was necessary to remake the mutants in an M13 clone containing the entire *Bal* I *pol* gene fragment. This clone, mpRT1/H, was constructed by inserting a 2.55-kb *Bgl* II–*Eco*RI fragment from HXB-2D into M13mp19 (Fig. 1). Single nucleotide changes were introduced using oligonucleotides as described (9). As clone mpRT1/H was designed to express RT activity in *E. coli* we were able to compare this activity with RT expressed by the newly constructed mutants. Relative RT activity and sensitivity to AZTTP and PFA were broadly as predicted from the properties of analogous mutants derived from mpRT4 (see Table 2 for comparison). The *Bal* I fragment from each mutant was purified and transferred into HXB-2D (Fig. 1) and constructs containing the fragment in the correct orientation were identified by restriction enzyme analysis.

Transfection of T Cells with HIV RT Mutant Clones. DNA prepared from the wt and mutant proviral clones was used to transfect the T-cell line MT-4 by electroporation. The CPE due to HIV replication was observed with the wt clone 4–5 days after transfection. Detection of supernatant RT activity and p24 antigen from wt cultures confirmed the presence of HIV (Table 3). The proviral clone pHIVΔ1, which contains a deletion in the RT coding region (nucleotides 305–846) but

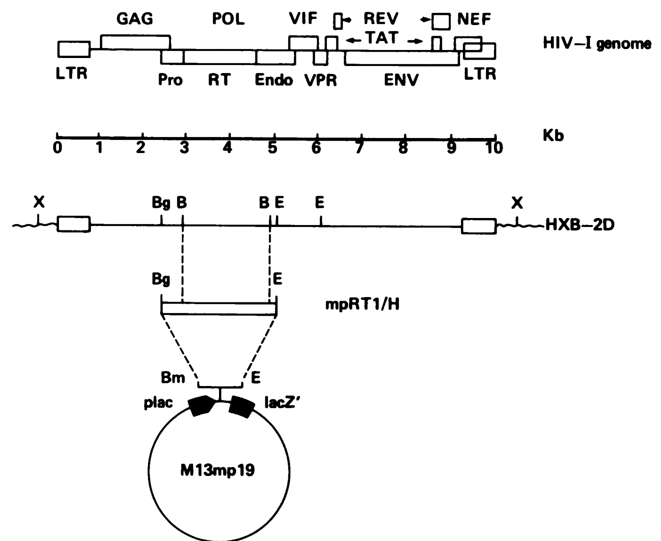

FIG. 1. Construction of proviral HIV clones with mutations in the RT gene. A 2.55-kb *Bgl* II–*Eco*RI fragment from the proviral clone HXB-2D was inserted into M13mp19 replicative form digested with *Bam*HI and *Eco*RI to give construct mpRT1/H that expresses HIV RT on infection of *E. coli*. Single-stranded DNA prepared from this clone was used as substrate for site-directed mutagenesis to introduce single nucleotide changes giving the amino acid substitutions in "RTM/H" mutants detailed in Table 1. The 1.9-kb *Bal* I fragment containing each mutation was isolated from RTM/H mutant RF DNA and transferred into HXB-2D previously digested with *Bal* I to remove the wt fragment. Mutant proviral clones were verified by digestion with restriction enzymes. The HIV-1 genome is shown at the top of the figure with sizes in kb shown below. Open boxes in HXB-2D represent the long terminal repeat (LTR) regions that are flanked by sequence derived from cellular DNA (wavy lines). B, *Bal* I; Bg, *Bgl* II; Bm, *Bam*HI; E, *Eco*RI; X, *Xba* I.

maintains the correct reading frame, as expected gave no CPE and no detectable supernatant RT activity or p24 antigen after transfection. Cells transfected with mutant clones containing single-amino acid substitutions in RT showed various signs of virus replication, depending on the RT activity expressed by the mutant gene. Clone pHIVRTM2 (1–2% wt RT) showed no signs of replication, but pHIVRTM16 (12% wt RT) and pHIVRTM22 (35% wt RT) showed low levels of p24 antigen, although they gave no CPE or supernatant RT activity (Table 3). This probably indicates abortive infection especially as no infectious virus could be recovered even after prolonged incubation and passage of these cultures. In contrast, cultures transfected with pHIVRTM6, which expressed more substantial levels of RT, were able to yield infectious virus, although appearance of CPE was delayed compared to wt (Table 3). In addition, transfection with pHIVRTM18 also gave infectious virus, although a delay in

Table 3. Cell transfection with proviral HIV clones

Clone	Day 6			Day 7			Day 8			Day 9		
	CPE	RT	p24	CPE	RT	p24	CPE	RT	p24	CPE	RT	p24
HXB-2D	+++	59.4	>12									
pHIVΔ1	−	0.16	<0.025	−	0.07	<0.025	−	0.22	<0.025	−	0.11	<0.025
pHIVRTM2	−	0.11	<0.025	−	0.14	<0.025	−	0.39	<0.025	−	0.20	<0.025
pHIVRTM6	±	0.9	>12	+	9.6	>12	++	104.5	>12	+++	33.9	>12
pHIVRTM16	−	0.16	0.025	−	0.17	0.025	−	0.21	0.08	−	0.25	0.06
pHIVRTM22	−	0.21	0.085	−	0.15	0.04	−	0.21	0.06	−	0.24	0.06

MT-4 cells were transfected with 10 μg of each proviral HIV DNA clone by electroporation, using a Bio-Rad Gene Pulser, and maintained in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum plus antibiotics. Samples were withdrawn from cultures transfected with mutant clones between days 6 and 9 and cells were assessed for the presence of HIV-1 p24 antigen by using the DuPont p24 ELISA kit. Levels of p24 are shown as ng of antigen per ml of culture. Virion-associated RT in culture supernatants was assessed by pelleting virus (40,000 rpm, 10 min at 4°C in a Beckman TL-100 before RT assay). Activity is shown as cpm/ml ($\times 10^{-3}$) of culture supernatant. CPE assessment: −, no CPE; ±, 25%; +, 25–50%; ++, 50–75%; +++, 75–100%.

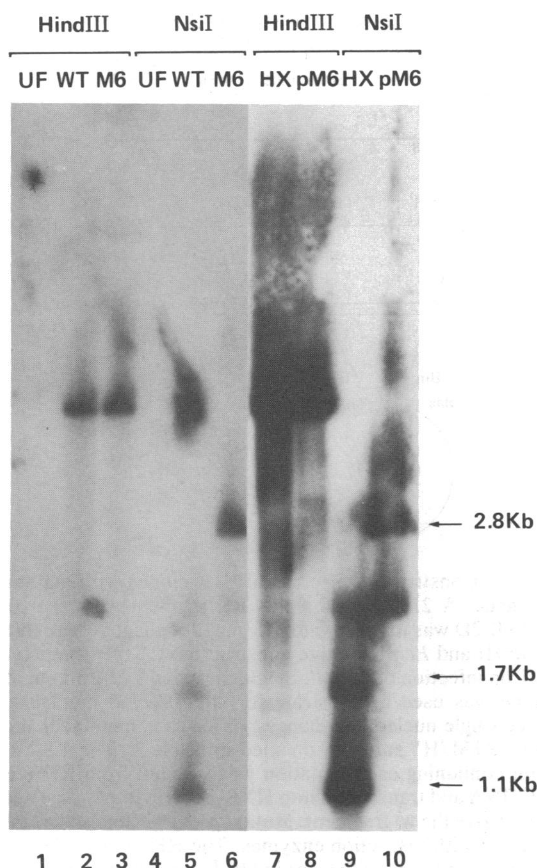

FIG. 2. Analysis of DNA from HIV-infected cells. DNA of high relative molecular mass was extracted from MT-4 cells 3 days after infection with wt HIV (WT) or mutant HIVRTM6 (M6) and from uninfected cells (UF) using standard procedures (13). DNA (10 μg) was digested with *Hin*dIII (lanes 1, 2, and 3) or *Nsi* I (lanes 4, 5, and 6) and electrophoresed in 1% agarose gels, transferred to nitrocellulose, and hybridized to a 2.55-kb *Bgl* II–*Eco*RI *pol* gene fragment purified from HXB-2D (see Fig. 1). Probe was labeled with ^{32}P using the random-primed oligonucleotide-labeling technique (Boehringer Mannheim kit). Recombinant plasmids HXB-2D (HX) and pHIVRTM6 (pM6) were also treated with *Hin*dIII (lanes 7 and 8) or *Nsi* I (lanes 9 and 10), electrophoresed on agarose gels, and hybridized to the same probe after transfer to nitrocellulose. Filters were washed sequentially in 4× SSC, 2× SSC, and 0.2× SSC at 65°C before exposure. Fragments derived from the *Nsi* I digestion of wt and HXB-2D DNA (1.7 kb and 1.1 kb) and the fused fragment (2.8 kb) resulting from loss of an *Nsi* I site in HIVRTM6 and pHIVRTM6 DNA are marked with arrows.

appearance of supernatant p24 antigen and CPE was also observed (data not shown).

Genomic Analysis of Infectious Virus Recovered from T-Cell Transfections. MT-4 cell cultures infected with virus after transfection were expanded and used to make virus pools. We wished to establish that these viruses (HIVRTM6 and HIVRTM18) contained the original single nucleotide change in the RT gene introduced by site-directed mutagenesis and had not reverted to wt. The original changes in each abolished an *Nsi* I restriction enzyme site within the RT gene and so we analyzed DNA extracted from virus-infected MT-4 cells. Southern blot analysis of DNA digested with *Nsi* I and probed with an HIV *pol* gene fragment demonstrated HIVRTM6 lacked the appropriate *Nsi* I site, giving a fused fragment similar to that seen with the proviral clone pHIVRTM6 (Fig. 2). The RT gene from DNA extracted from HIVRTM18-infected MT-4 cells was amplified by polymerase chain reaction using an oligonucleotide primer-pair flanking the RT coding region. Analysis by *Nsi* I digestion confirmed the loss of this restriction enzyme site (data not shown).

Sensitivity of Mutant Viruses to Antiviral Compounds. We next tested the sensitivity of the replication of HIVRTM6 and HIVRTM18 to inhibition by AZT or PFA by assessing reduction of CPE (in MT-4 cells) using a dye-uptake assay or by reduction of plaques (foci of multinucleated giant cells) in a CD4-expressing HeLa cell line susceptible to HIV infection. In addition, the sensitivity of supernatant virion-associated RT to AZTTP and PFA was determined by *in vitro* assay (Table 4). Decreases in sensitivity of HIVRTM6 and HIVRTM18 to PFA were observed by analysis of virus replication and direct inhibition of virus RT. These results were entirely consistent with the sensitivity of recombinant RT to PFA (see Table 2), indicating expression of altered RT can cause resistance to antiviral compounds. Surprisingly, HIVRTM6 and HIVRTM18 were not AZT-resistant, although virion RT showed decreased sensitivity to AZTTP. In fact, both viruses showed an unexpected increase in sensitivity to AZT (see Table 4).

DISCUSSION

Studies designed to identify functional regions of HIV-1 RT lead to the construction of a number of mutant genes expressing RT that retained enzyme activity but had reduced inhibitor binding. To assess the ability of these mutants to support HIV replication and to determine the drug sensitivity of potentially viable virus, we constructed proviral HIV clones with point mutations in the RT gene giving rise to expression of mutant RT with single amino acid substitutions in conserved regions of the enzyme. It was of little surprise that transfection of T cells with proviral clones expressing virtually inactive RT produced no infectious HIV. Interestingly, proviral clones with relatively active mutant RT (around 35% of wt in the case of pHIVRTM22) were unable to yield infectious virus after T-cell transfection, although low levels of p24 antigen detected in the cultures suggested transient replication had occurred.

Table 4. Sensitivity of HIV mutants to antiviral agents

Virus	ID_{50} for virus CPE, μM		ID_{50} for RT, μM	
	AZT	PFA	AZTTP	PFA
HIV (wt)	0.027 (1)	25 (1)	0.014 (1)	0.17 (1)
HIVRTM6	0.008 (0.3)	126 (5)	0.042 (2.8)	0.95 (5.6)

Virus	ID_{50} for virus plaques, μM		ID_{50} for RT, μM	
	AZT	PFA	AZTTP	PFA
HIV (wt)	0.03 (1)	20 (1)	0.006 (1)	0.09 (1)
HIVRTM18	0.004 (0.13)	100 (5)	0.028 (4.7)	0.45 (5)

Mutant HIV isolates recovered from MT-4 cell transfection experiments were assessed for susceptibility to AZT and PFA by CPE inhibition in MT-4 cells (for mutant HIVRTM6) or inhibition of plaque formation in HeLa cell line HT4-6C, which expresses the human CD4 gene. Control virus (HIV wt) was obtained from MT-4 cells transfected with the wt proviral clone HXB2-D. Multiplicity of infection in these experiments was controlled by titration of virus stocks in MT-4 cells by terminal dilution or plaque assessment in HT4-6C cells. ID_{50} values were derived from plots of cell viability or reduction in number of plaques versus inhibitor concentration (log_{10}). RT inhibition by AZTTP and PFA was determined using virion-associated RT precipitated from infected MT-4 cell culture supernatants with PEG. Solubilized virus extracts were used as enzyme source in standard RT assays and ID_{50} values were obtained from plots of percent RT activity versus inhibitor concentration. Numbers in parentheses indicate fold change in ID_{50} of mutants for inhibitors compared to wt virus.

In contrast, the two mutants expressing the most substantial RT activity (pHIVRTM6 and pHIVRTM18) gave rise to infectious virus with altered drug sensitivity. The virion-associated RT activity of both mutants showed similar impaired ability to bind AZTTP and PFA as the respective recombinant RT counterparts. In addition, the level of resistance of both viruses to PFA was consistent with the RT inhibition data. Surprisingly, instead of showing a degree of resistance to AZT in culture, we observed the mutant viruses were hypersensitive to the drug. It is possible that *in vitro* RT assays do not reflect precisely conditions within infected cells, particularly regarding deoxyribonucleotide and RT concentrations, variations in which are less likely to affect PFA binding. Alternatively, although the mutant enzymes appeared to bind AZTTP less well than wt enzyme, they might incorporate the inhibitor at a more rapid rate and thus to a greater degree than wt RT. These findings also imply that assessment of AZTTP binding by HIV RT from clinical isolates might not be a useful method to predict mutation has occurred to a drug-resistant phenotype.

This study has significant implications for HIV therapy with antiviral drugs acting on virus RT. We believe this is the first report of HIV showing a degree of resistance to an antiviral drug (in this case, PFA) and it raises the possibility of such an occurrence during therapy of patients with AIDS. Severe constraints are likely on changes within the RT gene that still enables efficient virus replication perhaps delaying the appearance of drug-resistant mutants. This hypothesis has been borne out in the extensive clinical experience with acyclovir during treatment of herpes simplex virus infections, as only a single herpes simplex virus mutant with altered DNA polymerase has so far been reported (20). In addition, attempts to select AZT-resistant HIV by repeated passage in culture in increasing concentrations of AZT have so far proved unsuccessful (ref. 21 and unpublished observations). However, due to the ability of HIV to mutate at a high rate during natural infection (22, 23), the appearance of drug-resistant mutants would not be a surprise. It is thus essential that HIV isolates are monitored during drug therapy to identify a decrease in sensitivity, allowing the possibility of dose alteration or alternative therapy to combat mutant strains should they arise.

Note. A report describing the isolation of AZT-resistant HIV from individuals treated for prolonged periods of time with AZT (24) was published after submission of this paper. Although the clinical significance of these observations is unclear at present, this report demonstrated the potential for HIV to become resistant to AZT in addition to the PFA-resistance described in the current study.

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