Reverse Transcriptase Fidelity and HIV-1 Variation

Mark A. Wainberg *et al.* (*1*) hypothesize that increased reverse transcriptase (RT) fidelity may account for the slowed emergence of epitope variants observed in patients treated with (−)2′,3′-dideoxy-3′-thiacytidine (3TC) and the failure to detect secondary drug-resistant variants during growth of 3TC-resistant human immunodeficiency virus–type 1 (HIV-1) in culture. A relatively simple model of virus population dynamics (*2–4*) permits an indirect test of this hypothesis. The model shows that delayed variation of 3TC-resistant HIV-1 is likely a result of decreased fitness rather than enhanced fidelity.

Coffin argues convincingly (*4*) that the proportion of variant genomes (P_n) in a large, rapidly replicating population of HIV-1 in vivo (*5*) is reasonably modeled as a function of virus mutation rate (μ), fitness of the variant relative to that of wild-type (s), and number of replication cycles (n). On the basis of half-life values ($t_{1/2}$) reported by Wainberg *et al.* (*1*), the fitness gained from loss of neutralizable epitopes in individuals treated with azidothymidine (AZT) is calculated to be 100%, or $s = 2.0$ (*6*). When applied to the model of Coffin, this increased fitness predicts a rapid accumulation of epitope variants with $t_{1/2} = 14$ cycles ≈ 5.2 weeks (Fig. 1A, thick solid line). Thus, the model closely approximates the 5.6-week $t_{1/2}$ measured by Wainberg *et al.* The model also provides a good fit to the data from the report (*1*) for AZT resistance in culture when $s = 7.5$ (Fig. 1B, thick solid line).

At these high s values, reductions in mutation rate are expected to minimally delay the emergence of HIV-1 variants. If one makes the arguable assumption that RT fidelity is rate-limiting for virus mutation (*7*), a threefold decrease in mutation rate [as predicted from the report (*1*)] will result in only a 3- to 4-day delay in epitope loss in vivo (Fig. 1A, thin dashed line) and about a 2-day delay in AZT resistance in culture (Fig. 1B, thin dashed line). Even large reductions in mutation rate are predicted to only modestly affect the timing of variant emergence (Fig. 1, A and B, thin dotted lines). Assuming that all virions achieve similar fitness gains from neutralization escape and AZT resistance, then the delays observed by Wainberg *et al.* for 3TC-resistant HIV-1 cannot be explained by a simple decrease in virus mutation rate.

These modeling results strongly suggest that other rate-limiting steps are involved. Calculations based on growth competition experiments (*8*) reveal a reduced fitness of 3TC-resistant mutants relative to wild-type HIV-1 ($s = 0.91$ and 0.73 for the M184V and M184I mutants, respectively). Although a 9% reduction in relative fitness ($s = 0.91$) may not seem significant (*1*), its impact on the composition of a virus population after multiple cycles of replication is potentially enormous (*3, 4*). This may contribute, in part, to the observed delays in epitope loss in vivo and outgrowth of drug-resistant variants in culture. Thus, a more realistic scenario for epitope loss in 3TC-treated patients is modeled assuming that 3TC reduces overall viral fitness within the host (Fig. 1A, thick dashed line). In the culture experiments (*1*), population size is also a potentially important consideration. If fitness is further reduced during the drug-selection protocol, the size of the virus population may become diminishingly small (relative to mutation rate), and the probability of selecting drug-resistant variants will decrease.

This analysis reaffirms several important points previously emphasized by Coffin (*3, 4*) and others (*9*). The frequency of genetic variants arising in an HIV-1 population is influenced by many factors. These include mutation rate, fitness, population size, the number of replication cycles, competition, and random sampling. Although high retroviral mutation rates presumably facilitate variation by continuously generating pools of mutant genomes (*7*), the overall composition of a large, rapidly replicating HIV-1 population is likely determined primarily by relative fitness and not mutation rate.

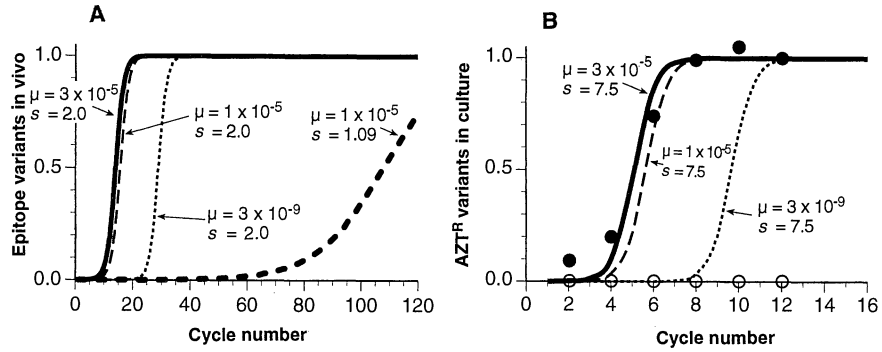

Fig. 1. Predicted effects of mutation rate (μ) and relative fitness (s) on HIV-1 variation. (**A**) Emergence of epitope variants in vivo. Thick, solid line, model of data from the report by Wainberg *et al.* (*1*) for AZT-treated individuals ($t_{1/2}$ = 14 replication cycles ≈ 5.2 weeks). Thin, dashed, and dotted lines, effects of changing mutation rate while holding relative fitness constant ($s = 2.0$). Thick, dashed lines, model of data from the report (*1*) for 3TC-treated individuals ($t_{1/2}$ = 108 replication cycles ≈ 40 weeks). Population size = 10^{10} virions with replication cycle = 2.6 days (*5*); wild-type mutation rate = 3×10^{-5} per nucleotide per cycle (*10*); 3TC-resistant mutation rate = 1×10^{-5} per nucleotide per cycle (*1*). Curves are essentially the same, starting with either zero or 10^5 preexisting variants in the population (data not shown). (**B**) Emergence of AZT-resistant (AZT^R) variants in culture. Filled and open circles, data from the report (*1*) for wild-type (HxB2) and 3TC-resistant (HxB2-M184V) HIV-1, respectively [figure 1 in (*1*)]. Thick, solid line, model of wild-type data. Thin, dashed, and dotted lines, effects of changing mutation rate while holding relative fitness constant ($s = 7.5$). Population size = 10^6 virions; replication cycle = 3.5 days (*11*); wild-type and 3TC-resistant mutation rates as above. Modeled s value of 7.5 represents the average fitness gain during the course of the multistep drug selection protocol (*1*). Slightly better fit of the wild-type data is obtained if one assumes that AZT-resistant mutants preexist in the starting virus population (data not shown). All curves were generated for single-locus variants using the equation

$$P_n = \frac{(s - \mu_r)M_{n-1} + \mu_f W_{n-1}}{sM_{n-1} + W_{n-1}}$$

where P_n is the proportion of variant genomes in the population (ordinate) at replication cycle n (abscissa), s is the fitness of the variant genome relative to that of wild-type, μ_f and μ_r are the forward and reverse mutation rates, respectively (assumed here to be equal) and M_{n-1} and W_{n-1} are the number of variant and wild-type genomes, respectively, at cycle $n-1$ (*2–4*).

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Wainberg *et al.* (*1*) found increased fidelity for lamivudine (3TC)-resistant HIV-1 RT mutants harboring a methionine-to-valine substitution at codon 184 (M184V). They postulated that increased RT fidelity of M184V variants might have clinical implications by delaying the generation of double-resistant viruses during treatment of 3TC-resistant viruses with additional drugs.

To investigate whether the observed differences in fidelity measured for the 184Val RT enzyme in cell-free nucleotide insertion experiments is effective in vitro, we performed selection experiments in cell culture with the wild-type (184Met) and 3TC-resistant (184Ile and 184Val) viruses. The selection experiments were performed either with nevirapine, delavirdine, or ritonavir (*2*). In all of these experiments, viruses with reduced susceptibility to the second drug were selected without the loss of the 184 genotype. For instance, genotypic analysis of nevirapine-resistant viruses after five cell culture passages revealed the presence of the Y181C nevirapine resistance mutation (*3*). These results indicate that increased fidelity could not prevent or significantly delay the generation of additional mutations.

We also analyzed four patients who received zidovudine-lamivudine combination therapy after 1 year of 3TC monotherapy. At the start of combination therapy, the virus population of all patients harbored the 184Val variant. Genotypic analysis of the RT gene obtained after 1 year of combination therapy revealed the presence of all known zidovudine resistance mutations (*4*).

We conclude from these in vitro and in vivo data that increased RT fidelity of the 184Val variant is not sufficient to prevent the generation of double-resistant HIV-1 viruses. Consistent with this idea, Coffin (*5*) suggested that, after sufficient number of replication cycles, differences in fidelity are less crucial for the appearance of mutations than difference in viral fitness. Further clinical research is necessary to evaluate whether increased enzyme fidelity may have implications for the strategy during combination therapy.

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2. The in vitro selection experiments were initiated by infection of 10^6 SupT1 cells with equal amounts of virus based on HIV-1 CA-p24 (100 ng). After infection, the culture medium was supplemented with either 0.1 μM nevirapine, 0.05 μM delavirdine, or 0.05 μM ritonavir. During the selection procedure, cell-free supernatant and infected cells were harvested after the observation of large syncytia. The second passage was performed at the same drug concentration with 0.5 ml of cell-free supernatant from the previous passage. In the following passages, 0.5 ml of cell-free virus supernatant of the previous passage was passaged at doubled drug concentrations. After several passages, proviral DNA was isolated, amplified, and sequenced directly.
3. At passage 5, all 184 variants harbored the nevirapine resistance mutation Y181C, which was absent in the fourth passage.
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Wainberg *et al.* state that enhanced fidelity of nucleotide insertion by the 3TC-selected mutant HIV-1 RT (which contains the 184 Met → Val amino acid mutation) represents the molecular basis for the sustained (partial) suppression of plasma viral burden in HIV-1–infected individuals treated with 3TC, despite the development of virus resistance to the compound (*1*). In support of their hypothesis, they found that 184 Met → Val RT mutant HIV grown in cell culture in the presence of several nucleoside RT inhibitors (NRTIs), the protease inhibitor saquinavir, and several non-nucleoside RT inhibitors (NNRTIs) did not select for variants displaying resistance to these compounds (*1*). The latter observation could be ascribed to increased fidelity of the 184 Met → Val RT, resulting in a markedly lower flexibility of the mutant virus to efficiently escape drug pressure, or to a potential incompatibility of the 184 Met → Val RT mutation with any other mutation selected for by either NRTIs or NNRTIs.

In attempting to clarify these issues, we tried to obtain resistance to 3TC with a number of HIV-1 strains containing the NNRTI-specific mutations (103 Lys → Asn, 106 Val → Ala, 138 Glu → Lys, and 181 Tyr → Cys) in their RT genes. In all cases, we found a drug dose–dependent emergence of virus within 13 to 36 days after 3TC treatment, virus that contained, in addition to the preexisting NNRTI-specific mutation, the 184 Met → Ile mutation in its RT. Our data show that a mutation at the 184–amino acid position of RT is compatible with the NNRTI-specific mutations, and thus, potential incompatibility among the different mutations cannot explain the lack of resistance of the 184 Met → Val RT mutant virus to NNRTIs, as observed by Wainberg *et al.* (*1*).

When we characterized the mutant virus strains (that originated from the wild-type HIV-1 or 184-Val RT mutant virus strains, and were exposed to the NNRTIs) for the presence of amino acid changes in their RT, we found that (i) in all cases, the 184-Val mutation was retained during the NNRTI resistance selection process in the absence of 3TC, and (ii) the NNRTI-specific amino acid changes (*2*) were found in both the wild-type and 184-Val RT mutant viruses. Thus, the 184-Val RT mutant virus acquired the NNRTI-specific mutations in its RT (Table 1), retained pronounced resistance to 3TC, and became resistant to the NNRTIs. This resistance to the NNRTIs depended on the nature and location of the amino acid mutation in the RT and did not markedly differ from the resistance profile of the corresponding single-mutant virus strains that did not contain the 184-Val mutation (*3*).

Because our data did not support sequential chemotherapy of 3TC and NNRTIs as a strategy to delay the development of drug resistance in HIV-1, we combined a variety of NNRTIs with 3TC at different drug concentrations in a concomitant treatment schedule. This therapy afforded a marked delay of virus-induced cytopathicity at drug concentrations that were at least 10- to 50-fold lower than those required for the individual drugs to prevent virus replication. For example, 3TC given at 0.02 or 0.05 μg/ml as a single drug prevented virus emergence for only 4 to 6 days, whereas the concomitant presence of 3TC with an NNRTI such as BHAP, TSAO-m^3T, or MKC-442 (0.02 or 0.04 μg/ml) delayed the emergence of virus-induced cytopathicity for 19 to more than 52 days, or even cleared the virus from the infected cell cultures (*4*); the latter NNRTIs were able to delay virus appearance for only 6 to 15 days when used as single agents (*4*, *5*).

Because we have also demonstrated that combinations of 3TC-TP and various NNRTIs merely have an additive inhibitory effect on HIV-1 RT activity (*4*), we believe that the pronounced antiviral efficacy of the concomitant combination of 3TC and NNRTIs may be attributed to the efficient

Table 1. Emergence of wild-type HIV-1(III_B) and mutant HIV-1/184-Val strains in CEM cell cultures in the presence of a variety of NNRTIs.

Compound	Initial concentration (μg/ml)	HIV-1 wild type		HIV-1 184-Val	
		Virus breakthrough*	Mutations in the RT	Virus breakthrough*	Mutations in the RT
Pyridinone L-697,661	0.25	5 (3)	none	5 (3)	184-Val
Nevirapine	0.5	20 (14)	106-Ala	23 (11)	184-Val + 188-Cys/WT§ + 190-Ala/WT§
Delavirdine (BHAP U-90152)	0.5	†	NA‡	34 (32)	184-Val + 236-Leu
8-Chloro-TIBO	0.1	†	NA	30 (25)	184-Val + 106-Ile
Loviride (α-APA)	0.1	23 (11)	181-Cys	23 (18)	184-Val + 181-Cys
Quinoxaline HBY 097	0.005	33 (21)	101-Glu	†	N.A.
TSAO-m^3T	0.5	16 (11)	138-Lys	13 (7)	184-Val + 138-Lys/WT§
Thiocarboxanilide UC-781	0.01	20 (11)	106-Ala/WT§	18 (14)	184-Val + 181-Cys
MKC-442 (I-EBU)	0.05	23 (11)	181-Cys	25 (14)	184-Val + 188-His
	0.25	†	NA	48 (35)	184-Val + 188-His

High multiplicity of infection of HIV-1(III_B) and HIV-1/184-Val was subjected to several passages in 5-ml CEM cell cultures (~3.5×10^5 cells per milliliter) in the presence of fixed concentrations of NNRTIs in 25-cm^2 culture flasks to produce mutant virus strains that were able to grow in the presence of the individual compounds. Initial virus input into each cell culture consisted of 750 μl of fresh supernatant that was obtained from an HIV-1–infected cell culture when the virus was most abundantly present (~4 days after infection). Passages were performed every 3 to 4 days through the addition of 0.5 to 1.0 ml of the infected culture supernatants to 5 ml of a suspension containing 3.5×10^5 uninfected CEM cells per milliliter. *Data indicate the mean day required for development of 50% cytopathicity (after initiation of the experiment). Values in parentheses represent the first day of microscopically visible virus breakthrough. †Virus breakthrough was completely suppressed in the presence of the drugs, even after further subcultivation of the cell cultures in the absence of the drugs. ‡NA, not applicable. §188-Cys/WT, 190-Ala/WT, 138-Lys/WT, and 106-Ala/WT represent mixtures of wild-type (WT) virus and HIV-1 strains containing a mutation in their RT at the indicated amino acid position.

complementary suppression of the NNRTI- and 3TC-specific resistance mutations by 3TC and NNRTIs, respectively. Myers *et al.* (6) and Gulick *et al.* (7) found a profound suppression of viral replication and efficient suppression of resistance when HIV-1–positive individuals were concomitantly treated by a drug "cocktail" containing AZT and ddI and nevirapine, or AZT and 3TC and indinavir, respectively (6, 7).

Given the enormous virus replication dynamics, virus plasma load and virus turnover in vivo (8), it is expected that mutations that may arise during such an intensive virus replication may easily counteract any potential decreased mutation rate that results from the higher fidelity of the mutant RT. Our results caution against strategies aimed at the accumulation of drug resistance mutations in the HIV-1 RT genome through the administration of single compounds in a sequential therapeutic treatment schedule. Such sequentially acquired mutations would make the virus easily and highly resistant to multiple drugs. Our observations strongly argue for the use of a combination of different HIV inhibitors such as 3TC and NNRTIs to suppress virus replication and to delay the emergence of drug-resistant virus in HIV-1–infected individuals. From a comparative side-by-side study of nine different NNRTIs (4), the quinoxaline HBY 097 and the thiocarboxanilide UC-781 emerged as prime candidate drugs to be included in a concomitant combination strategy with 3TC.

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Response: Keulen *et al.* and Balzarini *et al.* demonstrate that NNRTI-resistant variants of the 3TC-resistant HIV (with the M184V alteration) can be generated under in vitro drug selection conditions. In our report, we demonstrated that the fidelity of 3TC-selected RT containing the M184V substitution was increased as measured in cell-free experiments (*1*). We also showed that viruses containing the M184V alteration could not easily be selected for resistance against a variety of compounds including the non-nucleoside inhibitors, nevirapine and delavirdine, and the protease inhibitor, saquinavir. Those experiments were performed with relatively steep increases in drug concentrations, so as to maximize potential differences in behavior between wild-type and mutated recombinant viruses. More recent studies in the laboratory of one of us (*2*), using less stringent selection conditions (akin to those used by Balzarini *et al.*) than those initially used by us, have led to the appearance of HIV variants that display resistance against nevirapine or delavirdine in studies performed with 184V-containing viruses. These findings are consistent with results described in the comments: namely, viruses containing the 184V substitution can apparently undergo further mutation so as to become resistant to NNRTI and probably to protease inhibitors as well.

One possible reason that delayed kinetics of virus breakthrough, resulting from an increase in RT fidelity, is not easily demonstrated in cell culture under certain selection conditions is that increased fidelity may result in a significant decrease in numbers of lethal mutations that are then generated during viral replication. To test this, one of us (M.A.W.) measured the TCID50:p24 ratios of HXB2-derived wild-type virus during 10 successive rounds of selection in the presence of AZT or 3TC. The ratio remained around −0.4 for AZT-selected viruses even after 10 cycles, while those undergoing 3TC-selection gradually increased by about 15-fold to about 6.5. This result confirms that a greater proportion of virion particles released from cells infected with M184V variant are viable (and therefore infectious), as compared with virion particles produced by infection with wild-type virus. Even though 184V-containing HIV should generate a less diverse population than would wild-type HIV, presence of viable, infectious virus in increased numbers might facilitate selection under conditions of selection pressure from a drug.

We did not argue (*1*) that the increase in RT fidelity associated with the 184V substitution precludes further mutagenesis. The notion that an increase in RT fidelity may have clinical importance will require anal-

ysis of viral variability in patients undergoing treatment with drugs such as 3TC. Such studies should be performed on patients with relatively high CD4 T cell counts, in contrast to those performed by Keulen *et al.*, because the fidelity hypothesis invokes a role for the immune system in dimunition of viral burden (*1*, *3*). Conceivably, other mutations that confer an even greater increase in fidelity than that conferred by M184V might be selected by other drugs. Combinations of drugs and mutations that significantly increase RT fidelity could form a basis for future chemotherapeutic strategy.

Preston has attempted to model the impact of polymerase fidelity on the rate of epitope loss and appearance of drug-resistant variants. He models virus population dynamics using experimental values for fitness and fidelity determined for the M184V variant. The data available are insufficient to generate a model of virus population dynamics that can appreciate the role of polymerase fidelity on viral variation.

1) *Mutation rates*. To model the rate of generation of viral variants for two viruses that differ in their polymerase fidelities, one would need the mutation rates for both viruses: the wild-type and the variant virus. The number utilized by Preston for wild-type virus, 3×10^{-5} per nucleotide per viral replication cycle, is valid (*4*). The use of 1×10^{-5} per nucleotide per cycle as the mutation rate for 3TC-resistant virus, however, is not. We stated, based on in vitro measurements, that the RT of 3TC-resistant HIV displays a 2- to 16-fold decrease in the efficiency of misinsertion of deoxynucleotide triphosphates, depending on the type of misinsertion involved (*1*). The threefold increase in fidelity that we found for M184V RT is an average rate of formation of mispairs. HIV-1 RT can create mutations through multiple types of errors: misinsertions, mispair extensions, and slippage-mediated errors. Our measurements quantitated only one of these components, the misinsertion. Thus, the differences in the overall error rates can be vastly different between the two viruses and need to be quantitated by single cycle infectious experiments, as we had originally stated (*1*). One cannot substitute the average misinsertion efficiency for the in vivo mutation rate.

2) *Replication rates and fitness*. Preston argues that the reason for slow emergence of variants is likely explained by a lower fitness of M184V virus. While fitness may be a factor (*5*), the argument is flawed. As cited above, the measurements of $TCID_{50}$:p24 ratios reveal that the decreased mutation rate increases the proportion of viable virus particles in a population. These results point to a key impact of increased fidelity on the biology of HIV and reveal pitfalls in the methods currently used to measure fitness. Preston uses a value of 0.91 for M184V on the basis of the competition between wild-type and M184V viruses (*5*). Those experiments were based on mixed infections of wild-type and M184V viruses followed by several serial passages and measuring the amount of each virus at each point. While the genotyping shows that the wild-type virus constitutes two-thirds of the virus population in the dish after 11 passages, it is not clear what portion of it is viable. Also, the curves plotting the proportions of the M184 and V184 genotypes in the mixture [figure 1B in (*5*)] reveal that the mutant genotype may be slowly on the rise, accompanied by a consistent drop in the proportion of the wild-type genomes. Whether further passages will result in the dominance of the M184V variant remains to be seen. A recent study shows that the fitness measurements of M184V in different cell types yield dramatically different results (*6*). Therefore, it seems improper to apply the fitness values derived from experiments performed with one cell line (MT-4 cells) to the events occurring in another cell type (PBMCs). Furthermore, attempts to measure fitness (*5*, *6*) have used $HXB2_{HIV-1}$, which contains a deletion in the *nef* gene (*7*). In view of the fact that NEF can influence HIV replication and fitness, it is desirable to repeat these experiments with a clinical isolate or at least with an isolate without any lesions in essential genes.

Thus, it appears that the two important values required to model the impact of fidelity on virus mutation rate—virus replication fidelity and virus fitness—are currently not available. Therefore, both the modeling and the conclusions drawn from it are premature.

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