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REVIEW

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Virtues of being faithful: can we limit the genetic variation in human immunodeficiency virus?

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Abstract Human immunodeficiency virus (HIV) infections are characterized by a high degree of viral variation. The genetic variation is thought to be a combined effect of a high error rate of reverse transcriptase (RT), viral genomic recombination, the selection forces of the human immune system, the requirement for growth in multiple cell types during pathogenesis, and persistent immune activation associated with HIV disease. This hypermutability gives the virus an ability to escape mechanisms of innate immune surveillance and therapeutic interventions. Indeed, HIV variants that are resistant to drugs that antagonize both the HIV protease and RT enzymes are well described. Furthermore, there are seemingly no procedures to restrict this disarming property of HIV to mutate rapidly. Recently we have shown that some of the drug-resistant RTs display an increased in vitro polymerase fidelity. The question is whether this finding will stimulate new approaches that will not only help the immune system to deal with the virus more efficiently but also to reduce or delay resistance to various classes of anti-HIV drugs. The pros and cons of this concept and the influence of viral replication rates and viral fitness on HIV variability are discussed.

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Abbreviations *AZT* 2',3'-dideoxy,3'-azathymidine · *DDDP* DNA-dependent DNA synthesis · *dNTP* Deoxynucleotide triphosphate · *dUTP* Deoxyuridine triphosphate · *HIV* Human immunodeficiency virus · *RDDP* RNA-dependent DNA synthesis · *RNA* pol II RNA polymerase II · *RT* Reverse transcriptase · *3TC* (–)2', 3'-Dideoxy,3-thiacytidine

Introduction

Human immunodeficiency virus (HIV) infections are characterized by a high degree of genetic variation [1–4]. The presence of multiple genetic variants coexisting within the same patient has led HIV to be termed a quasispecies. The ability of HIV to rapidly vary its genetic information allows it, during the course of pathogenesis, to infect various cell types (macrophage/monocytes, T lymphocytes, and central nervous system cell types), to alter its biological phenotypes, and to evade immune response and drug treatment. There are no procedures to counter this disarming rate of variation. Based on the discovery that some of the drug-resistant reverse transcriptases (RTs) display an increased polymerase fidelity [5–8], we have conjectured

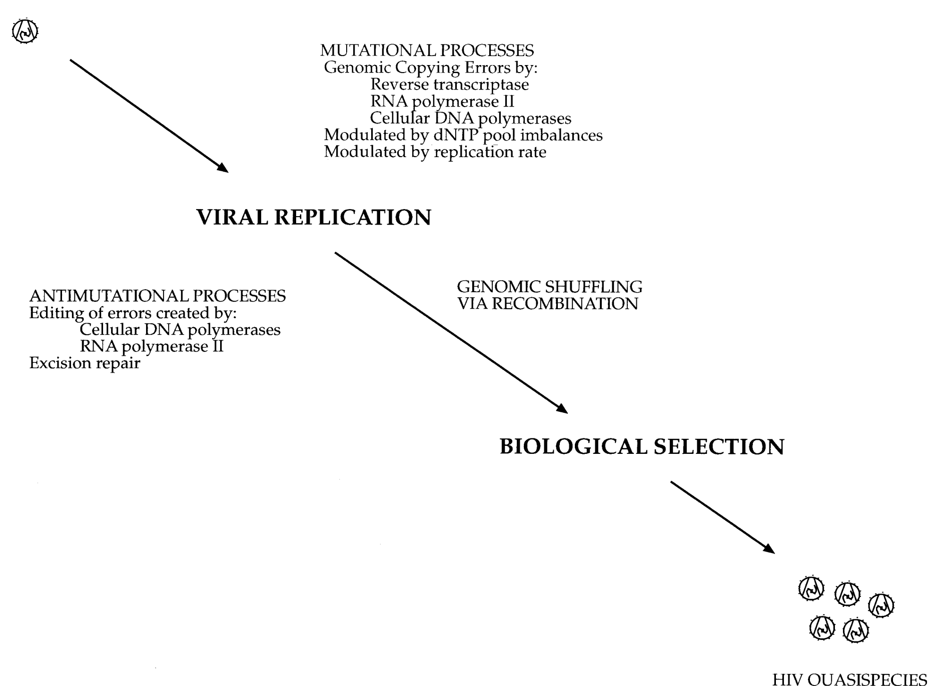

Fig. 1 The various factors that contribute to the origin of HIV quasispecies. The single particle shown represents a virus particle prior to replication (and not the initial viral inoculum in a patient). The processes that promote or suppress mutations during the viral replication, those that further accentuate or reduce variation in an individual are shown

that this finding may lead to new approaches that might help the immune system to deal with the virus more efficiently and possibly reduce or delay resistance to various classes of anti-HIV drugs [7, 9]. In this review we discuss various factors that contribute to genetic variation in HIV, its advantages to the virus, and the feasibility of approaches to limit such variation.

Factors that influence HIV variation

Genetic variation in HIV provides an effective means for the virus to successfully establish infection and pathogenesis. On the one hand, variation allows the virus to evolve from a slowly replicating, macrophage-tropic strain to a rapidly replicating T-cell tropic virus. On the other hand, it provides a means of escape from immune surveillance and drug therapy.

Several factors contribute to the high rate of variation in HIV and include: (a) copying by RT, the viral DNA polymerase, (b) error repair, (c) the rate of viral replication, (d) genomic recombination, and (e) biological selection, including immunological surveillance. Copying errors by polymerase during DNA synthesis play a major role in the generation of mutations. The rate of such mutations can be increased by mutagens as well as by changes in deoxynucleotide triphosphate (dNTP) precursor pools and decreased by error avoidance/error repair mechanisms. Also, a high rate of viral replication can enhance minor variants in a relatively short time. Furthermore, one must then overlay the mutation-shuffling effects of genomic recombination on the overall error rate. Finally, consideration must be given to the biological effect of each mutation on the phenotype of the gene product and its selective value (Fig. 1). Some mutations may be lethal, causing the loss of the mutant virus from the population, and others might confer an advantage that leads to its overrepresentation in the population. Thus, selective forces adjust the number of mutations created at the level of genomes to reflect losses and gains. We consider below the contribution of each of these factors to the overall mutation rate of HIV.

Error-prone processes

Genomic mutagenesis via copying errors can result from the action of three polymerases. These include the viral RT that performs reverse transcription, the host cell DNA-dependent DNA polymerase which replicates the integrated provirus, and the cellular RNA polymerase II which transcribes the integrated viral DNA into RNA. Of these, contribution of the host cell DNA polymerase is thought to be negligible. The contribution of RNA polymerase II (RNA pol II) to error has been perceived to be significant since this enzyme has no proofreading activity. However, accurate measurements of mutation rates by RNA pol II are not available. There is in vitro evidence that appears to indicate that the eukaryotic elongation factor TFIIS substantially enhances RNA pol II transcription fidelity [10] in a manner analogous to the role played by the bacterial Gre A protein in maintaining the fidelity of *E. coli* RNA polymerase-mediated transcription [11]. Hence the actual fidelity of RNA pol II catalyzed polymerization in vivo is likely to be much higher than originally perceived. Therefore, although errors at the level of cellular RNA polymerase II may also occur and contribute to HIV genetic diversity, the low fidelity of HIV RT is generally thought to be a key cause of the high mutation rate in HIV [12, 13].

RT is responsible for replication of the viral genome, first by the synthesis of a DNA copy from the RNA genome (RNA-dependent DNA synthesis; RDDP), then by synthesis of a second DNA strand (DNA-dependent DNA synthesis; DDDP). The result is a double-stranded DNA which is transported to the nucleus and integrated into the host genome. Errors can potentially occur at each of the two levels of DNA synthesis (RDDP and DDDP). In contrast to most cellular DNA polymerases, retroviral RTs neither possess a proofreading activity nor are found to associate with a protein that possesses such an activity. A comparison of HIV-1 RT with other retroviral RTs has shown a lower fidelity for HIV RT using in vitro kinetic studies of nucleotide misinsertion [14] or mispair extension [15] as well as by genetic assays designed to measure reversion and forward mutation frequencies [14, 16]. These studies show that HIV-1 RT both inserts incorrect nucleotides and extends mismatched termini at a relatively high efficiency. However, several studies have demonstrated that the rates for specific mutations can vary significantly in a template sequence-dependent manner [17–19]. These results indicate that using a single template sequence to compare two polymerases is insufficient to determine the role of polymerase fidelity on mutation rates. Other studies performed to compare the fidelity of RDDP and DDDP activities of HIV-RT have found that both processes are subject to high error rates [19, 20]. Whether these reflect mutation rates during viral replication in vivo remains to be seen. Recently an in vivo method for determining overall mutation rate was developed, and it has shown that the mutation rate of HIV-1 during a single cycle of infection is 20-fold lower than those estimated via in vitro fidelity assays using recombinant purified HIV-1 RT [21]. It is unclear whether this discrepancy is due to cellular repair processes, or whether the results reflect differences between the HIV strains used in the in vitro (NY5) and in vivo (NL4–3) assays.

The polymerization process is also subject to influences of dNTP pool composition. Imbalances in dNTP pools can affect the dNTP utilization kinetics of RT, which in turn can affect viral replication [22, 23]. In fact, the modulation of dNTP pools has been investigated as a strategy to inhibit HIV-1. For example, hydroxyurea is an inhibitor of cellular ribonucleotide reductase, a regulator of nucleotide pools, and can synergize with several nucleoside analogs to block replication of both wild-type and drug-resistant variants of HIV-1 [24–26]. It is also likely that nucleotide pool composition can affect the fidelity of viral replication by RT and its error specificity [27]. It has been proposed that the appearance of G→A hypermutations in HIV-1 viral genomes results from low levels of deoxycytidine triphosphate in the dNTP pool [28], based on the observation that the frequency of these types of mutations increases in the absence of deoxycytidine triphosphate. However, other reports indicate [29, 30] that this explanation does not hold for all types of G→A hypermutations.

Error avoidance and repair

A major mechanism of error avoidance by polymerases is the 3'-5' exonuclease activity, which is lacking in RTs. A second common error avoidance mechanism utilized by many DNA polymerases, the inefficient extension of mispaired termini, is also not well used by HIV-1 RT [15].

While errors made during the second strand synthesis (DDDP) are likely subject to cellular repair processes, the design of retroviral reverse transcription precludes repair of errors created during the first DNA strand synthesis (RDDP). The rapid removal of the RNA strand from the RNA.DNA hybrid results in a DNA product that is single stranded (or is in a duplex state only transiently). Hence it would seem that mispaired regions due to nucleotide substitution, deletion, or insertion events do not exist long enough to be recognized and corrected. Moreover, these mispaired intermediates are presumably not accessible to repair enzymes since they occur within a nucleoprotein complex.

Retroviruses must also avoid the misincorporation of deoxyuridine triphosphate (dUTP) present in the host cell into their genomes as dUTP can substitute for deoxythymidine triphosphate during the polymerization process. Intracellular concentrations of dUTP can influence mutation rate by affecting the level of dUTP incorporation into DNA which results in T→C transitions mediated by G.U basepairing. Cellular mechanisms that counterbalance this process involve two key enzymes: dUTP pyrophosphatase (dUTPase) and uracil-*N*-glycosylase. While dUTPase degrades dUTP in the cell, uracil-*N*-glycosylase removes the uracil residues from DNA. Some type D retroviruses and lentiviruses including equine infectious anemia virus, feline immunodeficiency virus, and caprine arthritis-encephalitis virus encode a dUTPase within their pol open-reading frame [31]. The presence of dUTPase in several lentiviruses appears to be an adaptation to facilitate replication within macrophages which contain high intracellular levels of dUTP [32]. Interestingly, however, HIV-1 and the related human immunodefiency virus type 2 (HIV-2) or simian immunodeficiency virus do not encode a dUTPase. This led to the question of how these lentiviruses remain unaffected by dNTP pools in macrophages. Suggestive evidence that HIV-1 has evolved alternative mechanisms to ensure low incorporation of dUTP has come from a recent study showing that HIV-1 Vpr associates with the cellular uracil-*N*-glycosylase [33]. Co-immunoprecipitation studies also show an association between these proteins [33]. Since Vpr is known to be a part of the preintegration complex, the associated uracil-*N*-glycosylase might be targeted to the integrated or unintegrated proviral DNA where it can remove the inserted uracil residues. A role for Vpr in error avoidance is further supported by a report that a variant of HIV-1 deficient in Vpr function displays a fourfold increase in mutation rate [34].

Genomic recombination

Retroviruses are well known to undergo deletions, insertions, and more complex genetic rearrangements during the process of reverse transcription [35]. The presence of two copies of viral genomic RNA per virion facilitates recombination between genetically distinct strains during coinfection. RT-mediated recombination is thought to involve strand transfer or template switching by RT during the copying of genomic RNA [35]. Additionally, in vitro evidence suggests that HIV RT-catalyzed strand transfer events are highly error-prone [36–39] However, many of the mutations seen in these in vitro studies were of the single nucleotide addition class and would lead to frameshifts and thus probably be lethal in vivo. Where base substitutions were observed, these were likely the result of strand transfer events that followed untemplated nucleotide additions onto blunt-ended RNA.DNA templates [36, 37, 39]. Since recombinogenic strand transfer events normally do not occur at blunt genomic termini but rather from internal template sites during viral replication, it is unlikely that base substitutions would happen in vivo via this type of mechanism [35]. Thus it appears likely that the predominant contribution of genomic recombination to genetic variation is to exchange or shuffle preexisting mutations that have resulted from uncorrected copying errors, to yield HIVs containing combinations of multiple mutations. Such variants may not have rapidly or readily arisen solely through polymerization errors. Of particular clinical significance is the emergence of multidrug-resistant recombinants that have been recently observed [41, 42], adding another dimension of complexity to the already difficult task of controlling HIV infection via drug therapy. In addition, the generation of the various subtypes or clades of HIV are thought to be the result of genomic recombinational events [43, 44].

Selective forces

Although the mutation rate per round of replication is defined as the sum result of copying errors and repair, the actual rate at which mutants accumulate in a population is influenced by selection and the rate of viral replication. Recent studies of in vivo viral dynamics [45–48] have shown that HIV-infected $CD4^+$ T cells undergo constant cycles of infection and clearance. By performing kinetic studies of viral outgrowth following drug treatment these groups were able to calculate the viral and $CD4^+$ T lymphocyte turnover rates in patients. They estimated the number of virions produced per day to be in the range of 10^7–10^9 within a single patient [48], with an average in vivo half-life of 2 days [45, 48]. Sustaining this large, short-lived population would require a high level of viral replication. Such a high level of replication would inevitably allow for rapid viral evolution. Calculations of probable viral growth rates and mutation frequencies [45, 49] suggest that this high level replication is enough to create a population of viruses of sufficient diversity to respond to any immunological or pharmacological challenge.

Another proposed mechanism that may influence viral population composition is the antigenic stimulation of infected lymphocytes or immune activation. This mechanism was proposed after a study of viral evolution in the spleen and peripheral blood of a single patient. In this study it was noted that 20% of the infected cells in the spleen harbored defective viruses [50], which suggests that the makeup and variability of the viral population within an infected individual can be partially attributed to the proliferation of infected lymphocytes. Subsequent studies [51, 52] which examined the spleens of four HIV-1 infected patients and one noninfected control patient revealed discrete compartments of HIV infected cells harboring the same mutations in the white pulp. These studies suggest that the proliferation of these defective viruses is mediated by the antigenic stimulation and subsequent cellular replication of infected cells in the spleen.

Biological consequences of variation and clinical implications

The genetic heterogeneity of the viral population provides an ideal set up for the dynamic evolution of the quasispecies as the disease progresses from early to late stage [53]. The variant subsets that are key to the pathogenetic process are classified on the basis of the host cell type they infect. Early in the disease course monocyte/macrophage tropic viruses predominate in the clinically latent stage of the disease [54]. Monocytotropic viruses are slowly replicating and non-syncytium-inducing [54]. During the progression to full-blown AIDS more rapidly replicating T-lymphocyte-tropic variants become the dominant quasispecies in an infected individual [53, 54]. It has been speculated that this shift in tropism is driven by immune pressure [55]. In the initial stages of the disease the rapidly replicating nature of T-cell-tropic viruses results in their presentation to the immune system and most likely to their rapid, selective clearance by the fully competent host immune response. Concurrently, as immune presentation of the more slowly replicating macrophage-tropic variants in the population is low at this point, these viruses emerge as the prevalent variants. Macrophage-tropic viruses persist and presumably contribute to gradual destruction of the host immune system during the asymptomatic phase. As a result, pressure from the immune response may eventually fall below the level needed to contain the more rapidly replicating and more cytotoxic T-cell-tropic viruses. The T-cell-tropic viruses presumably overrun the population, leading to the characteristic devastation of the immune system seen in late stage AIDS patients. At this point little significant immune pressure remains to drive the quasispecies development, and this may help to explain the relatively static nature of viral evolution late in disease [47].

HIV variation is also manifested in the antigenic diversity of the quasispecies population. Such diversity leads to the emergence of mutants that escape immunological neutralization [56]. The emergence of escape mutants has been demonstrated both in in vitro protocols using neutral-

izing antibodies to select for neutralization-resistant variants [57] and in neutralization studies with sequential, autologous viral isolates and sera from HIV-infected individuals [58]. Viral determinants recognized by neutralizing antibodies in vitro and possibly in vivo have been localized to epitopes contained in the viral envelope (env) proteins. In particular, neutralization determinants have been mapped to the hypervariable loops V2 and V3 of the HIV surface glycoprotein gp120 [59, 60]. In addition, V3 loop peptides have been found to be presented by the MHC class I and II of human antigen-presenting cells, implicating an important role for the V3 loop in cell-mediated immune clearance as well [61, 62].

The viral determinants involved in host cell tropism have also been mapped to the V3 and V2 loops [53, 63, 64]. Furthermore, it has been shown that some mutations in the V3 loop that lead to immune escape also produce shifts in tropism [65]. Such observations could indicate how tropism shifts simultaneously result in immune surveillance escape, allowing HIV successfully to evade host clearance.

The high level of genetic diversity in HIV-infected patients profoundly influences the outcome of clinical strategies for disease therapy and prevention. It is well documented that antiviral chemotherapies are frustrated by the eventual emergence of drug-resistant escape mutants that become selected for by the strong pressure imposed by the antiviral drug. In patients undergoing monotherapy with viral RT inhibitors, one of the two classes of currently approved anti-HIV drugs, resistant variants can generally be seen in 6–12 months [66]. The emergence of viruses resistant to the other approved class of HIV antivirals, those directed against the viral protease, is somewhat more delayed, taking at least 12 months before the appearance of escape mutants [67, 68]. The most hopeful chemotherapeutic approach currently being utilized to overcome the obstacle of drug resistance is the divergent combination strategy [69–72]. Drugs targeting different essential viral proteins are simultaneously administered. At present, encouraging results are being obtained in clinical trials that are underway employing simultaneous treatment with RT and protease inhibitors [71].

Even if chemotherapeutic combination strategies eventually prove successful, these approaches are prohibitively expensive and thus completely unavailable to the overwhelming majority of individuals at risk to HIV infection, especially those living in the underdeveloped countries of the Third World. For these individuals disease prevention via anti-HIV vaccines represents the most viable and cost-effective means of medical intervention against AIDS disease. Developing an HIV vaccine, however, poses a unique and daunting challenge. Some of the problems associated with this difficulty are due to a lack of knowledge as to what constitutes protective immunity and in identifying the protective epitopes [73, 74]. In addition, the immune response must be raised against multiple antigenic subtypes. Furthermore, the various clades of HIV found in different geographical regions of the world pose an additional challenge to the vaccine efforts [44].

Enhanced polymerase fidelity of drug-resistant viruses

The current understanding of HIV biology suggests that the most effective antiviral treatment regimens would be those which completely inhibit HIV replication [75]. The best approach currently to treat HIV disease, both in the short and the long term, is through successful combination anti-HIV therapy, employing compounds that display synergy in regard to inhibition of virus replication. In this light it is encouraging that potent triple and quadruple drug combinations have profoundly reduced the amounts of detectable viral RNA in patients' plasma [76, 77], suggesting that the lymph node reservoirs of HIV may have also been substantially affected. Ultimately the only sure way to prevent drug resistance may be to totally suppress virus replication such that resistance-conferring mutations do not have the opportunity to develop in the first place. Yet, as evident from the above discussion, genetic variation of HIV is key to pathogenesis and poses a severe hurdle for the clinician trying to treat the disease. Thus, if methods were available to reduce variation, it could further improve current treatment strategies.

Recent studies have shown that some of the nucleoside analog-resistance mutations confer increased polymerase fidelity on RT. For example, HIV-1 RT with the M184V alteration, derived from the (–)2', 3'-dideoxy,3-thiacytidine (3TC)-resistant HIV and an E89G variant shown to be resistant to several dideoxynucleoside triphosphates display a significantly enhanced dNTP insertion fidelity (2- to 45-fold increase) [5–8]. The interesting coexistence of high-level drug resistance and a decreased, biochemically demonstrable RT mutation rate, both due to a single mutation, may offer hope if this phenomenon can be translated into an ability to select for less mutable HIV variants (Fig. 2). First it will be necessary to show that the mutations that increase the in vitro fidelity also decrease the viral mutation rate during replication in cell culture. Recent studies by our group have shown, for example, that viruses containing the M184V mutation escape neutralization from neutralizing antibodies directed against the V3 loop more slowly and via a different mutation in the V3 region than the wild-type viruses [78]. It should also be noted that while initial research on the M184V mutation showed that this substitution results in an increase in fidelity of the DNA-dependent DNA polymerase step of RT, subsequent work has now extended this observation to the RNA-dependent DNA polymerization step as well [79, 80].

Coffin [49] has modeled the way in which changes in fidelity affect the rate at which a variant virus might appear. This model suggests that in order for increases in fidelity to make an impact on the rate at which a subsequent variant appears, increases in polymerase fidelity should be very large. As mentioned above, the high rate of HIV replication also contributes to variation, and may allow for the outgrowth of resistant viruses despite lower mutation rates. Modeling HIV population dynamics shows that the high replication rate and viral turnover can compensate for changes in mutation frequency [49]. It is also known that

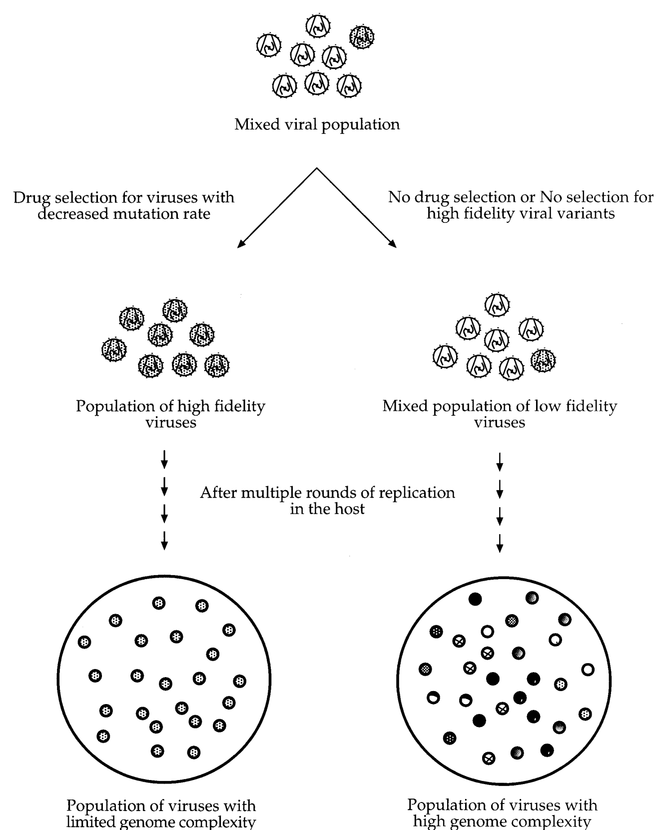

Fig. 2 The influence of drug-resistance mutations that also influence the viral mutation rate. The two mutually exclusive scenarios are depicted. In one, the drug selection leads to the enrichment of a virus containing certain nucleoside analog-resistance mutations (with proven increases in polymerase fidelity) and therefore resulting in a population of high fidelity viruses. In the other, no drug selection or selection for drug-resistant viruses that do not display any increase in replication fidelity lead to a mixed population of low fidelity viruses. Subsequent to this step, the viruses undergo multiple rounds of replication in the presence of selection forces within the host. This leads in the former case to a population of viruses with limited genome complexity and in the latter to a high genome complexity

mutations in a highly conserved gene, such as RT, can reduce the replicative fitness of the virus by driving viral replication at a suboptimal rate [49]. For example, in mixed infections containing equivalent inputs of wild-type and M184V variant viruses (derived from HXB2) followed by several passages in the absence of 3TC, it was shown that M184V is somewhat compromised in replication [23, 78]. Therefore it is likely that a decrease in the overall rate of replication combined with an increase in fidelity may together cause a greater decrease in viral mutation rate.

On a cautionary note, it appears that the currently available nucleoside analog drugs do not bring about the level of increase in fidelity that can be useful in a clinical setting. In all likelihood the modest increase in fidelity conferred by M184V and E89G substitutions will not be sufficient to impact significantly on the development of viral quasispecies under conditions of drug pressure. However, we should endeavor to find a means of increasing RT fidelity in a far more substantial way to accomplish our goal. In this context, a decreased error rate of 100-fold or more might lead to genuine diminution in quasispecies development. Thus the observation that RT fidelity is increased by the M184V and E89G substitutions may be a first step toward the goal of learning how to decrease the viral mutation rate in a biologically significant way.

Other considerations

Additional benefits may also be derived from decreases in patient viral load resulting from any reduction in replicative fitness stemming from fidelity-increasing mutations. For example, it has been demonstrated that individuals treated with 3TC over prolonged periods have lower overall viral burden in their plasma as measured by RT-PCR than persons treated with 2’,3’-dideoxy,3’-azathymidine (AZT), in spite of the fact that resistance to the latter compound develops more slowly and to a far lower degree than attained with 3TC [75]. One possible explanation for this is that the viruses selected by 3TC containing the M184V substitution are less able to replicate than wild-type or AZT-resistant viruses [81].

Further evidence in support of this hypothesis may come through studies that identify the presence of drug resistance-conferring mutations in viruses that cause primary HIV infection in the aftermath of sexual transmission. Some evidence now suggests that the T215Y substitution in RT, which encodes resistance to AZT, may be found in approximately 12% of cases of primary infection in Western countries [83]. This is likely in part a reflection of the widespread use of AZT in the treatment of HIV disease during the past decade. In recent years 3TC has also been used by tens of thousands of individuals, virtually all of whom should have developed the M184V substitution. It will now be important to identify this codon alteration in cases of primary infection and to determine the extent to which sexual transmission of viruses containing M184V may occur. If M184V-containing viruses are indeed attenuated, it is conceivable that the sexual transmission of these viruses may be less than what would normally be anticipated. Studies of this nature would need to be accompanied by follow-up of infected individuals and performance of quantitative viremia assays. These studies may shed important light on the extent to which viruses with increased fidelity may influence HIV treatment.

Finally, consideration should also be given to the notion that viruses with increased RT fidelity might constitute a part of an effective attenuated HIV vaccine strategy. One important issue with the use of attenuated HIVs as vaccines is their ability to revert to virulent status in the aftermath of vaccination, at least in a proportion of vaccinees. Safer vaccines might be developed by placing attenuating mutations and/or deletions (e.g., nef) into increased fidelity viral backgrounds. If the increase in RT fidelity is biologically significant, it is conceivable that such viruses may be unable to revert to a virulent form.

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