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Lethal mutagenesis of HIV

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Abstract

HIV-1 and other retroviruses exhibit mutation rates that are 1,000,000-fold greater than their host organisms. Error-prone viral replication may place retroviruses and other RNA viruses near the threshold of "error catastrophe" or extinction due to an intolerable load of deleterious mutations. Strategies designed to drive viruses to error catastrophe have been applied to HIV-1 and a number of RNA viruses. Here, we review the concept of extinguishing HIV infection by "lethal mutagenesis" and consider the utility of this new approach in combination with conventional antiretroviral strategies.

Keywords: HIV-1; Retroviruses; Lethal mutagenesis

1. Introduction

Variation in HIV populations results from the error-prone nature of retroviral replication and the rapid turnover of virus in infected individuals (Coffin, 1995). Mutations in viral genomes provide the genetic potential for immune escape, changes in cellular and species tropism, and the development of antiviral drug resistance (Domingo and Holland, 1997; Rambaut et al., 2004). However, the ability of HIV to adapt to environmental pressures is not without cost; the available evidence suggests that error-prone replication imposes a substantial genetic load on retroviral populations, as discussed in this review. Thus, it has been suggested that the mutation rates of retroviruses and other RNA viruses approach the maximal value that is compatible with sustained production of infectious progeny (Holland et al., 1990). Violation of this theoretical threshold is predicted to result in a sudden and irreversible collapse of the population structure due to an intolerable number of deleterious mutations (Eigen, 1971). The ensuing loss of replicative potential is referred to as "error catastrophe" (Eigen, 2002).

In theory, proximity to the threshold of error catastrophe should render HIV susceptible to extinction due to slight increases in the mutation rate. This concept is the basis of an antiviral strategy designed to specifically increase the error rate of retroviral replication. Here, we review the theoretical and experimental grounds for this strategy, termed "lethal mutagenesis" (Loeb et al., 1999). We begin by examining the sources of spontaneous mutations in retroviral genomes and the rate at which these errors are formed during viral replication. Next, we review data from in vitro studies suggesting that mutagenic compounds can increase the mutation rate of HIV-1 replication beyond the error threshold. We also address recent findings suggesting that specific cellular enzymes can induce a natural form of error catastrophe by directly altering the sequence of the HIV-1 genome. Finally, we examine the possibility of using virus-specific mutagens in combination with conventional antiretroviral drugs, and discuss potential challenges to this new therapeutic approach.

2. Retroviral mutagenesis

Genetic diversity and phenotypic variation are intrinsic properties of retroviral populations. This fundamental aspect of retroviral biology was appreciated as early as 1913, when Rous and Murphy demonstrated that chickens infected with

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serially-passaged strains of Rous sarcoma virus (RSV) often developed tumor types that differed from those produced by the parental virus isolate (Rous and Murphy, 1913). Phenotypic variation was also documented in pioneering studies by Howard Temin, who observed heritable differences in the morphology of cells infected with different strains of RSV (Temin, 1960). As other retrovirus species were isolated and characterized, it became apparent that independent isolates of the same species often varied greatly in tumorigenic and cytopathic potential, cell tropism, and drug sensitivity (Vogt, 1997). This inherent instability placed retroviruses among other RNA viruses, which exhibit similar propensities for phenotypic change (Temin, 1989).

DNA sequence analyses later demonstrated that retroviruses are subject to substantial genotypic variation, as extensively documented in HIV-1 (Coffin, 1986, 1995; Desai et al., 1986; Goodenow et al., 1989; Hahn et al., 1986). Estimates of the average number of nucleotide differences in pairwise comparisons of patient isolates range from 15 to 25% for portions of the *env* gene (Buonaguro et al., 1995; Learn et al., 1996; Murphy et al., 1993; Wang et al., 1995). Clonal analyses demonstrate that multiple subclasses of variants simultaneously coexist in HIV-1-infected individuals and that the relative frequencies of these genotypes often fluctuate during the course of natural infection (Goodenow et al., 1989; Liu et al., 2002; Meyerhans et al., 1989; Shankarappa et al., 1999). These findings have contributed to the widely-accepted view that retroviruses and RNA viruses exist as complex mixtures of related but genetically-distinct subtypes, frequently referred to as "swarms" (Temin, 1989) or "quasispecies" (Domingo et al., 1985; Domingo, 2003; Eigen, 1993).

2.1. Sources of mutations in retroviral genomes

The diversity of retroviral populations is a direct result of the error-prone nature of retroviral replication. Mutations in HIV-1 genomes primarily arise during three distinct polymerization steps in the retroviral life cycle (Fig. 1):

1) RNA-templated, minus-strand DNA synthesis by the viral reverse transcriptase (RT).
2) DNA-templated, plus-strand DNA synthesis by RT.
3) DNA-templated, plus-strand RNA synthesis by host-encoded RNA polymerase II (RNA pol II).

Measurements of the fidelity of RT-catalyzed DNA synthesis in vitro (reviewed in Menendez-Arias, 2002; Preston and Garvey, 1992; Preston and Dougherty, 1996; Svarovskaia et al., 2003) indicate that reverse transcriptases are substantially less accurate than cellular replicative DNA polymerases. Much of the difference in fidelity is due to the lack of an associated $3' \rightarrow 5'$ exonucleolytic proofreading activity in RT (Battula and Loeb, 1976; Roberts et al., 1988). The disparity in the error rates of RT and cellular replicative DNA polymerases suggests that mutations produced during copying of integrated proviral DNA are relatively rare and contribute little to the genetic diversity of actively-replicating retroviruses (Gojobori and Yokoyama, 1985). This view is supported by data showing that a large proportion of the mutations formed during a single cycle of viral replication in culture can be attributed to RT (Kim et al., 1996; O'Neil et al., 2002; Zhang, 2004). In contrast, much less is known about the role of RNA polymerase II in retroviral mutation. Experiments with prokaryotic and plant RNA polymerases suggest that transcription is a relatively error-prone process (Blank et al., 1986; de Mercoyrol et al., 1992; Libby and Gallant, 1991), though the fidelity of RNA synthesis can be modulated by other components of the cellular transcription machinery (Erie et al., 1993; Jeon and Agarwal, 1996; Koyama et al., 2003; Lange and Hausner, 2004; Shaw et al., 2002; Thomas et al., 1998). Analyses of mutations produced during retroviral vector replication also suggest that RNA pol II fidelity contributes to viral variation (Kim et al., 1996; O'Neil et al., 2002). However, the magnitude of this contribution remains unclear.

Other sources of errors are also likely to generate diversity in viral populations. Fluctuations in nucleotide pool levels (Julias and Pathak, 1998; Vartanian et al., 1994) and/or incorporation of dUTP (Chen et al., 2002; Lerner et al., 1995) may generate mutations during viral DNA synthesis. Spontaneous chemical decay of viral RNA or DNA produces aberrant bases that miscode during transcription or reverse transcription (Lindahl, 1993). RT incorporates damaged nucleotides during DNA synthesis in vitro (Bebenek et al., 1999; Feig et al., 1994; Furge and Guengerich, 1997; Hizi et al., 1997; Kamath-Loeb et al., 1997a; Preston et al., 1986) and inserts incorrect nucleotides across from damaged bases in RNA and DNA templates (Furge and Guengerich, 1997). These findings supported the suggestion that exposure of HIV-infected cells to damaged nucleosides might increase the viral mutation rate, thereby driving HIV to error catastrophe (Preston et al., 1988; see below).

Modification of bases in viral RNA or DNA by host-encoded enzymes also represents a potential source of mutations. Studies in both RNA viruses and retroviruses suggest that host enzymes other than DNA polymerases produce 'hypermutations', which occur as clusters of specific base substitutions in the viral genome (Cattaneo et al., 1988; Pathak and Temin, 1990a). For example, the pattern of $A \rightarrow G$ hypermutations observed in avian retroviruses suggests that these mutations result from the editing activity of double-stranded RNA deaminases (Bass, 1997; Felder et al., 1994; Hajjar and Linial, 1995; Pathak and Temin, 1990a). More recently, the cellular enzyme APOBEC3G has been shown to generate $G \rightarrow A$ hypermutations in HIV-1, (Bhagwat, 2004; Goff, 2003; KewalRamani and Coffin, 2003; Vartanian et al., 2003; see below). Thus, as with other RNA viruses (Cattaneo et al., 1988; Macnaughton et al., 2003; Murphy et al., 1991; O'Hara et al., 1984; Polson et al., 1996; Rueda et al., 1994), host-encoded enzymes influence the biological properties of retroviral genomes by directly altering the viral coding sequence.

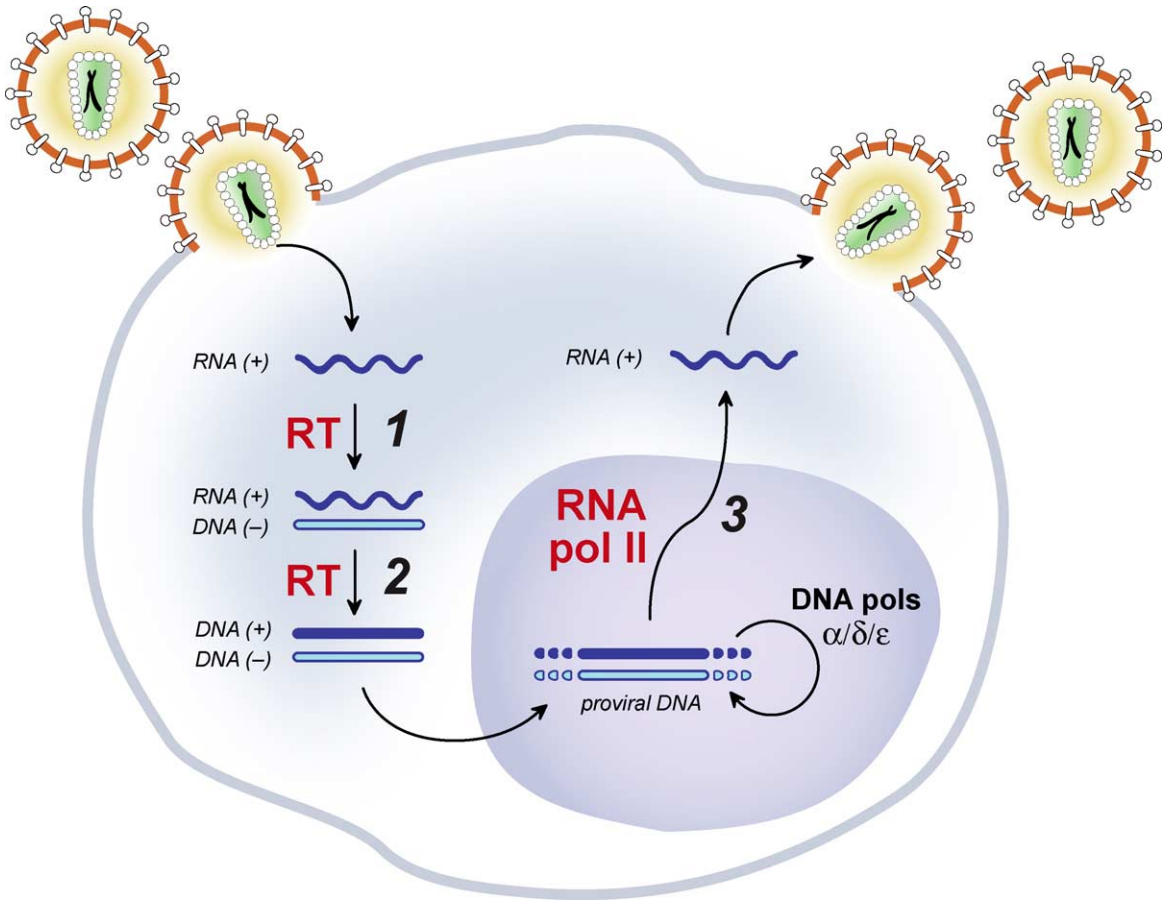

Fig. 1. Outline of the retroviral life cycle. Error-prone polymerization steps that substantially contribute to the viral mutation rate are numbered as described in the text. RT: reverse transcriptase; RNA pol II: cellular RNA polymerase II; DNA pols α/δ/ε: cellular DNA polymerases α, δ, and/or ε. (+) Plus-strand; (−), minus strand.

2.2. Retroviral mutation and recombination rates

Measurements of mutation rates require assays that constrain the virus to a known number of replication cycles. Early studies of murine leukemia virus (MLV) and RSV used specific host cell types and culture conditions to limit the virus to a single round of replication (Leider et al., 1988; Monk et al., 1992). These approaches were superceded by the use of retroviral vectors that are genetically restricted to a single replication cycle (Dougherty and Temin, 1988; Gabriel et al., 1996; Gao et al., 2004; Halvas et al., 2000a,b; Julias et al., 1997; Kim et al., 1996; Mansky and Temin, 1994, 1995; Mansky, 1996a,b; Mansky, 2000; O'Neil et al., 2002; Parthasarathi et al., 1995; Pathak and Temin, 1990a,b; Varela-Echavarria et al., 1992; Zhang et al., 2002). The majority of these assays measure mutation rates in nonessential target genes (*neo*, *gfp*, *LacZα* or *tk*).

Based on the results of vector virus experiments, retroviral mutation rates range from 5×10^{-6} to 9×10^{-5} (average = 5×10^{-5}) mutations per nucleotide per cycle of virus replication (Table 1). Given an average genome length of ~10,000 nucleotides, this equates to approximately 0.1–1 mutation per genome per replication cycle. The majority of errors are single base substitutions, which occur at an average rate of 3×10^{-5} per nucleotide per round of replication. A similar base substitution rate was also observed for the yeast retroelement TY-1 (Gabriel et al., 1996). Simple frameshifts and rearrangements are observed at average rates of $\sim 5 \times 10^{-6}$ per nucleotide per replication cycle (Preston and Dougherty, 1996). The average mutation rate for HIV-1 ($\sim 5 \times 10^{-5}$ mutations per nucleotide, Table 1) is comparable to the rates observed for several other retroviruses, and about a million-fold greater than the mutation rates of eukaryotic cells (Drake et al., 1998; Drake, 1999).

Vector viruses have also been used to determine the rates of recombination between the two strands of genomic RNA that are co-packaged in retroviral virions. For HIV-1, homologous recombination occurs throughout the genome at a rate of 2–3 crossovers per round of replication in cultured fibroblasts (Hu et al., 2003; Jetzt et al., 2000; Negroni and Buc, 2001; Onafuwa et al., 2003; Rhodes et al., 2003; Yu et al., 1998; Zhuang et al., 2002) and 10–30 crossovers per replication cycle in T lymphocytes and macrophages (Levy et al., 2004). Recombina-

Table 1
Forward mutation rates of retroelements

Element	Target	Mutations per base pair per replication cycle		References
		Base substitutions	All classes[a]	
Retroviruses				
HIV-1	*LacZα*	2×10^{-5}	3×10^{-5}	Mansky and Temin (1995) and Mansky (1996a)
	Viral genome	4×10^{-5}	5×10^{-5}	Gao et al. (2004)
	LTR	6×10^{-5}	9×10^{-5}	O'Neil et al. (2002)
MLV	*LacZα*	3×10^{-5}[b]	ND[c]	Halvas et al. (2000a,b) and Zhang et al. (2002)
	Gfp	3×10^{-5}[b]	ND	Zhang et al. (2002)
	Tk	3×10^{-5}[d]	3×10^{-5}	Parthasarathi et al. (1995)
SNV	*LacZα*	1×10^{-5}	2×10^{-5}	Julias et al. (1997), Kim et al. (1996) and Pathak and Temin (1990a,b)
HTLV-1	*LacZα*	4×10^{-6}	7×10^{-6}	Mansky (2000)
BLV	*LacZα*	1×10^{-6}	5×10^{-6}	Mansky and Temin (1994)
Retrotransposon				
TY-1	TY genome	2×10^{-5}	ND	Gabriel et al. (1996)
	Average	3×10^{-5}	5×10^{-5}	

[a] Includes base substitutions, frame shifts, genetic rearrangements (insertions/deletions) and hypermutations.
[b] As calculated in Svarovskaia et al. (2003).
[c] Not determined.
[d] As calculated in Drake et al. (1998).

tion also frequently results from template switching during obligatory primer strand transfer steps in the reverse transcription process (Hu et al., 1997; Yu et al., 1998; Zhang and Temin, 1993). Clearly recombination events contribute to viral diversity in HIV-infected individuals, as strains that are hybrids of differing subtypes are endemic in certain geographic regions (Kuiken et al., 2001). Recent analyses of splenocytes from HIV-1 infected patients show that cells harboring three or more genetically-distinct proviral copies are relatively common in vivo (Jung et al., 2002). These multiply infected cells provide ample opportunity for recombination-mediated rescue of defective genomes, as demonstrated in vitro (Dang et al., 2004; Li et al., 1992), and likely contributes to the sustained viability of HIV-1 and other retroelements that exhibit high mutation rates. Interestingly, HIV-1 is about 10-times more recombinogenic than several other retroviruses including spleen necrosis virus (SNV), MLV and human T-cell leukemia virus type 1 (HTLV-1) (Jetzt et al., 2000; Levy et al., 2004; Onafuwa et al., 2003; Rhodes et al., 2003; Yu et al., 1998). Lethal mutagenesis may be particularly effective against retroviruses with low recombination rates, due to their presumably diminished capacity for recombination-mediated repair of defective genomes.

Collectively, the aforementioned studies reveal many of the mechanistic details underlying the generation of diversity in retroviral populations. This diversity provides the raw material for viral adaptation, as witnessed by the rapid emergence of variants that thwart containment by the host immune system (Klenerman et al., 2002; Stebbing et al., 2003) and the common appearance of drug-resistant mutants during antiviral therapy (Shafer, 2002).

3. Error catastrophe in HIV

3.1. Evidence for an error threshold in HIV replication

The same error-prone replication strategy that provides retroviruses with exceptional adaptability also imposes a substantial load on the population by continually producing defective genomes. The highly defective character of retroviral isolates was appreciated in early studies of avian retroviruses, which revealed that a significant proportion of the proviral copies isolated from infected cells contained deletions, rearrangements or other defects in the viral genome (Fung et al., 1981; Hughes et al., 1978; O'Rear and Temin, 1981). Similar findings were also reported for other retroviruses (de Noronha et al., 1996; Hiramatsu and Yoshikura, 1986; Shields et al., 1978). For HIV-1, the frequency of defective proviruses in various tissue compartments ranges from 10 to 70%, based on analyses of specific portions of the genome (Li et al., 1991; Sanchez et al., 1997). Experiments with retroviral vectors have confirmed that deletions, insertions, duplications and frameshift mutations arise at high rates during retroviral replication (Gao et al., 2004; Mansky and Temin, 1994, 1995; Mansky, 2000; Parthasarathi et al., 1995; Pathak and Temin, 1990a,b). For example, as many as 50% of MLV proviruses produced during a single cycle of replication contain gross sequence rearrangements (Parthasarathi et al., 1995). Deleterious point mutations also arise at high rates in HIV-1 (Gao et al., 2004) and other retroviruses (Table 1). Thus, it is clear that spontaneous mutations are a significant cause of defective particles in retroviral populations.

Support for the idea that retroviruses exist near the error threshold can be found in experiments that examine the effects of mutagenic conditions on viral replication and mutation. Early studies of SNV showed that exposure of infected cells to 5-azacytidine (5-azaC) conferred a 15-fold increase in the viral mutation rate and a concomitant 95% reduction in titer, with minimal cellular toxicity (Pathak and Temin, 1992). Similarly, agents that alter the intracellular concentrations of dNTPs (i.e. hydroxyurea or thymidine) modestly increase the mutation frequencies and/or rates of SNV, MLV and HIV-1 vectors (≈5-fold), often with significant reductions in replication capacity (Julias and Pathak, 1998; Mansky et al., 2002, 2003; Pfeiffer et al., 1999). Chain-terminating nucleoside analogs with potent antiviral activities also perturb nucleotide pools and cause elevated mutation frequencies in cultured retroviruses (Jewell et al., 2003; Julias et al., 1997; LaCasse et al., 1996; Mansky and Bernard, 2000; Mansky et al., 2002, 2003). Together, these observations are consistent with the idea that as the error threshold is approached, small increases in mutation rates result in disproportionately large declines in viability (Domingo, 2003; Eigen, 2002).

Similar studies suggest that the high mutation rates of RNA viruses also approach the error threshold. In early experiments with poliovirus and vesicular stomatitis virus (VSV), exposure of virus stocks to increasing doses of chemical mutagens resulted in up to 100-fold reductions in infectious titer, with corresponding increases in mutation frequency of threefold (Holland et al., 1990). Similar results have been obtained in more recent studies of RNA viruses cultured in the presence of mutagenic nucleoside analogs (Airaksinen et al., 2003; Crotty et al., 2000, 2001; Grande-Perez et al., 2002; Pariente et al., 2001, 2003; Ruiz-Jarabo et al., 2003; Severson et al., 2003; Sierra et al., 2000; Zhou et al., 2003; see other articles this issue). In most cases, dramatic losses in titer were coincident with slight increases in mutation frequency. With the exception of the lytic bacteriophage Qβ, RNA virus and retrovirus mutation rates are similar (Drake, 1993; Drake et al., 1998; Drake and Holland, 1999). Thus, it appears that the spontaneous mutation rates of both retroviral and RNA viral genomes are close to the error threshold (Domingo, 2003; Eigen, 2002).

Other experiments have taken a genetic approach to examine the effects of increased mutation burden on retroviral replication. Amino acid substitutions that reduce HIV-1 RT fidelity in biochemical assays yield only modest (three- to four-fold) increases in virus mutation rates (Mansky, 2000; Mansky et al., 2002, 2003). Similar RT fidelity mutants introduced into MLV result in a maximal increase in virus mutation frequency of six-fold, with the majority of substitutions greatly reducing viral replication (Halvas et al., 2000a,b). This pattern of modest mutation rate increases coupled with substantial replication loss suggests error catastrophe. However, many amino acid substitutions that reduce RT fidelity also compromise enzyme activity, and it is likely that the reduced replication capacities observed in many of these genetically engineered viruses result from reduced RT activity and not error catastrophe. This is supported by recent studies showing that combinations of drugs and mutator RTs can increase HIV-1 mutation rates up to 30-fold (Mansky et al., 2002). Clearly, additional experiments are required to firmly establish the upper limit of the retroviral mutation rate.

3.2. HIV-1 restriction by APOBEC3G: host cell-mediated error catastrophe?

Recent studies of host cell factors that restrict HIV infection have revealed what may be a natural form of error catastrophe (reviewed in Goff, 2003; Gu and Sundquist, 2003; KewalRamani and Coffin, 2003; Vartanian et al., 2003). It has long been recognized that the viral protein Vif is essential for replication of HIV in primary $CD4^+$ T lymphocytes and some immortalized T cell lines (Chowdhury et al., 1996; Fisher et al., 1987; Strebel et al., 1987; von Schwedler et al., 1993). The diminished infectivity of Δ*vif* virions results from the packaging of host-encoded apolipoprotein B mRNA-editing complex 3G (APOBEC3G, formerly known as CEM15 (Sheehy et al., 2002). This enzyme is a member of the cytidine deaminase superfamily, which includes activation-induced cytidine deaminase (AID), adenosine deaminases that act on RNA (ADARs), and other APOBEC family members (Gerber and Keller, 2001). Incorporation of APOBEC3G into virions results in deamination of minus-strand cytidines, thereby leading to G → A hypermutation of the plus-strand of newly synthesized HIV-1 DNA (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003b). Thus, APOBEC3G appears to inhibit HIV-1 replication by direct modification of the viral genome. Vif counteracts this inhibition by interacting with APOBEC3G and preventing its incorporation into virions (Xu et al., 2004 and references therein).

The pattern of G → A hypermutations observed in Δ*vif* genomes suggests that APOBEC3G-catalyzed deamination occurs during and/or immediately following minus-strand DNA synthesis (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003b). However, the exact stage of the reverse transcription process that is targeted by APOBEC3G is unclear. One possibility is that APOBEC3G begins deaminating single-stranded DNA following the degradation of plus-stranded RNA by the RNase H function of RT. This would be consistent with the preferential ability of APOBEC3G to deaminate cytidine residues in single-stranded DNA in vitro (Harris et al., 2003; Suspene et al., 2004; Yu et al., 2004b), and would also be in agreement with the substrate specificities observed for related nucleic acid deaminases (Bhagwat, 2004; Gerber and Keller, 2001). For example, APOBEC1 deaminates cytosine in single-stranded DNA and RNA but not double-stranded DNA (Petersen-Mahrt and Neuberger, 2003; Teng et al., 1993), and activation-induced deaminase targets cytidine bases in single-stranded DNA but not double-stranded DNA or DNA–RNA hybrids (Bransteitter et al., 2003; Dickerson et al., 2003). However,

the possibility remains that APOBEC proteins act on RNA and/or DNA–RNA hybrid duplexes that are formed during reverse transcription (Bishop et al., 2004b). Further biochemical studies would reveal specific substrate requirements for deamination of the HIV genome.

While the evidence suggests that miscoding resulting from deamination is the primary mechanism of APOBEC3G-mediated restriction, other processes may also contribute to the antiviral action of this host cell enzyme. First, conversion of cytidine to uridine by APOBEC3G may result in aberrant initiation of plus-strand DNA synthesis by RT (Klarmann et al., 2003), which would presumably result in loss of viral infectivity. In addition, deoxyuridines generated by APOBEC3G are likely substrates for host-encoded uracil DNA-glycosylases, which generate abasic sites in the DNA strand (Krokan et al., 2002). Abasic sites are recognized and cleaved by apurinic/apyrimidinic endonucleases (Dianov et al., 2003), and the resulting strand breaks would likely result in degradation of the viral DNA. This may explain the reduced levels and increased lability of viral DNA formed after infection of cells with Δ*vif* virions (Fouchier et al., 1996; Mangeat et al., 2003; Mariani et al., 2003).

The discovery of APOBEC3G as a mechanism of host cell restriction provides an avenue for the development of agents that specifically enhance the antiviral activity of this editing enzyme (Harris and Liddament, 2004). It may be possible to design small-molecule inhibitors that interfere with the binding of Vif to APOBEC3G, resulting in lethal deamination of viral genomes in cells that are otherwise permissive for HIV infection. In addition, it is plausible that other cellular enzymes target the HIV genome for editing or modification, and that these activities are normally inhibited by viral proteins in a manner analogous to the interaction between Vif and APOBEC3G (see note added in proof). Thus, the induction of "lethal editing" (Mangeat et al., 2003) by promoting the enzymatic modification of viral RNA or DNA may provide an important therapeutic tool to control HIV infection.

4. Mutagenic nucleoside analogs as antiretroviral agents

Two strategies for lethal mutagenesis of HIV have been proposed, both involving nucleic acid precursors that are incorporated into the viral genome and mispair at high frequencies (Fig. 2). In the first scenario, a mutagenic ribonucleoside-5′-triphosphate analog is incorporated into newly-transcribed viral RNA by cellular RNA polymerase II (Fig. 2, Step 1), resulting in incorrect base-pairing during subsequent RT-catalyzed minus-strand DNA synthesis in newly infected cells (Step 2, (Loeb and Mullins, 2000)). Given the absence of RT-associated proofreading activity (Roberts et al., 1988) and the relatively poor activity of DNA repair enzymes on RNA–DNA hybrid duplexes (Kamath-Loeb et al., 1997b), these mismatched nucleotides are unlikely to be excised from the nascent viral DNA. Incorporation of the adducted base into host cell mRNA is expected to have relatively minor consequences, due to the short half-lives of these molecules (Ross, 1996) and the fact that mRNA copies do not become part of the cellular genome unless the RNA is retrotransposed by an endogenous RT (a relatively rare event). However, a fraction of the analog may be converted to the deoxy-form and polymerized into both viral and host DNA. The cellular genotoxicity of these adducts would likely be mitigated by the activities of specific repair enzymes, lesion bypass polymerases, and proteins that facilitate recombination-mediated repair of cellular DNA (McGowan, 2003). Thus, mutagenic analogs would presumably be significantly more deleterious to the virus than to the host cell.

In the second strategy, lethal mutagenesis is achieved using a mutagenic deoxyribonucleoside analog that is metabolized to its 5′-triphosphate by cellular enzymes and incorporated into nascent viral DNA by RT (Loeb et al., 1999). Analogs that are incorporated into viral DNA during RT-catalyzed minus-strand DNA synthesis (Fig. 2, Step 2) generate mispairs during subsequent plus-strand DNA synthesis (Step 3) and are likely to avoid repair in the precursor RNA–DNA duplex (Kamath-Loeb et al., 1997b). Similarly, analogs incorporated during plus-strand synthesis (Step 3) generate mispairs when copied by host DNA polymerases (Step 4). As noted above, analogs that are incorporated into cellular DNA are subject to repair, bypass or avoidance through recombination (McGowan, 2003). Although additional specificity can theoretically be accomplished using analogs that are better substrates for RT than for host polymerases, this important goal remains to be achieved.

4.1. Initial studies with 5-OH-dC

Efforts to develop strategies for lethal mutagenesis of HIV have focused primarily on the antiviral properties of mutagenic deoxyribonucleoside analogs. Initial experiments involved a series of deoxyribonucleosides known to generate mutations during DNA synthesis by RT and/or cellular DNA polymerases (Loeb et al., 1999). These analogs were screened for the ability to increase the viral mutation frequency during seven serial transfers of HIV-1 in culture. Two analogs that produced the largest increases in mutation frequency (three- to five-fold) were chosen for further study. Passage of virus stocks for an additional seven transfers in the presence of O^4-methyl-dT did not cause a significant reduction in titer, as measured by the concentration of HIV-1 capsid p24 in culture supernatants. In contrast, passage of the virus in the presence of 1 mM 5-hydroxydeoxycytidine (5-OH-dC) resulted in a sudden and dramatic drop in p24 levels in passages 15 and 16. No loss of titer was observed in parallel HIV-1 cultures passaged in the absence of the analog. Sequence analyses of PCR products amplified from passage 16 virus revealed a six-fold increase in the frequency of G → A substitutions in cloned DNA fragments from the analog-treated cultures relative to the untreated control cultures. This result is consistent with the incorporation of 5-OH-dCMP opposite template

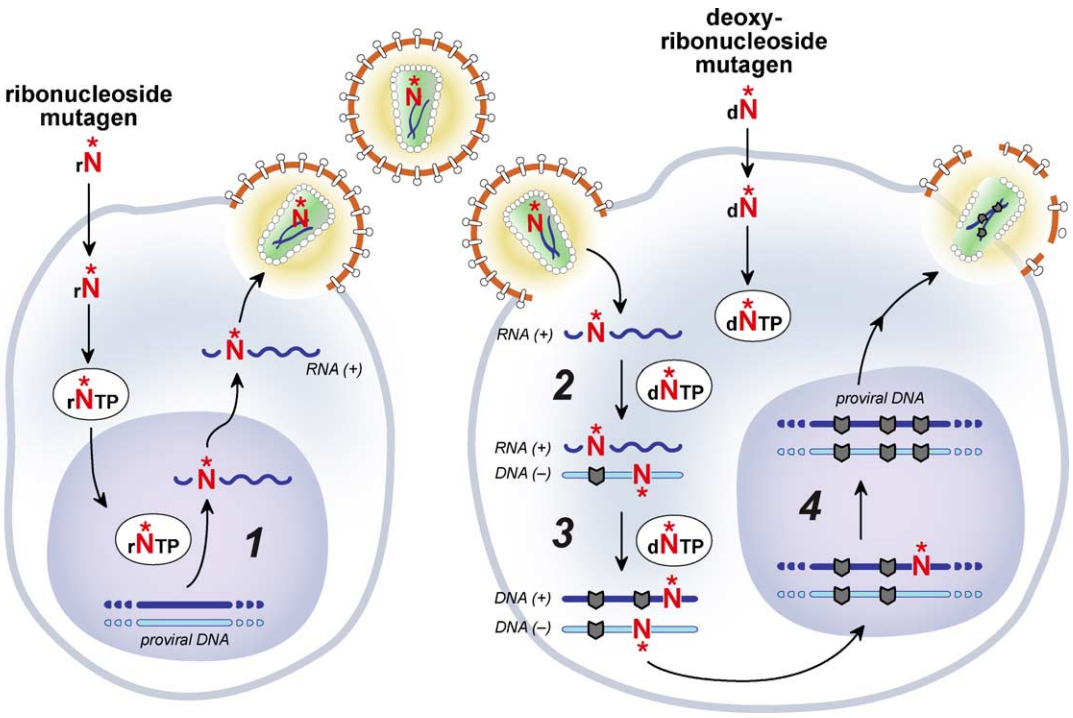

Fig. 2. Proposed strategies for lethal mutagenesis of HIV. Left: Incorporation of a mutagenic ribonucleoside-5′-triphosphate analog (r N* TP) into the nascent RNA genome of HIV by RNA polymerase II (RNA pol II). Right: Incorporation of a mutagenic deoxyribonucleoside-5′-triphosphate analog (dN*TP) into the viral DNA by RT and generation of mutations (chevron symbols) during subsequent polymerization steps. A portion of the analogs incorporated during RT-catalyzed plus-strand DNA synthesis are likely to be repaired by cellular enzymes, and thus would not result in mispair formation. Numbers refer to different steps in the replication cycle at which base analogs are inserted and/or copied by polymerases, as described in the text.

G residues during RT catalyzed minus-strand synthesis, followed by incorporation of dAMP opposite the lesion site during plus-strand synthesis (Kreutzer and Essigmann, 1998; Purmal et al., 1994). The mutator effect of 5-OH-dC was specific for G → A substitutions; no significant increase in overall virus mutation frequency was observed in the analog-treated cultures.

Two additional serial passage experiments were performed to verify the effects of 5-OH-dC treatment on HIV-1 replication. In four of the six analog-treated cultures, titers declined to levels below the limit of detection after 8–25 passages and were not recovered in additional passages of the culture supernatants. The remaining two analog-treated HIV-1 cultures retained detectable titers after 25 passages; detectable levels of virus in one of these were abolished after a total of 58 passages (Loeb, unpublished data). As in the first experiment, titer loss was coincident with an increase (three-fold) in the frequency of G → A substitutions. Viral titers were not significantly diminished in any of the untreated control cultures passaged in parallel.

Based on these data, we can conclude that exposure of HIV-1-infected cells to 5-OH-dC frequently results in loss of detectable levels of virus, with a concomitant increase in the G → A mutation frequency. These observations are consistent with the idea that the dramatic drop in viral titers resulted from the entry of HIV-1 into error catastrophe (Domingo, 2003; Eigen, 2002). Continued viral replication in one of the analog-treated cultures may have been due to the emergence of 5-OH-dC-resistant mutants, although this possibility remains to be addressed.

4.2. Other mutagenic nucleosides

The results obtained with 5-OH-dC suggest that specific nucleoside analogs containing modified bases are effective HIV mutagens. However, the relative high concentrations of 5-OH-dC used in these experiments (0.5–1.0 mM) and the long culture periods required to reduce viral titers make this particular compound a questionable candidate for clinical development.

To identify mutagenic deoxyribonucleosides with improved potency, purine and pyrimidine analogs containing aberrant bases have been screened for anti-HIV activity in cell culture. These experiments have produced two deoxycytidine compounds for further study. The first of these, 5-formyl-2′-deoxycytidine (5-fo-dC), is an oxidation product formed by exposure of 5-methyl-2′-deoxycytidine to ultraviolet or gamma radiation (Bienvenu et al., 1996; Privat and Sowers, 1996). 5-fo-dC readily base pairs with A or T, forming C → T or C → A mutations during cell-free DNA synthesis by *Escherichia coli* DNA polymerase I (Karino et al., 2001) and in mammalian cells (Kamiya et al., 2002). Treatment of cells with 5-fo-dC inhibited HIV replication within a single passage, with an EC_{50} (the concentration

of inhibitor required to reduce virus replication by 50%) of 3 μM (Daifuku, 2003). Inhibition of viral replication correlated with a two-fold increase in mutation frequency. No cytotoxic effects were observed at 5-fo-dC concentrations as high as 1 mM. The second compound, 5,6-dihydro-5-aza-2′-deoxycytidine (SN1212), provides even greater potency of inhibition, with an EC_{50} of 10 nM (Daifuku, 2003). The antiretroviral efficacy of SN1212 is currently being evaluated in an animal model of HIV-1 infection.

The potential for using mutagenic ribonucleosides to inhibit HIV infection has been largely unexplored. However, the observation that 5-azaC inhibits SNV replication and confers a substantial increase in mutation rate (Pathak and Temin, 1992) suggests that certain ribonucleoside base analogs may be effective retroviral mutagens. The antiviral activity of 5-azaC has been confirmed in conventional drug susceptibility assays with HIV-1 in vitro (EC_{50}s = 1 μM; (Bouchard et al., 1990). Thus, 5-azaC may represent a prototypic ribonucleoside for lethal mutagenesis of HIV.

Additional efforts to identify mutagenic nucleosides have used a cell-free reaction system that mimics the transcription and reverse transcription steps of the retroviral life cycle (Moriyama et al., 2001). In this assay, plasmid DNA serves as a template for T7 RNA polymerase-catalyzed transcription, and the resulting RNA copies are reverse-transcribed using avian myeloblastosis virus (AMV) RT. cDNA copies from the RT reaction are amplified by PCR, recloned into the plasmid vector and sequenced to score mutation frequency. This system was used to evaluate the mutagenic potential of nucleoside "P" which contains the tautomeric base analog 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (P) (Moriyama et al., 2001). The deoxyribose-5′-triphosphate form of this analog (dPTP) base pairs with template bases A or G (Negishi et al., 1997) and generates high frequencies of all four possible transition mutations in cell-free polymerase reactions (Zaccolo et al., 1996). Similarly, the ribonucleotide analog rPTP enhanced the frequency of error formation in the transcription/reverse transcription system, primarily due to an increase in C → T and T → C mutations (Moriyama et al., 2001). The ability of rP or dP to increase retroviral mutation frequencies and/or inhibit viral replication has not been reported.

4.3. *Antiviral mutagens in combination therapy*

Ideally, analogs that increase mutational loads would interact favorably with other drugs currently used to treat HIV infection. A beneficial synergistic action may result from therapeutic regimens involving combinations of mutagenic base analogs and conventional chain-terminating nucleoside inhibitors. The inhibitory potential of a chain-terminator depends in part on its relative concentration with respect to its corresponding physiological dNTP. Thus, improved potency can be achieved by diminishing the intracellular concentration of the competing substrate. This effect has been demonstrated in vitro for a number of inhibitor/pool modifier combinations (Ahluwalia et al., 1994; Balzarini et al., 1991; Borroto-Esoda et al., 2004; Foli et al., 1997; Gao et al., 1999; Johns et al., 1993) and is the mechanistic basis for the clinical use of hydroxyurea with chain-terminating RT inhibitors (Lori and Lisziewicz, 2000; Rossero et al., 2003; Stebbing et al., 2004). Interestingly, alterations of dNTP precursor pool levels also enhance error formation during retroviral replication (Julias and Pathak, 1998; Mansky et al., 2002, 2003; Pfeiffer et al., 1999), as discussed above. Thus, potent viral inhibition might be achieved using mutagenic nucleosides that simultaneously exhibit two modes of action; modulation of dNTP pools to enhance chain termination and induce a mutagenic state in the host cell, and enhancement of mispair formation by incorporation into the HIV genome.

5. Potential challenges to antiretroviral mutagenesis

Many of the same barriers to conventional antiretroviral therapy are likely to present obstacles for lethal mutagenesis. A primary concern is the issue of viral latency. Several cellular and anatomical reservoirs contribute to the long-term persistence of HIV-1, even in patients whose peripheral viral loads are suppressed to undetectable levels (reviewed in Pierson et al., 2000). Of these, the latent population of integrated, replication-competent genomes in resting $CD4^+$ T-cells is perhaps the most worrisome (Chun et al., 1995). This reservoir is believed to be extremely stable ($t_{1/2}$ ∼1–4 years) and may be continually reseeded by low levels of ongoing viral replication during suppressive therapy. Activation of resting $CD4^+$ T-cells by antigenic or mitogenic stimuli results in the production of infectious virions in vitro, and is likely to contribute to the resurgence of viral loads following interruption of drug treatment in vivo (Pierson et al., 2000). Therefore, the maximum therapeutic benefit that can be realized from lethal mutagenesis is probably durable suppression of viral load, rather than complete clearance.

Another potential obstacle to antiviral mutagenesis is the development of drug resistance. HIV-1 variants resistant to all currently approved drugs have been extensively documented and characterized (Parikh et al., 2001; Shafer, 2002), and patients failing combination therapy frequently harbor viruses resistant to one or more drugs in the regimen (Englund et al., 2004; Gallego et al., 2001; Havlir et al., 2000). In addition, detection of resistant variants prior to initiation of treatment is often an independent predictor of therapy failure (Little et al., 2002; Van Vaerenbergh et al., 2000). Thus, the development of drug resistance is generally associated with a poor clinical outcome. The use of mutagenic nucleosides in combination with conventional drugs is unlikely to prevent the development of resistance, as similar treatments of cultures infected with the RNA virus FMDV readily select for inhibitor-resistant variants (Pariente et al., 2003). The identification of ribavirin-resistant mutants in culture (Pfeiffer and Kirkegaard, 2003; Scheidel et al., 1987; Scheidel and Stollar, 1991) and in patients infected with hepatitis C virus (Young

et al., 2003) suggests that resistance to mutagenic analogs is also a likely outcome. These findings raise the intriguing question of how HIV might evolve to acquire a mutagen-resistant phenotype. One possibility is that mutations in RT would compromise the binding and/or insertion of dNTP base analogs at the primer terminus, thereby diminishing their incorporation during DNA synthesis. In the case of ribonucleoside analogs, resistance may be more difficult to achieve since incorporation is dependent on the host cell RNA polymerase. However, substitutions in RT that favor insertion of the "correct" nucleotide opposite adducted bases in the RNA template strand could potentially produce resistance to the mutagen. While evidence for such a mechanism is lacking, the ability of HIV to tolerate substantial genetic change in RT (Smith et al., 2004) suggests that resistance to mutagens will not be prevented by functional constraints in the viral polymerase. Selection and characterization of mutagen-resistant HIV mutants in cell culture could readily address this issue.

6. Perspectives

Although lethal mutagenesis has been demonstrated in HIV-1 and a variety of other RNA viruses, several important questions concerning this strategy remain unanswered. Perhaps the most important experimental task is delineating the exact mechanism by which mutagenic nucleosides exert antiviral effects. Specifically, the possibility that viral mutagens reduce titers by blocking other steps in viral replication should be more thoroughly addressed. Important examples of the pleiotropic effects of viral mutagens can be found in studies of ribavirin, a broad-spectrum inhibitor of RNA virus replication. Ribavirin treatment of infected cells induces elevated error frequencies in several viruses, suggesting that drug-induced mutagenesis contributes to loss of viral viability (Airaksinen et al., 2003; Crotty et al., 2000, 2001; Kanda et al., 2004; Severson et al., 2003). However, a number of other modes of action have also been proposed for ribavirin (reviewed by Benarroch et al., 2004; Crotty et al., 2002; Zhang et al., 2003a). It therefore is likely that the drug exerts several antiviral effects, even in cases where enhanced mutagenesis is presumed to be the principal mechanism. In addition, ribavirin and other viral mutagens may perturb host cell homeostasis by interfering with RNA splicing (Ghoshal and Jacob, 1997) or post-transcriptional RNA modification (Longley et al., 2003). Simple measurements of cytotoxicity are likely to underestimate the subtle cytostatic effects of viral mutagens on host cell nucleic acid biosynthesis and metabolism.

Finally, the issue of clinical utility must be addressed. Viral latency is a serious obstacle to conventional antiretroviral therapy (Pierson et al., 2000) and is likely to present similar barriers to clearance by lethal mutagenesis. However, the recent observation that viral mutagens prevent persistent arenavirus infection in mice (Ruiz-Jarabo et al., 2003) is encouraging and suggests that similar inhibitors of HIV can be identified that are safe and effective. Mutagenic analogs may be useful both for the treatment of drug-naïve patients and for salvage therapy of individuals harboring virus resistant to conventional antiretroviral drugs. Similarly, strategies that induce a mutagenic state by altering nucleotide pool levels, enhancing the lethal editing activity of APOBEC3G, or activating other nucleic acid-editing enzymes represent novel approaches to antiretroviral therapy. Further research in the emerging field of viral error catastrophe should provide valuable insights into the effects of elevated mutation rates on viral replication and inspire promising new approaches for treating HIV infection.

Note added in proof

While this review was in preparation, several other deaminases in the APOBEC family have been shown to generate G → A hypermutations in HIV-1 and/or simian immunodeficiency virus (Bishop et al., 2004a; Liddament et al., 2004; Wiegand et al., 2004; Yu et al., 2004a; Zheng et al., 2004). For a review of these and other recent discoveries related to APOBEC-mediated restriction, see (Harris and Liddament, 2004).

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