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A combinatorial ledge: reverse transcriptase fidelity, total body viral burden, and the implications of multiple-drug HIV therapy for the evolution of antiviral resistance

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Abstract

The chronicity, high mutation rates, and high circulating titers of HIV during the 'stable' phase of infection make rapid evolution of resistance mutations a key predictor of antiretroviral efficacy. Recent advances in measurement of viral RNA titers, turnover dynamics and the in vivo spectrum of resistance mutations allow realistic in vivo estimates of important kinetic parameters of within-patient evolution of viral resistance. First-order estimates of the frequency of viral genotypes necessary for resistance to many antiretroviral combination regimens indicate that many such genotypes pre-exist in patients prior to initiation of therapy. The combinatorial nature of observed multiply-resistant genotypes, however, along with current estimates of total-body viral load and viral turnover dynamics, imply a strikingly sharp transition associated with the change from two-drug to three-drug antiretroviral regimens: pre-existing resistance being near-certain in the first instance but highly unlikely in the second. This abrupt change, a 'combinatorial ledge', carries with it a number of important implications for the understanding and control of HIV infection and other potential targets of antiviral therapy.

Keywords: Antiretroviral resistance; Combination therapy; Polymerase fidelity

1. Introduction

Sustained efficacy of an antiretroviral drug regimen is limited in practice by the emergence within the host of drug-resistant virus (Smith et al., 1994; Richman, 1995; Japour et al., 1996; Kuritzkes, 1996; Wolf et al., 1996). The replacement of sensitive by resistant genotypes occurs in several steps: initial generation of mutations principally by reverse transcriptase (RT) errors, amplification of mutant genotypes by differential selection, and fixation of the mutant population

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by integration into the chromosomal DNA of long-lived cells within the host. In marked contrast to most other infectious agents, HIV combines a very high mutation rate (on average more than one new mutation for each newly transcribed viral genome) (Mansky and Temin, 1995; Mansky, 1996) with an enormous number of total virions produced in the course of natural infection (Katzenstein and Holodniy, 1995; Haase et al., 1996). These properties qualitatively alter the process of emergence of clinical resistance since many potentially resistant genotypes are present in the host prior to initiation of any antiretroviral therapy. If genotypes resistant to a proposed drug regimen exist in the host even before starting therapy, the eventual emergence of clinical resistance is a foregone conclusion, awaiting only the amplification by selection of existing resistance mutants. By contrast, if resistance to some drug or combination of drugs requires sufficiently many changes from the wild-type genotype such that pre-existing resistant viral genomes are unlikely, there exists at least the potential for long-term suppression or even eradication of virus from the host.

The estimation of the probability of pre-existing resistant genotypes depends upon several key variables recently well-quantified in vivo: the rate of production of new virions within the host (Perelson et al., 1993, 1996, 1997; Ho et al., 1995; Coffin, 1996), the in vivo mutation rate per virion, and the number of potential viral mutations which would if present convey high-level antiretroviral resistance (Brown and Richman, 1997; Stilianakis et al., 1997). Experimental understanding of each of these variables has progressed substantially and in this setting it may be valuable to reassess models describing how resistance arises in order to better guide future therapy. In particular, the current shift to multiple-drug antiretroviral regimens (Bray, 1995; Ferre et al., 1996; Hammer, 1996; Hammer et al., 1996; Carpenter et al., 1997) and the combinatorial nature of the expected frequency of resistant genotypes (Colgrove and Japour, 1997) predicts a rather sharp transition where addition of a single new drug can shift the probability of existing resistant genotypes from near certainty to great improbability. Reasonable estimates of the variables outlined above suggest this transition should occur with the switch from two-drug to—optimally chosen—three-drug therapy. As three or more antiretroviral agents become standard therapy, there may ensue a fundamental change in the dynamics of the virus-host interaction wherein the within-patient evolution of the HIV quasispecies is dominated no longer by the huge pool of circulating, rapidly cycling virus already containing resistant genotypes but rather by the persistence of stable reservoirs of much lower levels of active virus replication in specialized sites such as lymphoid tissue (Haase et al., 1996; Cavert et al., 1997). Within this new realm very likely reside complex and as yet unexpected interactions between host, virus and antiretroviral agents.

Sophisticated and elegant models have been developed recently to better explore and understand the in vivo dynamics of HIV infection (Essunger and Perelson, 1994; Coffin, 1996; Perelson et al., 1996). Indeed, the combinatorial transition is implicit in the mathematical formulation of several of these models. Nonetheless, explicit description of the effect and thorough analysis of its implications have not been widely reported. Further, the marked qualitative change in the kinetics of the evolution of resistance is quite robust and independent of the details of the models and is thus likely to remain a useful insight even as increasing complexities of the underlying biology emerge. The basic observation is quite simple and straightforward but its ramifications into many areas of antiviral therapy are sufficiently far-reaching to merit detailed consideration. Current rapid changes in clinical practice and in the epidemiology of antiretroviral resistance impart a particular urgency to these considerations as we seek the most durably efficacious strategies in the new regime of highly potent, multi-drug antiretroviral regimens.

2. The in vivo rate of virion production and whole body viral burden

As accurate serological tests for HIV infection became widely available, it was apparent that

large numbers of infected individuals were asymptomatic. The most sensitive nucleic-acid detection techniques in use in the mid-1980s showed only a small fraction of circulating T-cells harboring virus during this asymptomatic phase (Ho et al., 1989) and prospective studies of asymptomatic HIV-positive cohorts found many individuals remaining well for years before progressing to AIDS. Thus arose the concept of a viral quiescent phase of presumed low-level viral reproduction and minimal host response. Rapid and simple polymerase chain reaction (PCR)-based assays of viral RNA levels began to change this impression by demonstrating high-titers of circulating virus even in asymptomatic individuals (Bray, 1995; Katzenstein and Holodniy, 1995; Hammer, 1996; Saag et al., 1996). With the advent of combination antiretroviral therapy including protease inhibitors, it became possible at least in a subset of patients to effect such a profound drop in viral titers that to a first order approximation, production of new virus was essentially zero (Cao et al., 1993; Perelson et al., 1997). Ho et al. (1995), Wei et al. (1995) and others realized that the initial rate of decline of viral RNA levels after instigation of such combination therapy provided an important new window into the dynamics of infection during the 'plateau' phase. The rapid fall in viral RNA indicated that the half-life of circulating virions in this setting was on the order of 2 days and therefore that, far from quiescence, the stable phase of infection was characterized by furious but balanced competition between virus and host. For a half-life of 2 days, if the viral load at time t is given by $f(t)$ then:

$$f(t) = 2^{(-t/2)} \Rightarrow f(t) = e^{(-t\ln(2)/2)} \therefore f(1)/f(0)$$
$$= e(-\ln(2)/2) \approx 0.7$$

Therefore, after 1 day, only 70% of the original virions present at the outset remain and the other 30% have been 'turned over' presumably destroyed through a variety of mechanisms by the host immune response. Thus, in a little over 3 days, the host destroys (and proviruses create) a quantity of virus roughly equal to the total body load of virus at any given time. In 1 year, circulating virus will turn-over more than 100-fold (365/3) and over the course of a 10 year plateau phase of infection infected host cells will produce a total number of virions on the order of a thousand times the average number of virions present at any given time.

The total number of virions present in an infected host will depend not only upon the titer of virions in various physiologic compartments but also upon the absolute size of these compartments set by the total effective volume of distribution therein available to the virus. This will vary considerably from patient to patient and will change depending on the clinical state. However, the presence of virus in diverse fluid compartments such as semen and CSF and the presence of virus within cells of the lymphoid system, gut and CNS show how widely distributed are HIV virions in natural infection and allow an order of magnitude approximation of the whole body viral burden and its distribution within various compartments (Korber et al., 1994; Zhu et al., 1996; Wong et al., 1997).

For the purposes of this analysis, there exist two distinct relevant measures of whole body viral burden: the total number of proviral copies of any sort and the subset of those that are actively producing new virions. For HIV infection, both dynamic models of viral turnover and direct quantitative measurement of viral sequences in lymphoid tissue (Haase et al., 1996) demonstrate the presence of an enormous reservoir of viral copies, ranging from 10^{11} to 10^{12} in an infected individual but not actively replicating and only very slowly mixing with the population of virus circulating in plasma. By contrast, the total body viral burden of actively replicating circulating virus can be estimated as the viral titer at any given time multiplied by the volume of distribution in total body extra cellular fluid, roughly 25–30% of body mass. It is this circulating compartment that will provide that 'library' of genotypes available to the virus for the rapid emergence of clinically apparent resistance through the selection of pre-existing resistant genotypes. Thus, a 100-kg man (roughly 100 000 ml volume) with a viral RNA 'load' of 100 000 copies/ml would harbor approximately three billion circulating virions ($100\,000 \times 100\,000 \times 0.3$) and produce between 1 and 10 trillion virions over

Table 1
Expected resistance mutation frequencies

(A) Total body circulating virion number

		Body mass (kg)					
		2	5	10	20	50	100
Viral load	2×10^{1}	4×10^{4}	10^{5}	2×10^{5}	4×10^{5}	10^{6}	2×10^{6}
	2×10^{2}	4×10^{5}	10^{6}	2×10^{6}	4×10^{6}	10^{7}	2×10^{7}
	2×10^{3}	4×10^{6}	10^{7}	2×10^{7}	4×10^{7}	10^{8}	2×10^{8}
	2×10^{4}	4×10^{7}	10^{8}	2×10^{8}	4×10^{8}	10^{9}	2×10^{9}
	2×10^{5}	4×10^{8}	10^{9}	2×10^{9}	4×10^{9}	10^{10}	2×10^{10}
	2×10^{6}	4×10^{9}	10^{10}	2×10^{10}	4×10^{10}	10^{11}	2×10^{11}

(B) Expected number of circulating mutant genotypes
(Example shown: 50-kg mother/5-kg baby assuming RT error rate of 10^{-4})

		No. mutant nucleotides		
		Single	Double	Triple
Viral load	2×10^{1}	$10^{2}/10^{1}$	0/0	0/0
	2×10^{2}	$10^{3}/10^{2}$	0/0	0/0
	2×10^{3}	$10^{4}/10^{3}$	1/0	0/0
	2×10^{4}	$10^{5}/10^{4}$	10/1	0/0
	2×10^{5}	$10^{6}/10^{5}$	$10^{2}/10^{1}$	0/0
	2×10^{6}	$10^{7}/10^{6}$	$10^{3}/10^{2}$	0/0

the median 10 year progression from infection to AIDS. By the same reasoning, a smaller host would carry a smaller number of virions and a 2-kg neonate on perinatal AZT following the ACTG 076 protocol (Connor et al., 1994) might have as few as 10–100 million total virions (Srugo et al., 1991). An antiretroviral-naive adult beginning triple-drug therapy can experience a three-orders-of-magnitude drop in viral titer and by the same token in total-body virion burden. Since pre-therapy RNA load can vary anywhere from 10^2 to 10^6 copies/ml with median numbers at 10^4 copies, and volume of distribution can vary from a 1-l neonate to a 100-l large adult, total body viral copy number can vary from 10^5 to 10^{11}, with more typical ranges from 10^7 to 10^9. Over the course of the 10-year median progression to AIDS, these typical-range viral loads would produce 10^{10}–10^{12} virions. These order of magnitude estimates are tabulated in Table 1A and shown graphically in Fig. 1 for a hypothetical maternal-infant pair in order to illustrate the range of observed variation.

3. The in vivo viral mutation rate

The rate at which resistance mutants arise will depend upon the product of the total number of virions produced and the mutation rate per virion. Estimation of the true rate of HIV mutation has been fraught with difficulty due to the complexity of the retroviral life-cycle, the difficulty of disentangling the mutation rate from the rate of fixation of mutations in vivo, and by the enzymological differences between purified recombinant RT and the enzyme within an infected cell. Recent clearer understanding of the natural mutation rate not only give us a more accurate picture of the within-patient evolution of HIV genotypes under selection by antiretroviral drugs but also, as will be discussed in this analysis, have profound implications for the expected combinatorial effects of drugs used in multi-drug combinations.

Unlike DNA-dependent DNA synthesis, where GTP-burning $5'\rightarrow3'$ exonucleolytic proofreading of the DNA polymerase complex allows an error

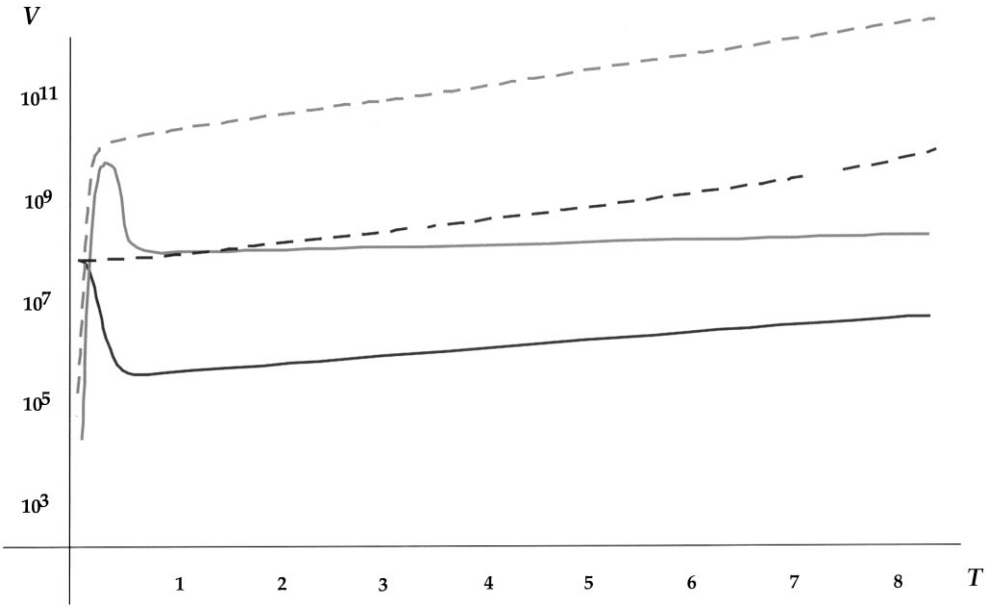

Fig. 1. Schematic diagram indicating the total instantaneous (solid lines) and cumulative (dashed lines) whole-body virion burden, V, from time of infection through time, T, in years during the plateau phase of infection for a hypothetical 50-kg adult woman (red) and an infant (blue) with perinatal acquisition of virus, both assumed to have average viral loads of 10 000 copies/ml once plateau phase is reached. Cumulative number of virions, V_c, assumes turnover of circulating virus with $t_{1/2} = 2$ days and therefore: $V_c = T \times V \times (\ln 2)/2$.

rate of less than 1 in 10^9 nucleotides, RNA-dependent polymerases both in mRNA transcription and viral RNA reverse transcription lack proofreading and show much higher error rates, typically on the order of 1 per 10^4 nucleotides (Preston et al., 1988; Patel and Preston, 1994). DNA polymerases trade speed and metabolic energy for higher fidelity, proofreading each newly added nucleotide and editing any misincorporations detected. RNA polymerase and RTs, by contrast, lack this energy-requiring mechanism and are limited in fidelity by the free-energy difference between correct and incorrect nucleotide base-pairing. Eigen and others have argued that this limitation in the fidelity of replication imposes strict limits on the size of RNA genomes such that the expected number of mutations per genome per replication cycle cannot be much greater than one if the replicon is to escape genomic collapse (Biebricher et al., 1985; Eigen, 1993a,b). Naturally occurring RNA viruses follow this rule closely; the only RNA virus families with genome sizes much above 10^4 nucleotides are those with segmented genomes (where the expected number of mutations per segment is less than 1). No RNA viruses approach the huge genome sizes of DNA viruses such as the Poxviridae, Iridoviridae and Baculoviridae, which can span hundreds of kilobases.

Measurement of RNA polymerase error rates can be made directly in in vitro systems but RT error rates are more complex to estimate. Viruses that replicate by reverse transcription actually undergo two separate error-prone polymerization steps: first, RNA polymerase transcription of a viral genomic template from the DNA provirus, then RT-mediated reverse transcription of viral DNA from the virion RNA template. Further, the presence of a diploid RNA genome within the virion and the requirement for a complex series of strand-switching and strand-displacement events to carry out reverse transcription adds further potential for novel types of replication errors. Finally, the mutations observed in the sequencing

of virus from clinical samples reflect, as noted above, a complex mix of the effects of mutation rate and of mutation fixation.

For these reasons, the initial measurements of HIV RT fidelity were made in vitro with purified recombinant RT on artificial templates. Careful biochemical analysis (Preston et al., 1988) gave mutation rates of 1/1700–1/3000 depending on the exact template and reaction conditions. These numbers were 20-fold higher than what had been measured for other RT enzymes and seemed too high to prevent the catastrophic accumulation of genome errors predicted by Eigen and validated by the genome sizes of RNA viruses. In vivo, however, the RT reaction takes place within the viral nucleocapsid inside the infected cell, an environment markedly different from the in vitro setting and presumably an environment for which the native RT had been optimized by evolution. To circumvent these limitations, Mansky and Temin (Mansky and Temin, 1995) created a set of vectors carrying portions of the HIV genome designed so that when co-transfected into a permissive cell line, only a single round of virion production was possible. When these viruses were used to infect a second round of cells, a marker gene on the resulting proviral DNA could be sequenced to estimate directly the in vivo error rate per round of replication. These rates also varied—by less than an order of magnitude—depending on template sequence and nucleotide substitution bias but the overall rate of nucleotide substitutions (leaving aside for this analysis more complex mutations), was $3.4 \times 10^{(-5)}$, much lower than the in vitro rates and much more in line with the error rates of other non-proofreading polymerases. Similar in vitro methods had been used to study the appearance of antiretroviral resistance under selection (Stair et al., 1993) with analogous findings of site-dependent frequency of pre-existing resistance mutations.

The combination of the total number of virions in an infected host and the mutation rate per virion produced allows a calculation of the expected distribution of mutant genotypes. For HIV, with a genome size of approximately 10^4 nucleotides, with an RT error rate of roughly $3.4 \times 10^{(-5)}$ per nucleotide, in a host producing, for example, 3×10^8 virions/day, there would be expected on average $(10^4) \times (3 \times 10^8)(3.4 \times 10^{-5}) \approx 10^8$ mutations/day, an average of 10^4 mutations at each possible nucleotide site. The exact number might vary depending upon mutational hotspots and nucleotide substitution biases and inter-nucleotide interactions but even given these effects, the infected host will contain many copies of every possible point mutant—including those conferring antiretroviral resistance—under these very ordinary conditions of viral load. Any therapy which can be overcome by a point mutant is thus doomed from the outset by the vast cloud of viral quasispecies mutants.

If the chance of a mutation at any given site is 3.4×10^{-5}, then the chance of any particular double mutant is (assuming independence of mutational events and neglecting hotspots and biases): $(3.4 \times 10^{-5}) \times (1/3) \times (3.4 \times 10^{-5}) \times (1/3) = 1.3 \times 10^{-10}$/virion (since at any site there are three possible nucleotide substitutions). At this rate, patients with the highest total body load ($> 10^{10}$) will thus approach a 'saturating' amount of virus so that all possible double mutants will be present. A patient with a more typical total body burden of 10^9 would still over the course of a year produce 10^{10}–10^{11} virions, and would thus stand a good chance of growing any given double mutant.

The probability of an infected host having zero of a particular mutant in this simple model is estimated by the Poisson distribution $p(0) = 1 - e^{(-M)}$ where $M =$ (total number of virions) $\times$ (frequency of mutant genotype) so that when $M = 1$, $p(0) = 1/e \approx 0.63$. Because of the exponential nature of this equation, $p(0)$ shifts very rapidly from near 0 to near 1 as M goes from $\gg 1$ to $\ll 1$. At $M = 3$, $p(0) = 1 - e^{-3} = 0.95$. At $M = 10$, $p(0) = 0.99995$. Thus, relatively small shifts in total viral burden around $M = 1$ can dramatically alter the chance that the genotype needed for high-level resistance lies waiting already in the host even before therapy starts.

This phenomenon is made abundantly clear when one considers the chance of any triple point mutant being present. By the same reasoning as above, the frequency of triple mutants should be $((3.4 \times 10^{(-5)})/3)^3 \approx 1.5 \times 10^{-15}$, a number so low

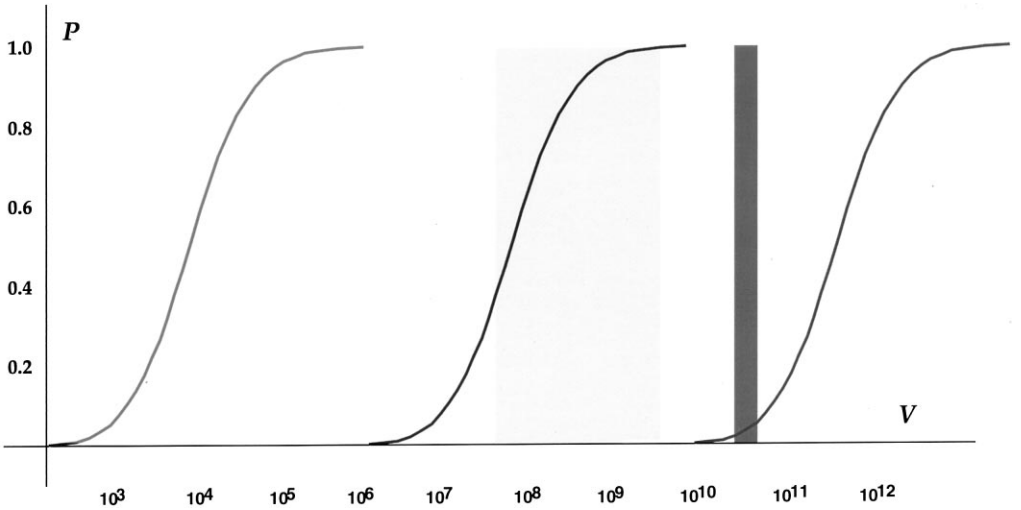

Fig. 2. Probability, P, ranging from 0 to 1 of any given single (red), double (purple), or triple (green) nucleotide substitution mutant existing before institution of antiretroviral therapy as a function of total body virion number V. Typical ranges for number of circulating virions (yellow) and estimate of cell-attached follicular dendritic cell virions (blue) show that all single mutants, nearly all double mutants but few of the possible triple mutants would be present. Probability is given by the zero term of the Poisson distribution assuming a mutation, μ, rate per nucleotide of 3.4 per 10 000: ($P = 1 - e - mV$).

that even patients with the highest recorded viral burdens would have a negligible chance of seeing any given triple mutant arise by chance. The estimates of the probabilities for various combinations of mutation number and total viral burden are tabulated in Table 1B and shown graphically in Fig. 2. The chance is rendered even more remote by the fact that the total body burden (and thus the available pool of potential mutants) can fall by several orders of magnitude at the instigation of triple therapy. In this setting the rate of production of new mutants will fall by a commensurate amount and therefore it may be possible to maintain the combinatorial improbability of pre-existing multiple mutants with fewer agents than required to instigate successful therapy.

Thus, the transition from an anti-retroviral regimen for which high-level resistance can arise in two mutations (in the simplest case a two-drug regimen) to a regimen requiring three independent nucleotide substitutions for high-level resistance (e.g. suitably chosen three-drug combinations) results not only in log-linear additive increase in the potency on the regimen (the greater drop in viral load) but also in a qualitative, highly non-linear switch to a new regime of potentially much greater durability of efficacy. (This would not have been the case had the initial in vivo mutation rate estimates held true in vivo. If, for example, the in vivo rate had been 1/2000, then triple mutants would have been present at a rate of approximately $1/(8 \times 10^9)$ and thus would have been expected to occur frequently in natural infection.) The initial experience with the marked difference in character of response between two- and three-drug regimens (Carpenter et al., 1997) is consistent with the combinatorial level occurring at this point as this model and the lower in vivo mutation rate would predict.

4. The spectrum of available resistance mutations

Whether the predictions of the simple combinatorial model presented above matter in the in vivo development of clinical resistance depends upon one final parameter: the spectrum of possible resistance mutations available to the virus. On the one extreme, in the unlikely event that some viral gene product can tolerate no mutation at all and still retain its function, resistance to an antiretro-

viral drug inhibiting this gene product would be impossible. At the other extreme, if large numbers of mutations can be tolerated by the gene product and can convey resistance while allowing the gene product to function (or equivalently, if a small number of mutations could convey resistance to a large number of available antiretroviral agents) then resistance is inevitable and long-term suppression is futile. At the outset of the era of antiretroviral therapy, there was no way to predict a priori how the virus would respond and there was no way to know whether resistance phenomena that could be generated rapidly in vitro would bear any resemblance to what would arise more slowly in the much more complex in vivo setting. Now, with almost a decade of widespread clinical experience with antiretroviral drugs and with nearly a dozen antiretroviral agents either in use or in advanced clinical trials, a much clearer estimate of the size of the pool of potential resistance mutations is possible. Unlike the situation in vitro, where one can generate a variety of resistance mutations either by selection or by saturation mutagenesis, only a very narrow spectrum of these are observed in vivo (D'Aquila, 1994; Arts and Wainberg, 1996; Kimberlin and Whitley, 1996; Richman, 1996). In every case, high-level resistance to a given agent arises again and again in multiple patients from the same single or small number of mutations. Continued selection results in ancillary mutations which increase resistance on the background of the initial mutant but do not themselves confer high-level resistance. With AZT (Mayers et al., 1992, 1994; D'Aquila et al., 1995; Japour et al., 1995), modest resistance arises first from a point mutation at codon 70 in the RT gene and then later high-level resistance ensues from a single amino-acid change at position 215 that (in accordance with the mutation frequency model presented above) appears more slowly because it requires a two-nucleotide change. Similarly small numbers of key mutations are seen for the other nucleoside-analog RT inhibitors (Johnson, 1995; Arts and Wainberg, 1996) with only modest (and sometimes antagonistic) cross-reaction between them. Complex second-order effects such as the lower catalytic activity and apparent higher RT fidelity seen with the RT codon 184 Methionine to Valine mutation (that arises in response to 3TC and may suppress the effects of codon 215 AZT resistance mutations) further limit the mutational flexibility of the virus. Protease inhibitors and non-nucleoside analog RT inhibitors select out a somewhat broader range of resistance mutants as would be expected since their larger binding surfaces offer greater numbers of amino acid contact sites whose alteration could affect the KM of the inhibitor. Even with these agents, however, naturally-occurring resistance is dominated by a few key mutations in each case with no single mutant providing resistance to all members of a given class of inhibitors. Given the set of available drugs and the observed spectrum of resistance mutations to individual agents, a number of available combinations of antiretrovirals may thus require from the virus three or more independent point mutations to achieve high-level clinical resistance.

5. Implications

Taken together, these analyses suggest that the existing armamentarium of antiretroviral agents is sufficient in appropriate combination to push the circulating HIV quasispecies within a patient into a qualitatively new realm. Here development of resistance depends not simply upon amplification of existing genotypes within the quasispecies but rather upon generation from a much reduced pool of virions of entirely new genotypes. This indeed corresponds well to the initial clinical experience with triple therapy including protease inhibitors where at least an appreciable subset of patients demonstrate a remarkable clinical recovery and disappearance of detectable circulating virus. The actual appearance of clinical resistance then depends upon complex and difficult to gauge effects such as the debilitating effect upon the viral gene product's normal function of the accumulation of multiple mutations. The effects of a reconstituted immune system in patients with rising CD4 counts may also shift the balance of the virus-host conflict. On the other hand, minor populations of virus in areas shielded from immunological and/or pharmacological attack such as the CNS now

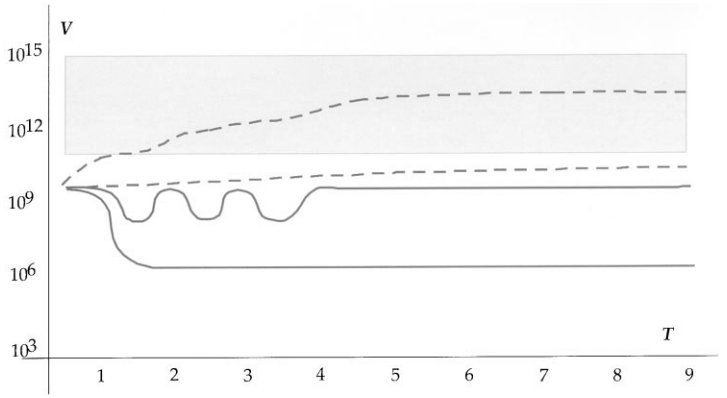

Fig. 3. Instantaneous (solid lines) and cumulative (dashed lines) number of virions, V, produced during years of time, T, for idealized models of initial 'up front' (green) or sequentially-added (red) triple anti-retroviral therapy, assuming initial viral burden of 10^9, each agent contributing an additive one log drop in viral titers, resistance arising to each agent by a single point mutant, and circulating virion half-life remaining constant at 2.5 days. Yellow hatched area shows excess viral production as a consequence of delayed combination therapy. Blue stippled area shows the range of estimated $(1-1/e)$ probability of any given triple mutant arising at random within range of mutation rates from 3×10^{-4} (lower border) to 3×10^{-5} (upper border).

may assume critical importance as circulating virus falls to very low levels. Of absolutely crucial importance will be public health efforts to limit the between-patient spread of partially-resistant viral strains for all the combinatorial advantages of multiple therapy are lost if the virus enters the host already resistant to some agents. In this setting, not simply RNA quantitation but also explicit sequencing may become clinically valuable to track the appearance of particular mutants and direct therapy accordingly. In some situations where the total viral number within the host is quite low, for example during the peripartum vertical transmission of HIV, it may be possible to cross the 'ledge' with only two agents where three would be needed in a larger host.

The true kinetics of resistance arising in the multiple drug era will also depend critically upon the 'fitness landscape' of partially resistant single-mutants. If a single mutant recovers enough replicative potential to grow out even in the face of triple therapy, or if antiretroviral agents are added sequentially over a large enough interval to allow selection of partially resistant mutants, then even multiple-drug regimens will eventually fail since they will allow slow but inexorable sequential accumulation of multiple mutants (Fig. 3) (except in the extreme case where total viral burden can be held so low—less than 10^4 virions/host, less than 1 virion/ml—that even single mutants become unlikely). Whether the virus has a smooth, stepwise path to high-level multiple drug resistance will hinge upon the precise combination and sequence of antiretroviral agents chosen (Fig. 4), giving renewed impetus to the study of the interaction between different resistance mutants. Further, if mutant virus replicates more poorly in the absence of selection, then removal of selection pressure—for example in changing to different antiretroviral drugs—may result in significant rates of revertant mutations to wild-type, resulting in even more complex patterns of quasispecies evolution. Finally, both the rate of occurrence of mutations and the overall fitness of established mutations will be influenced by features of the viral RNA such as secondary structure (Schinazi et al., 1994) as well the amino acid sequences of the translated gene products. These more subtle effects, however, should only modestly affect the essential character of the orders-of-magnitude combinatorial effects described by this model.

Within this simple, first-order model of multiple-drug therapy, whereas the initial potency of

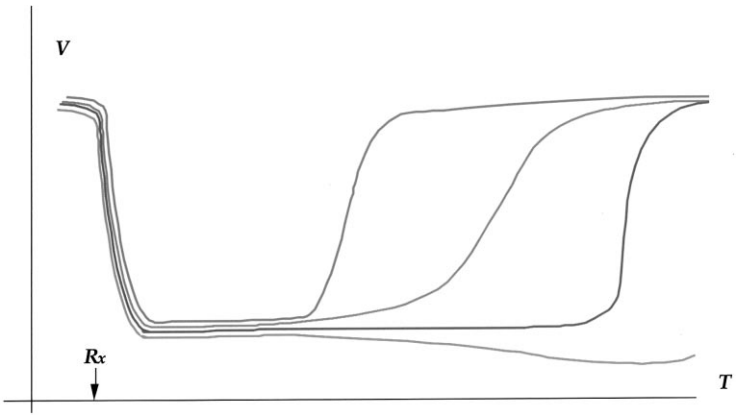

Fig. 4. Schematic illustration of the expected rate of acquisition of multiple resistance mutations as a function of the level of resistance of the partial mutants. At the institution of multiple drug therapy, R_x, viral burden, V, falls to a low plateau. If a single mutation is sufficient to confer the bulk of the resistant phenotype (red), there is rapid rebound to pre-therapeutic virus levels. If the phenotypic effects of multiple mutations are additive (green), there is delayed but accelerating recovery of virus. When the partial mutants remain fully sensitive (blue), there is prolonged delay but sudden rebound when the appropriate multiple mutant finally arises. If partial mutants demonstrate less-than-wild-type growth rates in the presence of the drug regimen (purple), viral levels may continue to fall.

therapy depends upon the degree of enzymatic inhibition achievable by a given drug combination, the potential durability of suppression hinges upon the balance between the absolute size of the total-body viral load and the expected frequency of resistance mutations. The population biological parameter, $R_{(0)}$, denotes the average number of newly infected hosts produced by a single already-infected host. The between-patient $R_{(0)}$ has long been used to measure the success of efforts to contain and reverse epidemics. When $R_{(0)}$ is held to less than 1, the epidemic eventually dwindles and disappears. The between-patient $R_{(0)}$ for HIV has always been estimated to be disturbingly high, often greater than 10, when the epidemic first moves into any new population. The combinatorial model suggests that the special characteristics of HIV infection make the within-patient $R_{(0)}$ (Nowak et al., 1997), where the 'hosts' are cells rather than organisms, a critical variable in the effort to subdue HIV in an infected individual.

This consideration of the frequency of viral mutations, the whole body viral burden, and the spectrum of available resistance mutants suggests that in the transition from two- to three-drug therapy (or more precisely to therapeutic regimens of any drug number where three or more independent mutations are required for high-level resistance), we may be entering a qualitatively new setting. Here it may be possible to lower the within-patient $R_{(0)}$ from approximately 2.6–3.5 in the absence of efficacious antiretroviral therapy (Little et al., 1998) to less than 1 with triple therapy for long enough that the virus is permanently held at bay. The simple but robust multiplicative effects underlying this sharp combinatorial ledge show how the molecular details of viral replication can have profound consequences for antiviral therapy. The addition of one new independently active antiviral drug to a standard initial regimen can cause an abrupt shift in the typical clinical response from modest and transient efficacy on one side of the 'ledge' to profound and durable viral suppression on the other. This principle should be generally applicable as multiple-drug therapy becomes possible for other high mutation-rate, high viral-load, chronic infection-producing pathogens such as Hepatitis C Virus and Hepatitis B Virus.

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